Systematics and Evolution of New Caledonian *Araucaria*

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ABSTRACT

New Caledonia is a global biodiversity hotspot and contains more than 2300 endemic species including 7% of the world's conifers. No other region in the world with such a small area possesses such a rich and distinctive conifer flora, and 13 of the world's 19 *Araucaria* species are endemic to New Caledonia. This thesis has investigated the evolution and systematics of this group.

A molecular phylogenetic study based on sequence data from two chloroplast regions resolved all 13 New Caledonian species as a monophyletic group, sister to the Norfolk Island Pine (*A. heterophylla*). The relationships between the New Caledonian species was not fully resolved as little sequence variability was detected, however, three main groups were defined. The species with bigger leaves occupied a basal polytomy, whereas the vast majority of species with smaller leaves were grouped together in a clade. Within this 'small leaved' clade, the three New Caledonian species with a coastal distribution formed another monophyletic group.

The timing of the radiation of all these species was tested via a molecular clock approach using different calibration tools (fossil data, geological events, substitution rates). The precise dating of the New Caledonian radiation remains uncertain because different calibration methods give different dates. However, it seems likely to have occurred between 10 and 43 *mya*. What can be said is that the limited sequence divergence between these species (which in other groups would be typical of <3 million years divergence), does not tally with the fossil record and geological events. This suggests a reduction in evolutionary rates in *Araucaria*.

A combination of molecular and morphological approaches was used to assess species limits and population identities. This resulted in re-determination of the identity of several populations and the distributions of some species. The current state of knowledge of the taxonomy of the New Caledonian species was summarised.

Finally, the distribution of chloroplast haplotypes among 468 individuals from 49 populations representing all New Caledonian *Araucaria* revealed strong taxonomic signal, and high genetic diversity among the species with bigger leaves, and low diversity in the coastal species. The distribution of genetic variation is discussed in the context of the evolution and conservation of the New Caledonian *Araucaria* spp.

Declaration

I hereby declare that this thesis is composed of work carried out by myself unless otherwise acknowledged and cited and that this thesis is of my own composition. Research towards this thesis was carried out between October 2001 and October 2004. No part of this dissertation has been previously submitted for any other degree.

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This work is the result of the collaboration between a great number of people and I hope that if I forgot some of them here, they will forgive me.

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4.3 *Araucaria* diversity on New Caledonia

4.3.1 New Caledonia: a biodiversity hotspot.

New Caledonia (Fig. 1.1) is a small island (19,103 km^2) located in the south Pacific Ocean some 11,000 km off the east coast of Australia. Despite its small size, it has an unusual and rich flora with 3002 native species of vascular plants, 77.3% of which are endemic (Jaffré, 2001). No other region in the world with such a small area possesses such a rich and distinctive flora (Watt, 1999), and it is the third highest level of island endemism, behind Hawaii and New Zealand. Particularly high levels of endemism on New Caledonia (97%) are associated with the metal-rich ultramafic soils (Jaffré, 1992).

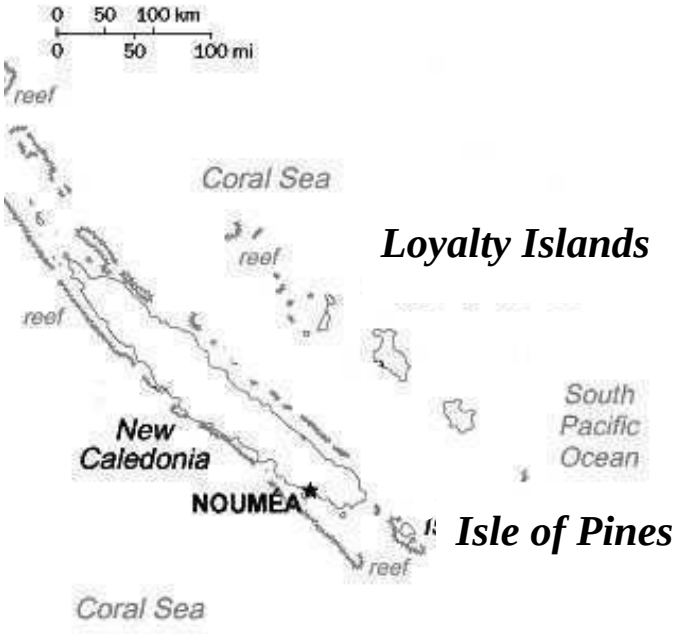


Figure 1.1: Map of the island archipelago of New Caledonia

The New Caledonian flora is characterised by the presence of primitive dicots such as species in the Winteraceae or Amborellaceae (Soltis *et al*., 1999). It also has a globally important conifer flora (43 species, all endemic; representing 7% of the

world's conifers; Watt, 1999). As New Caledonia once belonged to the supercontinent Gondwana, its flora is considered to be a Gondwanan relict (Jaffré, 1995; Balgooy, 1996; Pintaud, 1999).

One remarkable component of the New Caledonian flora is the genus *Araucaria* (Araucariaceae) which has 13 species, out of a global total of 19, located in New Caledonia. *Araucaria* species occur in several ancient Gondwanan localities (Australia, South America, New Guinea, New Caledonia) and fossil evidence suggests it was present on Gondwana before its break-up. It therefore provides a good model to study a putative Gondwanan element of the New Caledonian flora, as well as assess the factors that led to high species richness on the island. Moreover, *Araucaria* species are important in New Caledonia from a cultural perspective (male fertility symbols in the Melanesian culture), from an economic perspective (major landscape features important for tourism; the trees are also exploited for timber; Nasi, 1982; Manauté *et al.*, 2003), and also from a conservation perspective (they are facing threats from mining, fire and agriculture; Jaffré, 1995; Watt, 1999). This makes New Caledonian *Araucaria* a key genus both from an evolutionary/systematics perspective, but also with regards to its conservation.

4.3.2 Araucariaceae (Gymnosperma, Coniferales):

The Araucariaceae contains 33 of the world's *ca.* 630 species of conifer. It consists of three genera of which one, *Wollemia* W. G. Jones, K. D. Hill and J. M. Allen, was described as recently as 1995. The two others are *Araucaria* and *Agathis* Salisbury and have been known for more than a hundred years. *Wollemia* comprises one species and is restricted to New South Wales, Australia. The genus *Agathis* has 13 species which occur in the Malaysian region, Vanuatu, Fiji, Australia and New Caledonia. Though some *Agathis* species appear north of the equator in Malaysia, the genus is considered of southern origin and seems to have migrated north during the Plio-Pleistocene (Florin, 1963 cited in Setoguchi *et al.*, 1998). There is current phylogenetic uncertainty regarding the relationships among the three Araucariaceae genera. Attempts to resolve this uncertainty using *rbc*L sequences have resulted in

contradictory conclusions. Setoguchi *et al.* (1998) recovered the following well-supported topology (*Wollemia* (*Araucaria*, *Agathis*)); however Guilmore and Hill (1997) recovered (*Araucaria* (*Wollemia*, *Agathis*)).

4.3.3 *Araucaria* (Monkey-puzzle trees)

4.3.3.1 Extant species.

The name *Araucaria* is derived from “Arauco”, a region in Central Chile where the Araucani Indians live. The 19 extant species of *Araucaria* are arranged into four sections (*Araucaria*, *Eutacta* (Link) Endl., *Bunya* Wilde & Eames, and *Intermedia* C. T. White) (Table 1.1). This classification is essentially based on morphology (leaves, attachment of pollen cones and ovulate cones, cone scales, vascular system cone-scales complex, type of seedling germination, and seedling morphology). However DeLaubenfels (1988; 2002) has suggested two modifications to this treatment. Firstly, he only recognized two living sections, not four: sections *Bunya* and *Intermedia* were treated as synonymous with section *Araucaria* that was therefore broadened to include extra-American species (DeLaubenfels, 1988). However, Stockey (1986) preferred to retain sections *Bunya* and *Intermedia* on the basis of fossil and cuticular evidence. Secondly, DeLaubenfels (2002) argued that the species in section *Eutacta* were distinct enough to warrant generic status, on the basis of the morphological gap existing between the species of this section and the other *Araucaria* species (unique features of section *Eutacta* include four cotyledons and epigeal germination).

Section	**Extant species**	**Location**
Araucaria	*A.angustifolia (Bertol.) Kuntze*	Brazil
	A. araucana (Molina) K. Koch	Chile
Eutacta(Link) Endl	*A. bernieri* Bucholz	New Caledonia
	A. biramulata Bucholz	New Caledonia
	A. columnaris (Forster) Hooker	New Caledonia
	A. humboltensis Bucholz	New Caledonia
	A. laubenfelsii Corbesson	New Caledonia
	A. luxurians (Brongn. & Gris) Laubenfels	New Caledonia
	A. montana Brongn. & Gris	New Caledonia
	A. muelleri (Carr.) Brongn. & Gris	New Caledonia
	A. nemorosa Laubenfels	New Caledonia
	A. rulei Müll.	New Caledonia
	A. schmidii Laubenfels	New Caledonia
	A. scopulorum Laubenfels	New Caledonia
	A. subulata Vieill.	New Caledonia
	A.cunninghamii Aiton ex D. Don in Lambert	Australia
	A.cunninghamii Aiton ex D. Don var. *papuana* Lauterb.	New Guinea
	A.heterophylla (Salisb.) Franco	Norfolk Island
Bunya Wilde & Eames	*A. bidwillii* Hook	Australia
Intermedia C. T. White	*A. hunsteinii* K. Schum.	New Guinea

Table 1.1: Extant *Araucaria* species with their geographical locations and sectional affinities

The genus is currently distributed throughout the southern hemisphere in New Caledonia (13 species), Chile (*A. angustifolia*), Brazil (*A. araucana*), Norfolk Island

(*A. heterophylla*), Australia (*A. bidwillii, A cunninghamii*), and New Guinea (*A. hunsteinii, A. cunninghamii* var. *papuana*) (Fig. 1.2).

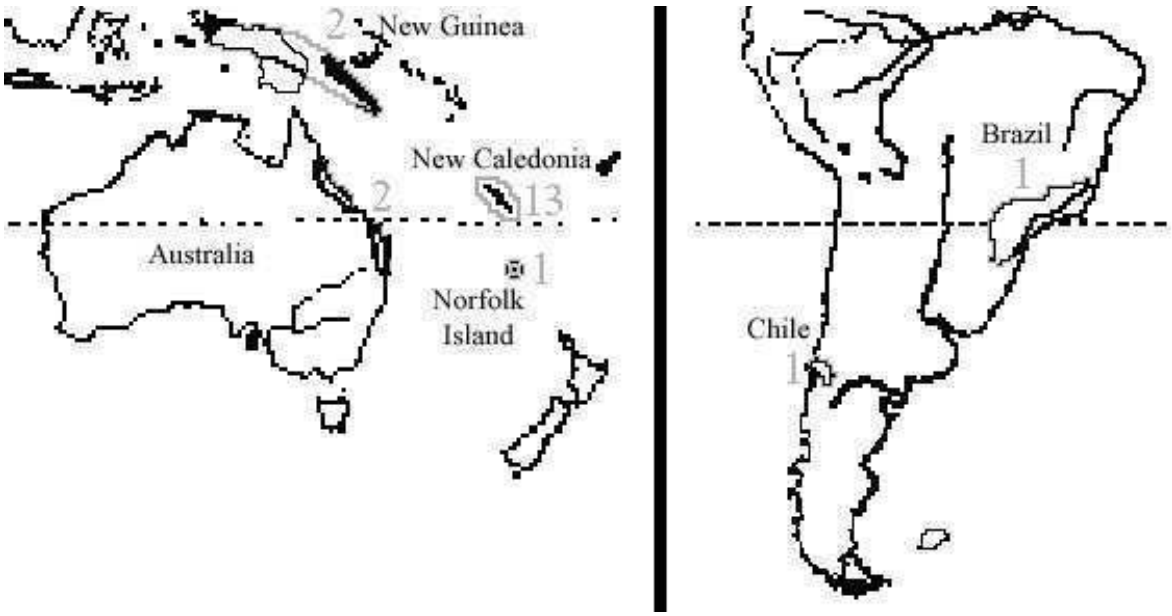


Figure 1.2: Distribution of extant species of *Araucaria*. Numbers represent the number of species present in each named geographical area

4.3.3.2 Extinct species.

An extensive fossil database is available for *Araucaria* (Hill and Brodribb, 1999). More than 40 species of extinct *Araucaria* are known and a few fossils exist which are as yet undetermined. The extinct species of *Araucaria* includes two additional sections, section *Perpendicula* M. Pole and *Yezonia* (Stopes & Fujii) T. Ohsawa *et al.* The geographical distribution of *Araucaria* fossils suggest that despite it now being restricted to Gondwanan fragments in the southern hemisphere, the genus was once widespread in both hemispheres (Setoguchi *et al.*, 1998).

4.3.4 New Caledonian *Araucaria*

New Caledonian *Araucaria* species belong to section *Eutacta* which also includes *A. heterophylla* (Norfolk Island) and *A. cunninghamii* (Australia, New Guinea). The Flora of New Caledonia account (DeLaubenfels, 1972) recognises thirteen species, which have been classified on the basis of their architecture (habit) into two groups (Fig. 1.3). One of these groups follows the Massart model (trees with plagiotropic branches and presence of partial reiteration) which includes the species with small leaves (<1cm long in adults), and the other group follows one the Rauh model (trees with orthotropic branches and no reiteration) containing the species with bigger leaves (Veillon, 1980). Veillon (1980) states that some alteration of the model may be observed when a tree is damaged (e.g. in the Rauh model a new axis may be generated but the vertical tendency of the new branch remains).

The species can show clear differences in height. *A. bernieri*, *A. laubenfelsii*, *A. subulata*, and *A. columnaris* can grow as tall as 50 m, with the record height being 60 m in some individuals of *A. columnaris* on the Isle of Pines and the Loyalty Islands. *A. humboldtensis* and *A. scopulorum* are the shortest species, usually not exceeding 15 m in height. The species can be classified into four different types of habitats, and 11 out of the 13 only occur on ultramafic soils (Table 1.2). This association with ultramafic soils creates a direct conservation problem. The richness of these soils in heavy metals, particularly nickel, means that several species are endangered by mining activities (the major source of the island's export income). Most species also have a restricted or fragmented distribution and also face severe threats from fire (Watt, 1999; Manauté, 2003). The IUCN conservation status of the species is summarised in Table 1.2.

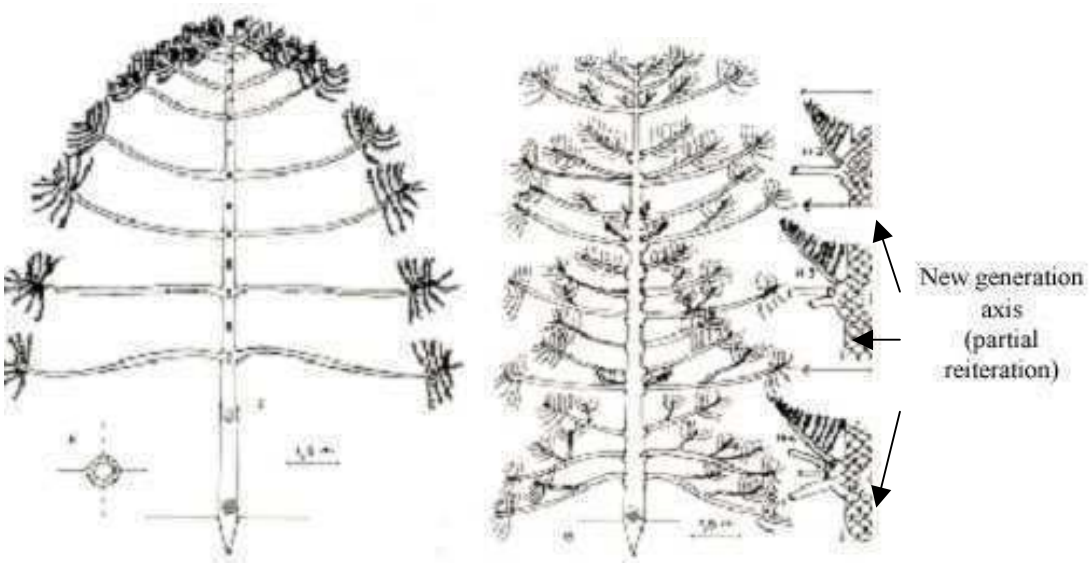


Figure 1.3: Growth models of New Caledonian *Araucaria*: Rauh (on the left), Massart (on the right), taken from Veillon (1980)

Species	Leaf type	Model	Soil type	Altitude range	Vegetation type	CN Status
A. columnaris	Small leaves (<1cm)	Massart	Non ultramafic rocks	0-50m	Coastal limestone forests	NT
A. schmidii		Massart		1400-1628m	Dense evergreen montane rain forest on acid soil	VU
A. bernieri		Massart	Ultramafic rocks	100-700m	evergreen rain forests at low and medium altitude	LR
A. humboltensis		Massart		800-1600m	Dense evergreen montane rain forest	LR
A. luxurians		Massart		0-200m	Rainforest and maquis	EN
A. nemorosa,		Massart		0-50m	Rainforest and maquis	CR
A. scopulorum		Massart		0-600m	Rainforest and maquis	EN
A. subulata		Massart		300-1000m	Evergreen rain forests at low and medium altitude	LR
A. biramulata		Rauh (with alteration)		150-1100m	Evergreen rain forests at low and medium altitude	LR
A. laubenfelsii	Big leaves (>1cm)	Rauh (with alteration)		400-1300m	Rainforest and maquis	LR
A. montana		Rauh (with alteration)		300-1350m	Rainforest and maquis	LR
A. muelleri		Rauh		150-1000m	Rainforest and maquis	LR
A. rulei		Rauh		150-1000m	Rainforest and maquis	EN

Table 1.2: New Caledonian *Araucaria* morphology, altitude, vegetation type and IUCN conservation status based on data in Nasi (1982); Manauté *et al.* (2003); Watt (1999). NT = Not Threatened, LR = Low Risk, VU = Vulnerable, EN = Endangered, CR = critically endangered

4.4 Hypotheses on the origin of *Araucaria* diversity in New Caledonia

The existence of two main architectural models led Nasi (1982) to raise the hypothesis that there were two ancestors for the New Caledonian species (one for each growth model). An alternative hypothesis, namely that the New Caledonian species are monophyletic, was raised based on *rbc*L sequence data (Setoguchi *et al.*, 1998). The Setoguchi *et al.* (1998) phylogeny indicated that the New Caledonian *Araucaria* were a monophyletic group (bootstrap value of 94%). The phylogeny had virtually no resolution among the New Caledonian species, but suggested that the sister species to New Caledonian *Araucaria* was *A. heterophylla* from Norfolk Island (Fig. 5). This is intriguing as Norfolk Island is relatively young (<3 million years old) raising a question as to the age of the New Caledonian species, and suggesting a conflict with a Gondwanan origin.

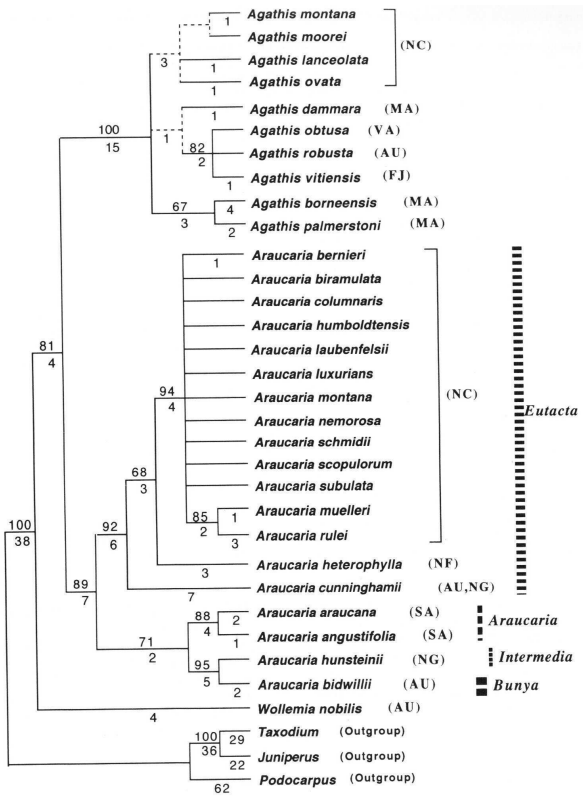


Figure 1.4: Consensus of 20 most parsimonious trees for Araucariaceae based on chloroplast *rbc*L sequences (Setoguchi *et al.*, 1998)

A. heterophylla is a small leaved species, and is the only *Araucaria* species on Norfolk Island. This suggests that the common ancestor of *A. heterophylla* and the New Caledonian species might have had small leaves too. This is supported by the fact that *A. cunninghamii*, the second closest species, also has small leaves. The presence of the large leaved species in New Caledonia would therefore be a convergence as this phenotype occurs in *A. araucana* from Chile. Regardless of which hypothesis on the number of origins of *Araucaria* on New Caledonia is correct (monophyly – Setoguchi *et al.,* 1998; versus two origins – Nasi, 1982), it is pertinent to consider whether the genus' presence on the island is attributable to vicariance or dispersal.

4.4.1 Dispersal attributes.

Araucaria seeds are small and possess wings, and are considered to be dispersed by wind. However, the wings size varies among species and for example it is larger in *A. columnaris* than in *A. scopulorum* (DeLaubenfels, 1972). In normal conditions (no cyclones) seed dispersal range doesn't exceed 1 or 2 km (Chauvin, pers. comm., 2002). However, the period of cone maturity matches the cyclone season in New Caledonia (January to May-June). Cyclones can be extremely strong in the Pacific, and hence there is the possibility for seed to disperse over long distances. However, while long distance dispersal may be possible for *Araucaria,* the lack of the species on other Pacific islands suggests there may be some limitation to this. In this respect it is important to note that some unpublished observations suggest that *Araucaria* seeds do not survive sea-water immersion (McCoy, pers. comm., 2002) and this must limit the potential for overseas seed dispersal. Having said this, the presence of species such as *A. columnaris* growing on numerous coral islets in the New Caledonian archipelago, indicates that at least some dispersal over water is possible, and the proximity of these trees to the sea indicates some level of salt and immersion tolerance.

4.4.2 Vicariance

The affinities of the New Caledonian flora and the flora of the surrounding ancient Gondwanan fragments (Australia, New Guinea, New Zealand) (Morat *et al*., 1994) suggest that much of the floristic composition of the island is most simply attributable to Gondwanan vicariance, although of course this need not necessarily apply to *Araucaria.* One of the key questions when dealing with vicariance hypotheses is whether the island has always remained above sea level since continental fragmentation? The presence of sediments of different ages covering all of New Caledonia, suggest that at one point or another, various parts of the island have been submerged (Picard, 1999). However, this does not exclude the possibility of some refuges remaining above sea level at any one time ensuring survival of a Gondwanan element of the flora. The fossil record in the region is unfortunately poor and insufficient to address vicariance versus dispersal hypotheses (Kershaw and Wagstaff, 2001).

4.4.3 Diversification of the genus in New Caledonia

In understanding the processes that have given rise to the evolution of such high levels of diversity of *Araucaria* on New Caledonia, it is worth considering the conditions under which differentiation could occur. The New Caledonian *Araucaria* represent a large number of closely related wind-pollinated taxa in a small country – how did they diverge in the first place?

Several hypotheses have been raised concerning their diversification. One key environmental variable that has been invoked is the obduction of ultramafic soils that occurred 37 million years ago (mya) and which now support high levels of endemism. Nasi (1982) and Jaffré (1995) have both suggested that this toxic metal-rich soil has promoted speciation events. The general point is that these soils strongly limit the growth of angiosperms, and hence favour the growth of gymnosperms, which are more tolerant of these edaphic conditions. Other key environmental changes that have been invoked as important are variation in sea level and climate changes during the glaciations in the last two million years. Although New Caledonia

was not glaciated during the Pleistocene, it, like other sub-tropical and tropical regions experienced climatic shifts. Pintaud (1999) showed that the variation in the rainfall levels during that period had a major impact on the distribution and diversification of palms. It is possible that *Araucaria* species were also affected.

Although it is not clear whether the ultramafic soils per se were the driving factor responsible for the diversification of New Caledonian *Araucaria* it is noteworthy that 11 of the 13 species occur on ultramafic soils. Of the two species that do not naturally occur on ultramafic soils, one grows on calcareous soils (*A. columnaris*) and one occurs only on non-ultramafic soils on the highest point of the island in the Mont Panie chain (*A. schmidii*). Nasi (1982) suggested that *A. columnaris* has diverged relatively recently with the emergence of the coral reef, and that the divergence of *A. schmidii* occurred prior to the deposition of ultramafic soils. Thus Nasi (1982) effectively proposes that *A. schmidii* is the most basally divergent New Caledonian *Araucaria* taxon.

In addition to major climatic fluctuations and ultramafic soil obduction, other potential factors that might have contributed to the diversification are:

- New Caledonia has a varied topography and steep gradients of climate and altitude.
- The ultramafic soils on the island are not homogeneous, there are in fact several different types of soils among which local adaptation may occur (Picard, 1999).
- The ultramafic soils are not continuously distributed; erosion following the initial obduction event has resulted in a network of ‘ultramafic islands’ of varying degrees of spatial separation.
- Some of the species tend to occur in valleys, separated from other valleys by mountain ridges (physical barriers).
- There is some evidence for inbreeding in New Caledonian *Araucaria* species (C. Kettle, unpublished, 2005).
- Two different kinds of pollen have recently been found in *A. araucana*, a winged type and a non-winged type (R. Mill, pers. comm., 2002). These may be

differentially adapted for long distance versus short distance dispersal. If there is some intra-specific variation in the frequency that either is produced, this may lead to an increased tendency for some populations to undergo differentiation.

- Previous meteorological evidence suggests that glaciation reduces the frequency and/or strengths of cyclones (Hoff and Pask, 1997), this may lead to periods of time in which seed and pollen dispersal efficiency is reduced.

4.5 Problems of species delimitation

Given that many populations of the extant species are geographically and topologically isolated it raises the question as to whether differentiation and speciation in this group is ongoing. The issue of the ongoing diversification is pertinent given the subtle morphological differences between some of the species. The ontogeny of the species are very similar during the first stage of seedling growth. All *Eutacta* species have 4 cotyledons, thin and long needle-like leaves (6-10 mm long x 1 mm wide) and a yellow/green colour. Differentiation occurs more rapidly in some species like *A. rulei* or *A. muelleri* where the leaves gets longer (up to 20 mm) early in development. However, in other species, the morphology remains similar for a few years, and even in adults the lower branches of the trees often keep their juvenile characteristics which are similar among the different species. This can make species identification and delimitation difficult and some doubts have been raised concerning the identification of some populations listed in floras or reports, and more fundamentally, concerning the distinction of some of the 13 species (Jaffré, pers. comm., 2002; Chauvin, pers. comm., 2002). Certainly, even a casual inspection of material in herbaria suggests that identification errors are frequent. This all adds up to an important practical problem for assessing relationships among species or evaluating the conservation status of a species: it is important to know that those species actually exist, and what their distributions are.

4.6 Thesis Objectives

The aim of this thesis is to use molecular data to investigate the evolutionary history and taxonomy of *Araucaria* on New Caledonia, focusing on the following topics, each presented as chapter:

I. What are the phylogenetic relationships among species of *Araucaria*?

Previous morphological studies and molecular phylogenies have respectively suggested multiple and single origins for *Araucaria* on New Caledonia. DNA sequence data will be gathered to test whether the New Caledonian species are monophyletic, and to place the relationships of these species into a broader phylogenetic context.

II. When did the diversity of *Araucaria* on New Caledonia arise, and how does this correlate with the history of the island?

Molecular phylogenetic data obtained in (I) will be examined in the context of the fossil record, geological dates, and estimates of nucleotide substitution rates to apply a molecular clock approach to *Araucaria*. The inferred dates for the diversification of New Caledonian species will then be compared with the known history of the island to evaluate which conditions are associated with the radiation.

III. Can molecular markers clarify the taxonomic status of populations and species of New Caledonian *Araucaria*?

Genetic data will be used to test the homogeneity of selected taxonomic units and to clarify the status of populations whose identification is uncertain. The genetic data will be compared with morphological data and the correlation between the different information sources examined.

IV. An overview of the taxonomy on the New Caledonian *Araucaria* species

The *Araucaria* account in the Flora of New Caledonia is now over 30 years old (DeLaubenfels, 1972), and since its publication, new information has come to light. Various authorities have made many observations, and not all of this has been published. In this chapter I will summarized the information available on the New Caledonian species and update it by adding some information obtained during the three years of the PhD, through field observation, herbarium and lab work, as well as literature reviews.

V. A broad scale investigation into the extent of intra-specific and inter-specifc genetic diversity among the New Caledonian *Araucaria* species

If the New Caledonian *Araucaria* species are still diversifying, then levels of intra-specific differentiation may be of a similar order of magnitude to the extent of inter-specific differences. In this chapter I will screen multi-population samples of all species for genetic markers to evaluate whether this is the case and also establish whether there are hotspots of genetic diversity for *Araucaria* species on New Caledonia. A second aim of this chapter is to understand the scales over which populations of these wind-pollinated species become genetically isolated as part of understanding how speciation may have occurred.

4.7 Summary of issues raised

There is a high level of morphological diversity among New Caledonia *Araucaria* species that is recognised as an important biological radiation. To understand this radiation, the following information is required.

1. A fully resolved phylogeny to allow a reconstruction of the evolutionary history of the genus, with reliably dated nodes. This will allow divergence events to be related to the biotic and abiotic history of New Caledonia, and the morphological and ecological transitions to be placed in context.

2. A clear-cut taxonomy with all species delimitation problems solved including an assessment of the magnitude of intra-specific versus inter-specific divergence. This will ensure that the units of analysis reflect natural biological units and will also contribute towards programmes concerned with their conservation.

4.8 Approaches: an overview of the different methods available

4.8.1 Sources of molecular data for plant taxonomy and phylogeny

Molecular data are increasingly being used in evolutionary biology to supplement observations that were previously restricted to morphological features. The popularity of molecular data is attributable to several features including:

- Environmental and developmental stability
- Practically unlimited number of characters
- Comparable homologous characters
- Range of different regions with differing mutation rates

In addressing a particular biological question, it is important that an appropriate molecular approach is chosen, as the source of DNA used will have an important effect on the results obtained (Page and Holmes, 1998). There are three genomes in plants available for study (nuclear, chloroplast and mitochondrial; Table 1.3.).

Attribute	Nuclear genome	Chloroplast genome	Mitochondrial genome
Structure	Linear chromosomes	Circular chromosome	Internally recombining circular chromosome
Ploidy level	At least diploid (higher plant)	Haploid	Haploid
Inheritance	Biparental	Uniparental Maternal (Angiosperm) Paternal (Araucariaceae)	Uniparental Maternal (Angiosperm) Paternal (Araucariaceae)
Recombination	Present	Absent	Absent
Mutation rate (mtDNA mutation rate at rate 1)	ca 10 x	ca 3.5 x	1 x

Table 1.3: Properties of the three plant genomes modified from Mogensen, 1996 and Ennos *et al.*, 1999

Mitochondrial DNA is found in the cytoplasm of plant cells. It is a haploid circular molecule of about 16 thousand base pairs (kbp) in animals, but is much larger in plants (up to 2200 kbp). It is present in multiple copies in the cells of eukaryotic organisms and is uniparentally inherited (in Araucariaceae mitochondrial DNA is thought to be paternally inherited (Mogensen, 1996)). Closely related taxa may have structural differences in their mitochondrial DNA due to the presence of numerous internal repeats and the capacity of substantial intra-chromosomal recombination that generate subcircles of the master chromosome (Ennos *et al.*, 1999). The study of this organelle genome is made easier by the numerous copies present in one cell, which makes it easy to isolate sufficient DNA from very small amounts of plant material.

Mitochondrial DNA has a very slow substitution rate in plants, which is 3-4 times slower than chloroplast DNA (Table 1.4). Soltis *et al.* (1998) described all of the major problems associated with its uses in plants, including the presence of foreign DNA, large duplications, short dispersed repeats and high level of rearrangements. Furthermore, the very slow substitution rate of mitochondrial DNA

in plant leads to difficulties in detecting sufficient variation at low taxonomic levels. As such it has received relatively little use in plants, which contrasts strongly with the situation in animals where its smaller size, rapid substitution rates, and conserved gene order have made it a useful source of genetic markers (Ennos *et al.* 1999).

Chloroplast DNA (cpDNA) is found in the cytoplasm of plant cells. It is also a haploid circular molecule that is uniparentally inherited as a single gene and is usually about 120-150 kbp. It is considered to be paternally inherited in the Araucariaceae (Mogensen, 1996). CpDNA evolves at an intermediate rate compared to nuclear and mitochondrial genes (Table 1.4). All of the molecules within individuals generally represent a homogenous assemblage, that is, there is limited evidence of heterogeneity in size or structure within a plant. The lack of major structural changes (inversions, transpositions, deletions and insertions) in the chloroplast genome makes it relatively easy to work with in comparative studies. This is true because restriction pattern differences between species usually result from mutations at restriction sites or small insertion/deletions rather than from structural changes. If the latter were frequent it would be difficult, tedious and time consuming to do comparative studies involving a number of taxa. Also, because changes such as large inversions are rare, they may prove to be phylogenetically informative for identifying monophyletic assemblages (Crawford, 1990). CpDNA variation also includes simple sequences repeats (SSRs or microsatellites). These regions are expected to have a high mutation rate due to slippage during replication. As cpDNA is inherited as a unit and not subject to recombination, cpDNA sequences and restriction sites can be readily combined. It can provide useful signal for species delimitation, but it should preferably be used in combination with nuclear markers as it is essentially just giving information on the maternal or paternal lineage.

Nuclear DNA is present in eukaryotic organisms in the nucleus of the cells.

It has a more complex history as each gene is present in at least two copies (more in a polyploid species, and more for duplicated genes) and shows allelic variation.

Nuclear DNA polymorphisms provide virtually unlimited opportunities for studying the mechanisms of evolution. Variability in nuclear DNA is due to recombination, inversions, transpositions, substitutions, insertion/deletion polymorphism, and can show gene-specific variation in rates and histories. The nuclear genome contains a lot of replicated DNA and has a faster rate of evolution than the organelle genomes (perhaps more precisely a greater heterogeneity of mutations rates). One source of nuclear DNA variation is SSRs. These regions are expected to have a high mutation rate due to slippage during replication.

The high rate of evolution of the nuclear genome, as well as it tendency to recombine, leads to some advantages and to some disadvantages. On the one hand the complexity of the genome leads to a range of suitable markers evolving at a range of speeds thus offering the potential of a suitable gene for any given question. On the other hand, problems of gene duplication and high rates of evolution can lead to problems in the determination of homology such as the confounding effects of paralogous genes or homoplastic nucleotide substitutions. Another problem with nuclear DNA is simply one of sampling. Universal primers do not exist for the nuclear genome, and true universal primers for ubiquitous single copy regions are unlikely to ever exist given recombination, changes in gene order among species, and gene duplication.

4.8.2 Choice of the appropriate methods and regions for taxonomic and phylogenetic studies

4.8.2.1 Different tools for different questions

The choice of the molecular methods to use is very important. It will be influenced by the scale of the study and the question to be addressed. It is therefore important to choose markers with an appropriate mutation rate, as this will determine the level of polymorphism retrieved. These choices are constraint by several elements: sequence information already available, work in related species, lab facilities, time and money.

4.8.2.2 Polymerase chain of reaction (PCR)

The development of the polymerase chain of reaction was a major break-through in genome analysis. The principle of PCR is to amplify a section of the genome by using a thermostable DNA polymerase (*Taq* polymerase) and to use the product of the duplication as template for further amplification. Successive cycles of duplication rapidly increased the number of templates available and thus the number of subsequent duplications. The region to amplify is selected by using two flanking primers. Depending on the selectivity of the primers, the portion of genome amplified will be more or less specific (Aert *et al.*, 1997).

Though PCR is a relatively robust technique, some factors can influence the success of the reaction. First the quality of the DNA extracted is important as poor quality DNA or low concentration DNA reduces the number of templates and makes the PCR more sensitive to contamination. Secondly, the nature of the template in terms of G/T richness, the length of the region, and its composition (presence or absence of repeats), will influence the binding with the primer and the efficiency of the DNA polymerase. Finally, the PCR process can be influenced by the conditions of the reaction, like the ionic environment (e.g. $MgCl_2$ or KCl concentrations) or the accuracy of the temperature of the thermal block. This can impact on the amount and specificity of the product.

4.8.2.3 PCR-Sequencing

The principle of PCR-sequencing is to determine the exact succession of nucleotides of an amplified fragment by using labelled dNTPs during the PCR process, and running the product through a automated sequencer. The resulting sequences can be aligned and compared from several individuals, in order to assess degrees of divergence and infer relationships. This method is the most exhaustive way to explore a given genomic region, but it is expensive and time consuming.

4.8.2.4 PCR-Restriction fragment length polymorphism (RFLP)

RFLP is based on the use of restriction enzymes to detect polymorphism (Brettschneider, 1997). A polymorphism in a restriction pattern occurs when (i) a substitution results in the gain or loss of a restriction site, (ii) an insertion or deletion (indels) in the DNA adds or removes a restriction site, or (iii) an indel changes the size of a restriction fragment. In PCR-RFLP, the amplified DNA region is digested by selected restriction enzymes and the restriction fragments are compared among individuals (Edward, 1997; Semerikov *et al.*, 2003; Ziegenhagen *et al.*, 1997).

4.8.2.5 Amplification fragment length polymorphism (AFLP)

AFLP analysis is based on the detection of multiple DNA restriction fragments by PCR amplification. The procedure begins with the restriction digestion of the entire genome with two different restriction enzymes. Amplification of restriction fragments is accomplished by the ligation of double-stranded adapter sequences to the ends of the restriction sites, which serve as “universal” binding sites for primer annealing in PCR. Restriction fragments of a particular DNA can then be amplified with “universal” AFLP primers corresponding to the restriction site and adapter sequence. Because the number of fragments detected this way will be too high to be resolved in any fragment analysis system, e.g. gels, the AFLP primers have at their 3’ end a number of selective bases that extend into the restriction fragment. This results in selective amplification of those fragments in which the primers extension matches the nucleotide flanking the restriction site. The number of selective bases modulates the number of fragments to be amplified. A limit to 50-100 fragments allows detection on denaturing polyacrylamide gels (sequence gels) or capillary based sequences. Restriction fragment patterns generated by the AFLP technique are called AFLP fingerprints. Their frequency is dependent on the sequence polymorphism between the tested DNA samples.

AFLPs are a very powerful DNA marker technique first designed to allow reconstruction of very high-density DNA marker maps (Vos *et al.*, 1995; Vos and Kuiper, 1997). Because of the quantity of markers obtained, its use has been widened

to resolving phylogenetic relationships among closely related species (Despres *et al.*, 2003; Semerikov *et al.*, 2003).

One of the main problems raised by the use of AFLP is the fact that AFLP data can be homoplastic (Despres *et al.*, 2003). The fact that fragments of the same size can be non-homologous cannot be ignored. As well, AFLP fragments are dominant markers, which means heterozygotes cannot be distinguished from the dominant homozygote. Finally each character takes only two states (0 or 1), increasing the risk of parallelism or reversion. One other problem seems to be more directly related to the family studied in the present work. While studying genetic variability in *Wollemia nobilis*, Peakall *et al.* (2003) did not recover any polymorphic markers using the AFLP technique. Other members of the Araucariaceae were also scanned, but little variability was retrieved, leading to the conclusion that the Araucariaceae might have very low levels of genetic variation.

Preliminary efforts were made to get this technique to work in the current study. Six species (*Araucaria rulei, A. subulata, A. columnaris, A. bernieri, A. schmidii*) were scanned using 64 different primers combination, but only three polymorphic markers were retrieved. Given these poor results and the fact that AFLPs are time consuming and relatively expensive, the method was discarded.

4.8.2.6 Simple sequence repeats (SSRs) = microsatellites

Microsatellite DNA consists of small repeat units, generally less than six nucleotides that generate repeating regions up to 100-250 bp. These regions are highly interspersed throughout eukaryotic genomes, which are thought to have a microsatellite sequence distributed once every 10 Kb (Ciofi *et al.*, 1998). Microsatellite variation is identified by PCR amplification of DNA using primer pairs which flank the microsatellite repeat. The size of the amplified fragment is then obtained by running it on a polyacrylamide gel or capillary sequencer. These methods can identify variants that differ by just one nucleotide in length.

The high level of polymorphism of microsatellite markers makes them a useful tool for biodiversity studies.

a. Nuclear microsatellite loci are usually highly polymorphic with alleles varying in the number of repeat units; they are codominant, considered selectively neutral and inherited in a Mendelian fashion.

b. Chloroplast microsatellites are uniparentally inherited. Some species have maternal inheritance of the chloroplast, others paternal. As cpDNA is a non-recombinant, all cpSSR loci are linked which enable them to be combined to retrieve haplotypes, composed of the combination of alleles found at each cpSSR locus.

Several authors (e.g. Navascues and Emerson, 2005; Provan *et al.*, 2004; Petit *et al.*, 2005) have highlighted the increased role of SSRs in the study of plant genetic diversity. Uniparentally transmitted markers have been shown to be highly informative for inferring population history and have several advantages compared with biparentally inherited markers. Multiple microsatellites on uniparentally-inherited chromosomes allows the study of haplotype genealogies uncomplicated by the problem of recombination (Gomez *et al.*, 2005). Combined analysis of uniparentally and biparentally transmitted microsatellites can provide additional insight into several population genetic parameters such as population structure and gene flow. However, as levels of divergence increase, concerns of homoplasy increase and this is likely to be a problem in phylogenetic studies (Provan *et al.* 2001).

From a practical perspective, the reproducibility of the technique is high (Jones *et al.*, 1997) and relatively low cost once the flanking primers are obtained.

However, Mueller and Wolfenbarger (1999) stressed that nuclear microsatellite primers developed for one species can rarely be used beyond the very closest relatives and therefore need to be developed *de novo* for each species or group of species.

4.8.3 Approach for each chapter

The study of the different aspect of the evolution of New Caledonian *Araucaria* has involved several molecular approaches. To establish a phylogenetic framework and to apply molecular clock approaches I have used sequencing of two cpDNA regions. For species delimitation I have used a combination of cpSSRs and morphological data. To undertake an assessment of broad scale genetic structure I have used cpSSRs.

2.1 Introduction:

Islands often contain large numbers of endemic species, and these are typically unevenly concentrated into particularly species-rich groups (Levin, 2000). Features of islands that are considered important in promoting endemism and species radiations include geographical isolation, a diverse set of local niches, and reduced competition compared to continental ecosystems. Recent radiations leading to high levels of island endemism have been well documented in oceanic island systems including Hawaii, the Canary Islands, and the Galapágos (reviewed in Baldwin *et al.* 1998). These island systems are all derived from volcanic activity, and thus the present day biota has been derived from over-water dispersal, colonisation of open habitat, and subsequent radiation and diversification.

In contrast to oceanic islands, those formed by continental fragmentation do not necessarily go through a phase involving large amounts of empty habitat. A land mass can fragment into constituent parts, and the biota of an island formed in such a fashion could diverge via conventional allopatric speciation. In this respect it is tempting to think of oceanic islands and continental islands as being fundamentally different entities. However, this may well be an oversimplification. Continental islands can themselves be subject to major disturbance, which may wipe out the existing vegetation over large areas. These areas may then become occupied by over-water dispersal, colonisation of open habitat, and subsequent radiation and diversification in much the same fashion as oceanic islands. This point is supported by the observation that species diversifications endemic to large continental islands such as Madagascar often show the same phylogenetic structure as those endemic to smaller oceanic islands (Lavin *et al.* 2004).

2.1.1 New Caledonia: a hot spot of *Araucaria* evolution.

New Caledonia is a Pacific Ocean island with high levels of plant endemism (77.3%). Geologically it has a continental origin, and it and New Zealand became isolated from a larger Gondwanan fragment containing Australia and Antarctica some 80 mya (Sanmartín & Ronquist, 2004). One particularly charismatic element of the New Caledonian flora is the island's 13 *Araucaria* species.

Araucaria was once a very widespread genus present in both hemispheres and it has an ancient fossil record. It is nowadays restricted to the southern hemisphere, on lands that previously formed the Gondwanan supercontinent. Six of the 19 extant species have a scattered distribution being found in Brazil (*Araucaria angustifolia* (Bertol.) Kuntze), Chile (*A. araucana* (Molina) K. Koch), Norfolk Island (*A. heterophylla* (Salisb.) Franco), Australia (*A. bidwillii* Hook, *A cunninghamii* Aiton ex D. Don), and New Guinea (*A. hunsteinii* K. Schum., *A. cunninghamii* var. *papuana* Lauterb.). The 13 remaining species of *Araucaria* are restricted to New Caledonia and all belong to section *Eutacta* (Link) Endl. (which also includes *A. heterophylla* and *A. cunninghamii*); the other species being arranged into three other sections (*A. araucana* and *A. angustifolia* in section *Araucaria*, *A. bidwillii* in section *Bunya* Wilde & Eames, and *A. hunsteinii* in section *Intermedia* C. T. White).

Araucaria in many ways represents a model group to test a series of important questions of plant evolution and biogeography. It is a small manageable genus with a good fossil record and a widespread distribution. The high diversity of species (13 out of 19) on a small continental island raises questions as to the age of these species and the timing and nature of their origins. In this chapter I explore the phylogenetic systematics of the group to provide a framework for subsequent molecular clock analyses and evolutionary inference in Chapter 3.

2.1.2 Previous work on New Caledonian *Araucaria*

Nasi (1982) inferred relationships between New Caledonian *Araucaria* species based on his own observations coupled with the previous morphological studies of Veillon

(1980) and DeLaubenfels (1972). His first conclusion was that the existence of two main architectural types in New Caledonian species might reflect two separate ancestors (e.g. two lineages colonising New Caledonia). Large leaved species typically show the Rauh growth form (orthotropic branches, no reiteration – classic examples of this model show a candelabra habit), whereas species with smaller leaves typically show the Massart model (plagiotropic branches with partial reiteration – these species show a more columnar habit). In order to explore relationships among species, Nasi (1982) further made comparisons based on three criteria: floristic affinity, architectural affinity, and ecological affinity, and a summary of these are shown in Table 2.1. What is immediately apparent from this informal analysis is the lack of correlation between similarities with respect to the different criteria. For example *A. humboldtensis* is considered ecologically similar to *A. muelleri* and *A. laubenfelsii,* but is more dissimilar with regards to floristic or architectural affinities. Likewise, *A. muelleri* has a very close match in architecture with *A. rulei,* but shows greater floristic differences. Whilst these data are useful in summarising known information about the species, they do not form a suitable dataset for inferring taxonomic or phylogenetic relationships.

A more formal analysis of relationships was carried out by Setoguchi *et al.* (1998) using *rbc*L sequence data. The resulting phylogenetic trees suggested that the New Caledonian *Araucaria* represent a monophyletic group, but there was little resolution among the New Caledonian species (other than the recovery of a weakly supported clade containing *A. rulei* and *A. muelleri*). The study suggested that the closest species to New Caledonian *Araucaria* was *A. heterophylla* from Norfolk Island (Fig. 2.1).

The study of Setoguchi *et al.* (1998) represents the most complete study available on the New Caledonian *Araucaria*. However, in addition to the lack of resolution among the New Caledonian species, there are some additional problems. Firstly, no precise locality details are available for several of the samples, which were based on cultivated material; there is thus some uncertainty over the source material used in the phylogeny. Secondly, only a single specimen was used per species, and hence there is a concern that even the limited resolution that was obtained may not hold up to further sampling.

The overall aim of the present study is to undertake further phylogenetic analysis on the New Caledonian *Araucaria*, based on material of known wild origin and multiple samples per species. To place the results of the New Caledonian species into a broader context, samples from all other species in the genus, as well as representatives from other genera in the family have been included in phylogenetic analyses.

Species	Growth model	Type of leaves	Type of soil	Altitude range	Type of vegetation
A. columnaris	Massart	Small leaves (thin needle)	Non ultramafic rocks	0-50m	Forest on calcareous soil
A. schmidii	Massart			1400-1628m	Rainforest on acid soil
A. humboltensis	Massart (alterated)		Ultramafic rocks	800-1600m	Rainforest
A. bernieri	Massart			100-700m	Rainforest
A. scopulorum	Massart			0-600m	Rainforest and maquis
A. nemorosa,	Massart (alterated)			0-50m	Rainforest and maquis
A. luxurians	Massart			0-200m	Rainforest and maquis
A. subulata	Rauh			300-1000m	Rainforest
A. biramulata	Rauh (alterated)	Big leaves (broader scale)		150-1100m	Rainforest
A. montana	Rauh (alterated)			300-1350m	Rainforest and maquis
A. laubenfelsii	Rauh (alterated)			400-1300m	Rainforest and maquis
A. rulei,	Rauh			150-1000m	Rainforest and maquis
A. muelleri	Massart			150-1000m	Rainforest and maquis

Table 2.1: New Caledonian *Araucaria* species, their growth form, leaf characters and habitat preferences, data taken from Nasi (1998), Manauté *et al.* (2003), and personal observations

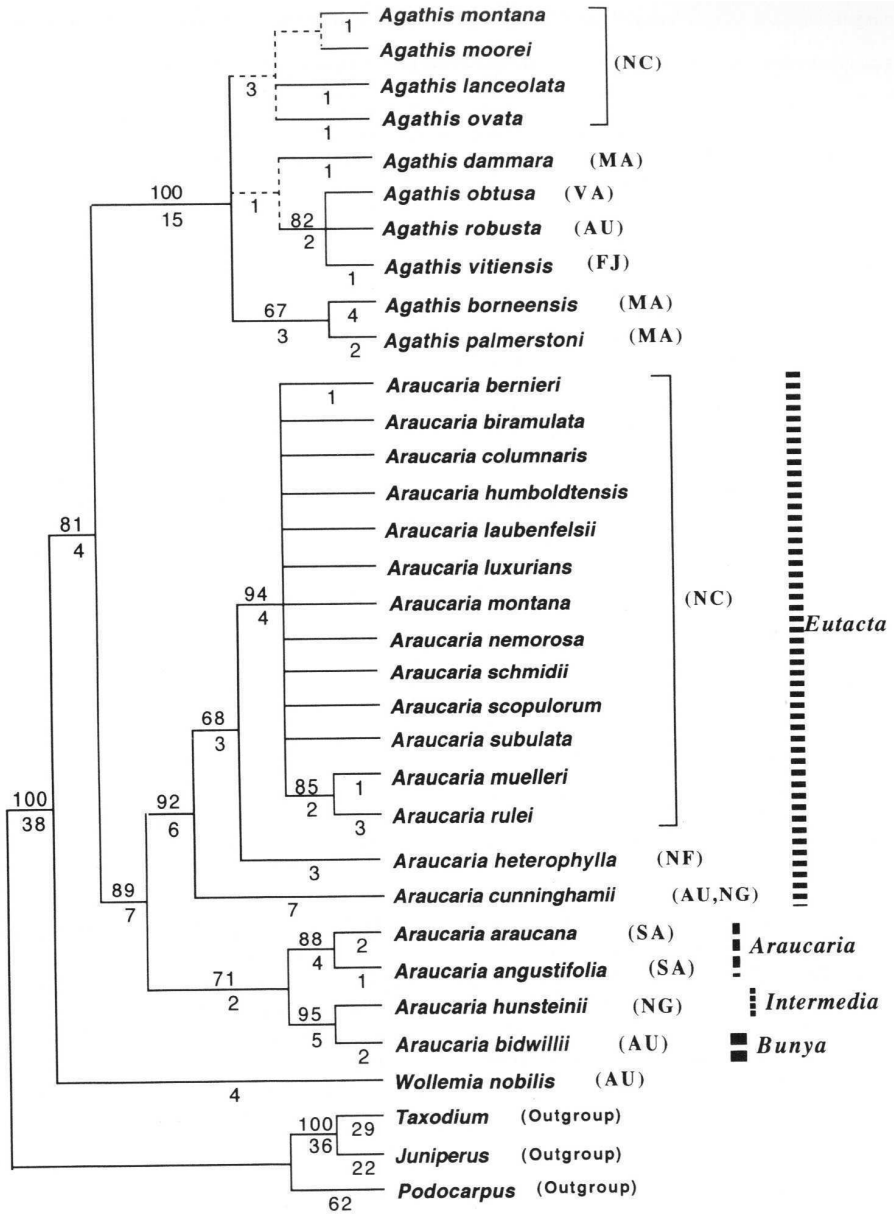


Fig. 2.1: Consensus tree of 20 equally most parsimonious trees for the Araucariaceae based on *cp*DNA *rbc*L sequences, taken from Setoguchi *et al.* (1998).

2.2 Material and methods

2.2.1 Materials: field sampling

Plant material of New Caledonian species was collected during three successive field seasons in December 2001, 2002 and 2003 (Fig. 2.2, Table 2.2). The sampling season was chosen to match the coning season in order to ensure that cones were present to confirm species identification. Populations were located using the flora (DeLaubenfels, 1972), and local knowledge. Species were determined from herbarium and field observations using both the key in the flora (DeLaubenfels, 1972) and comparison to other herbarium material. A total of 23 *Araucaria* populations were sampled, including all 13 species and two populations per species when possible (only one population of *A. humboldtensis*, *A. subulata,* and *A. schmidii* were obtained). For each individual 6 to 10 leaves were collected and pictures of the tree's shape, bark and leaves were taken. The leaf material was dried and preserved in silica gel. Herbarium specimens, including adult foliage and juvenile foliage (when possible) were made for most of the populations. Material sources for non-New Caledonian species and outgroup species are shown in Table 2.3. One sample per species was obtained.

After examining the phylogenetic position of Araucariaceae in the context of other conifers (based on the studies of Setoguchi *et al.,* 1998 and Quinn *et al.*, 2002), a representative of the sister family (Podocarpaceae) and the two sister genera to *Araucaria* (*Agathis* and *Wollemia*) were selected as outgroups (Table 2.3). The outgroups selected were *Wollemia nobilis* W. G. Jones, K. D. Hill and J. M. Allen, *Agathis lanceolata* Lindley ex Warb., *Agathis montana* Laubenf. (Araucariaceae) and *Dacrydium araucarioides* Brongniart and Grisebach (Podocarpaceae).

2.2.2 Approaches

The New Caledonian *Araucaria* show a high level of morphological variation and overlapping characters. Given the time consuming nature and practical difficulties of obtaining robust phylogenetic hypotheses from morphological data (Scotland *et al.*, 2003), I have not pursued morphological characters as an approach. I have instead focused on the search for more sequence-based characters.

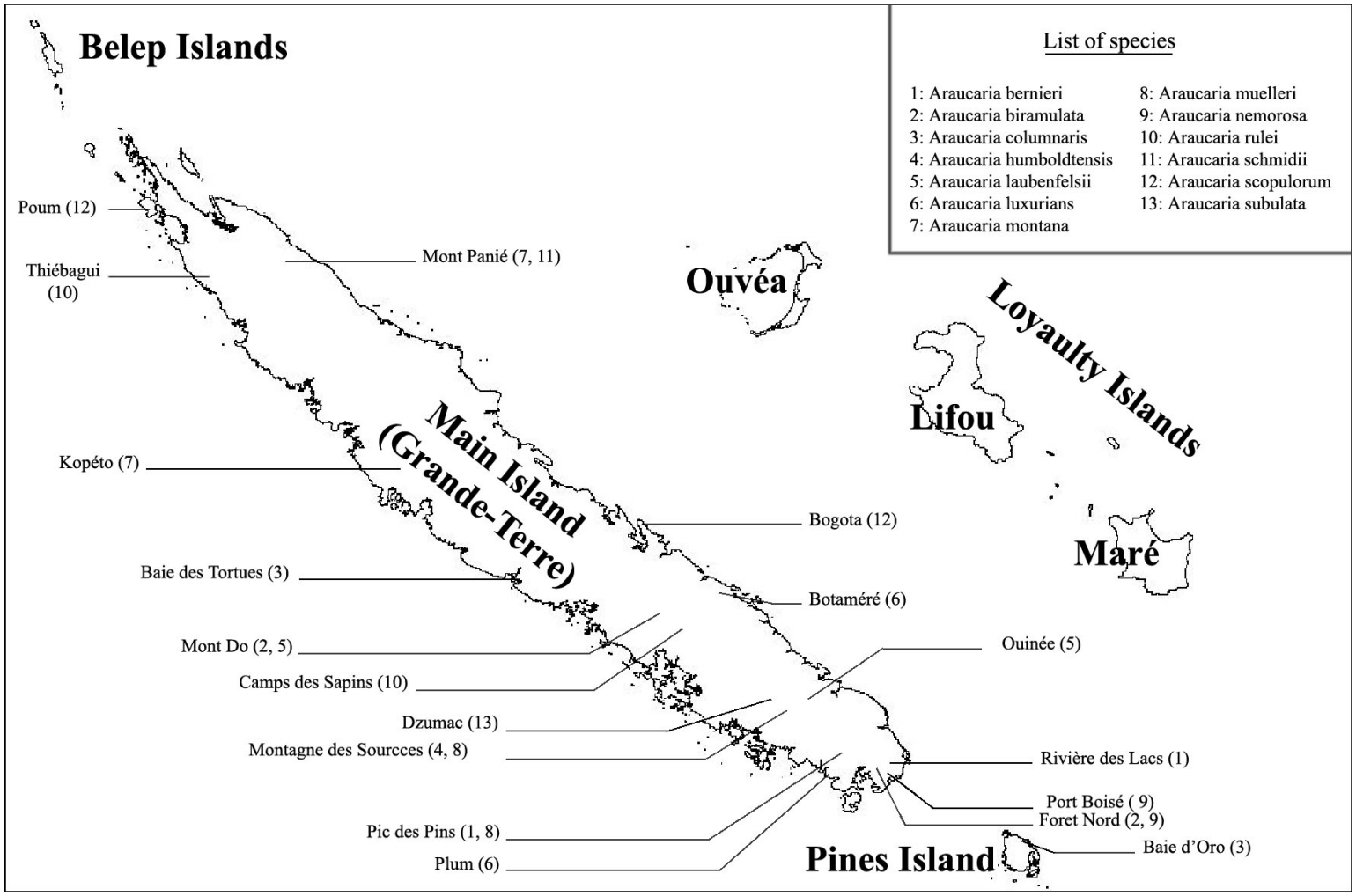


Fig. 2.2: Map of the location of the New Caledonian *Araucaria* populations sampled for the molecular phylogenetic analysis. Numbers by localities relates to the species collected at that locality, see legend for numerical codes

Species	*A. schmidii*	*A. columnaris*	*A. subulata*	*A. humboltensis*	*A. bernieri*	*A. scopulorum*	*A. nemorosa*	*A. luxurians*	*A. biramulata*	*A. montana*	*A. laubenfelsii*	*A. rulei*
A. columnaris	424											
A. subulata	414	424										
A. humboltensis	434	434	433									
A. bernieri	414	424	412	413								
A. scopulorum	424	424	423	433	423							
A. nemorosa	424	424	424	434	424	424						
A. luxurians	424	414	424	434	424	424	324					
A. biramulata	444	444	443	443	443	443	444	144				
A. montana	444	444	443	432	443	443	444	244	313			
A. laubenfelsii	444	444	443	431	443	443	344	144	223	211		
A. rulei	444	444	444	434	444	444	444	344	334	334	234	
A. muelleri	444	444	443	431	443	443	344	244	233	233	132	424

Table 2.2: Levels of affinity between the New Caledonian *Araucaria* species, based on data in Nasi (1982). The numbers represent (in order): floristic affinity, architectural affinity, and ecological affinity. 1=very strong, 2=strong, 3=low, 4=very low

Species	**Locality**	Abbreviation	Collector	Collector number	GPS	
A. bernieri	Riviere des Lacs	RDL	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	4009	S22°09.1937'	E166°35.2861'
A. bernieri	Pic des Pins	PDP	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	677	S 22°15'	E 166°49'
A. biramulata	Foret Nord	FND	Kettle C. J., Kranitz M. L.	100	S22°16.8053'	E166°53.5986'
A. biramulata	Mt Do	MDO	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	4082	S21°45.4344'	E166°00.0059'
A. columnaris	Ile Des Pins	IDP	Kettle C. J.	840	S22°34.9942'	E167°31.3994'
A. columnaris	Baie Des Tortues	BDT	Kettle C. J., Kranitz M. L.	22	545346.57	7610616.74
A. humboldtensis	Mt. Des Sources	MDS1	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	2001	S22°06.5528'	E166°36.0375'
A. humboldtensis	Mt. Des Sources	MDS2	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	2002	S22°06.5570'	E166°36.0348'
A. laubenfelsii	Mt Do	MDO	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	4050	S21°45.1489'	E165°49.'
A. laubenfelsii	Mine Bokaine	MBK	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	4132	S21°29.2593'	E165°53.5876'
A. luxurians	la Foa (Col d'Amieu)	LFA	Kettle C. J., Kranitz M. L.	63	S21°34.8555'	E165°49.5243'
A. luxurians	Plum	PLU	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	930	S 22°18'2155	E 166°40'2199
A. montana	Kopeto	KOP	Kettle C. J., Kranitz M. L.	49	S21°09.8671'	E165°02.1548'
A. montana	Mt Panie	MPA	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	4243	S20°33.4270'	E164°47.0274'
A. muelleri	Mt. Des Sources	MDS	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	3000	S22°07.4753'	E166°36.1668'
A. muelleri	Pic des Pins	PDP	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	635	S 22°09'2480	E166°35'3188
A. nemorosa	Port Boise	PBS	Kettle C. J., Kranitz M. L.	420	S22°21.5192'	E166°57.2949'
A. nemorosa	Foret Nord	FND	Kettle C. J., Kranitz M. L.	93	S22°19.4862'	E166°54.8365'
A. rulei	Camp Des Sapins	CDS	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	312	S21°46'12	E166°11'05
A. rulei	Thiebagui	THI	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	38	S20°28'39	E164°13'39
A.scopulorum	Poum	POU	Kettle C. J., Kranitz M. L.	12	S20°15.2670'	E164°02.1622'
A.scopulorum	Bogota	BOG	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	278	S 22°36'26	E 166°13'59
A. schmidii	Mont Panie	MPA1	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	4221	S20°35.2378'	E164°46.1209'
A. schmidii	Mont Panie	MPA2	Gardner M. F. , Hollingsworth M. L., Hollingsworth, P. M., Kettle, C. J. , Kranitz, M. & Thomas P.	4224	S20°35.2378'	E164°46.1209'
A. subulata	Dzumac	DZU1	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	990	S22°01.5882'	E166°28.4823'
A. subulata	Dzumac	DZU2	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	679	S22°01.5882'	E166°28.4823'

Table 2.3: Accession details for New Caledonian species used for the molecular phylogenetic study

Ideally, phylogenetic analysis should include information from both the nuclear and organelle genomes (Wendel & Doyle, 1998). Preliminary work was undertaken to develop AFLPs and nuclear ribosomal internal transcribed spacer (ITS) sequencing to provide information from the nuclear genome. However, the generation of AFLP profiles was unsuccessful (Chapter 1), and efforts to amplify single nuclear ITS products amenable to phylogenetic analysis was also unsuccessful (multiple products were recurrently amplified which would lead to paralogy issues in phylogeny reconstruction). Instead, I focused my efforts on sequencing non-coding chloroplast regions. These should be under less functional constraints than protein encoding regions like *rbc*L, and hence should be more variable. The starting point for this was the large single copy region (LSC), which is slightly less conserved than the rest of the chloroplast genome (Clegg *et al.*, 1994).

2.2.2.1 DNA extraction

DNA was extracted from 0.5g of silica dried leaf material using Plant DNeasy kit (Qiagen, UK). Leaf material was placed in a 1.5 ml eppendorf tube and frozen by immersion in liquid nitrogen. DNA was extracted following the manufacturer's instructions using all the steps.

2.2.2.2 Choice of cpDNA primers

In order to study sequences from the large single copy region, 13 primers pairs were tested: 12 described by Grivet *et al.* (2001) and 1 from Sang *et al.* (1997). As these were developed mainly on angiosperms, the first PCR attempt was conducted with the lowest annealing temperature in the ranges suggested in those publications. When double bands were obtained, temperature gradients, going from the lowest to the highest suggested in Grivet *et al.* (2001), were tested to try to obtain high quality single products (Table 2.4). Based on preliminary sequence data, two regions provided the vast majority of variable sites (*trn*S-*trn*FM and *psb*A-*trn*H) and these were sequenced for the full analyses (methodology described below). Other regions

investigated which were less informative, or presented technical difficulties (Table 2.5) are not discussed further.

Species	Origin	Source	BG Base accession number
A. araucana	Wild, Province de Malleco, Chile	Bekessy, Sarah. April 1999	19990741
A. angustifolia	Wild, Brazil	Gardner, Martin	GAR521 AM001534
A. bidwillii	Cultivated, Australia	Liverpool garden festival	19841690
A. cunninghamii	Wild, Queensland, Australia	CSIRO, Canberra	19762123
A. heterophylla	Cultivated		19885028
A. hunsteinii	Wild, Morobe, Papua New Guinea	Woods, P.	19623223
Agathis montana	Wild, New Caledonia	Gardner, Martin	CAGNC71 AM000467
Agathis lanceolata	Wild, New Caledonia	Gardner, Martin	CAGNC24 AM000455
Dacrydium araucarioides	Wild, New Caledonia	Gardner, Martin	CAGNC35 AM000447

Table 2.4: Collection/source data for non-New Caledonian *Araucaria* and outgroup taxa for the molecular phylogenetic study.

Region	**Size (bp)**	**Ta (°C)**	**Elongation time (Minutes)**	**PCR**	**Sequence obtained**	**Number of parsimony informative sites**	**Species tested ****
*trn*Q-*trn*S	1303	47	3	Good	Some (reverse only)	5	1,2,3,4,5,6,7, 8,9,10,12,13, 14,17,18
*trn*S-*trn*R	1814	47-55 *	3	Good	Some (Forward only)	1	1,2,3,6,7,8,9, 10,12,14,17
*ccmp4*_L-*atp*H	1274	47	2	Multi band or nothing	-	-	3,7,12,14,17
*atp*H-*atp*I	1228	47	3	Good	-	-	1,3,8,12
*rpoC*2-*rpoC*1	1879	47	3	Good	-	-	1,2,3,8,9,12
*rpoC*1-*rpo*B	1884	47	3	Multi band	-	-	2,3,9,12
*trn*S-*trn*Fm	1254	47.5	5	Good	Good	44	1 to 23
*trn*S-*trn*T	1386	57.5	2	-	-	-	1,3,8,12
*trn*T-*trn*F	1754	57.5	2	-	-	-	1,3,8,12
*psb*B-*psb*B	1512	47-51 *	3	Good	Good	None	3,6,7,8,9,10,14, 17
*pet*B-*pet*D	1618	47-55 *	3	Dbl band	-	-	1,2,3,8,10,14,17
*rps*8-*rpl*16	1162	46	2	Dbl band	-	-	3,7,12,14,17
*psb*A-*trn*H	1208	47.5	3	Good	Good	49	1 to 23

Table 2.5. Chloroplast regions, reaction conditions and results obtained in primer testing.

*Gradient tested, ** Species tested: *1 Araucaria bernieri, 2 Araucaria biramulata, 3 Araucaria columnaris, 4 Araucaria humboldtensis, 5 Araucaria laubenfelsii, 6 Araucaria luxurians, 7 Araucaria montana, 8 Araucaria muelleri, 9 Araucaria nemorosa, 10 Araucaria rulei, 11 Araucaria schmidii, 12 Araucaria scopulorum, 13 Araucaria subulata, 14 Araucaria angustifolia, 15 Araucaria araucana, 16 Araucaria bidwilli, 17 Araucaria cunninghamii, 18 Araucaria heterophylla, 19 Araucaria hunsteinii, 20 Agathis lanceolata, 21 Agathis montana, 22 Wollemia nobilis, 23 Podocarpus macrophyllus*

2.2.2.3 DNA Sequencing

- Polymerase chain reaction

For the polymerase chain reaction (PCR), 2 µl of DNA were added in a 50 µl PCR containing 5µl of 10X NH_4 buffer, 5µl of 2mM dNTPs, 2.5µl of 50mM $MgCl_2$, 1.5µl of each 10µM primer, 1.25units of Biotaq polymerase (Bioline, UK) and 32.5µl of distilled water.

The amplifications were performed in a MJ Research PTC-200 Thermal Cycler with a first denaturising step of 4 min at 94 ºC, followed by 30 cycles [45s of denaturing at 94ºC, 45s of annealing (Ta shown in Table 2.4) and 1-4 min of extension at 72 ºC, with a final extension step of 72 ºC for 10 min (Grivet *et al.*, 2001). PCR products were purified using the Qiaquick PCR purification kit (Qiagen, UK) according to the manufacturers' instructions.

- Sequencing reaction

Sequencing was performed in both directions using the same forward and reverse primers as the PCR. For the sequencing reaction, 1 µl of DNA was combined in a 10 µl PCR containing 4µl of Quickstart DTCS mix (Beckman Coulter, UK), 1 µl of 10 µM primer and 4 µl of distilled water. The PCR conditions were as follows: 35 cycles of [20 sec at 96°C, 20 sec at 50°C, 4min at 60°C].

- Sequencing PCR purification

For each reaction, 10 µl of distilled water was added to the 10 µl PCR product, which was then transferred to a fresh 0.5 ml tube containing 4 µl of "stop solution" (1.5M NaOAc + 50 mM EDTA) and 1 µl of 20mg/mL glycogen. 60 µl of 100% cold (-20ºC) ethanol was then added to each reaction, mixed throughly centrifuged in a microcentrifuge (~13 000 rpm) at 4 ºC for 15 mins to precipitate the DNA. The supernatant was removed, and 200 µl of cold ethanol (70%) were added to wash the pellet then the tubes were centrifuged in a microcentrifuge (~13000 rpm) for 5 mins. The ethanol wash was repeated a second time. The pellet was dried in a vacuum

centrifuge for 2-5 mins and resuspended in 40 µl of Sample Loading Solution (Beckman Coulter, UK).

- Sequencing electrophoresis, trace analyses, and matrix assembly

Sequences were run on a Beckman CEQ8000 sequencer and analysed using the Default Analysis parameters from the Analysis module of the CEQ8000 software version 8.0. Pre-peak reduction was applied when enzyme slippage occured. Analysed sequences were exported into Sequencher software version 4.5 for automated alignment. The alignment was then checked manually. The completed matrix was saved as a Nexus file. Gaps inferred from the sequence alignment were coded according to the methods described by Simmons et al. (2000). Gaps sharing 5' and 3' ends were coded as absence or presence of characters.

2.2.2.4 Data analysis

Maximum parsimony analyses were performed with Paup (Swofford, 2000) with heuristic searches. The tree bisection reconnection (TBR) branch-swapping algorithm was used alongside MULPARS and COLLAPSE options (collapse branch if minimum length is 0). Stability of the cladograms was then tested with the Goloboff fit criterion (with k=0, 2, 4, and 8), which allows individual down-weighting of noisy characters (Wenzel, 2002). Optimisation in the analysis was performed using Accelerated transformation (ACCTRAN). Bootstrap support measures were obtained by 1000 replicates of "Fast-Bootstraps". The consistency index and retention index were obtained using the "describe tree" option in Paup (Swofford, 2000).

In order to investigate whether different methods of analyses would result in different topologies, a maximum likelihood analysis was also carried out. Maximum parsimony is a method in which the optimal (most parsimonious) tree will be the tree with the smallest number of mutational changes (Doyle *et al*., 2001). On the other hand, maximum likelihood is a model-based method and uses a model of nucleotide substitution to calculate the likelihood of the observed data given a model of

evolution. Settings were obtained by running Modeltest 3.06 (Posada and Krandall, 1998) on the matrix. The HKY+G model was selected. In this model, base frequencies were set to (Lset Base) = (0.3080 0.1872 0.1854) and the Ti/Tv ratio set to 0.8610. The analysis was then run using Paup (Swofford 2000). Boostrap support measures were obtained by running 500 replicates of "Fast-Boostraps".

Datasets were analysed separately and then combined using the "total evidence" approach (Wenzel, 2002), which states that the greater number of characters that are used in an analysis, the higher the likelihood of the true tree being recovered, or of increased support being found for specific relationship within the tree. Moreover, because the chloroplast genome is uniparentally inherited as a unit and not subject to recombination, multiple cpDNA sequences can be readily combined (Soltis *et al*., 2000).

2.3 Results

2.3.1 Sequence characteristics

Both forward and reverse sequencing of the *trn*S-*trn*Fm region gave high quality sequence reads and alignment was straightforward. Because all sequences did not start at the same point and were not the same length, sequences were trimmed for the final analysis. The region of *trn*S-*trn*Fm analysed was 928 base pair long; 866 characters were constant, 18 parsimony-uninformative and 44 were parsimony-informative. Three microsatellite motifs were present in the sequence. One was a multi-A repeat microsatellite which ranged from 7 to 11 repeat units, one was a multi-C repeat microsatellite ranging from 8-11 repeat units, and finally one was a AT repeat microsatellite ranging from 6 to 8 repeat units. A complete matrix for the 19 species of *Araucaria* was obtained, as well as the four outgroup species. In total, 36 accessions were represented in the analysis.

For *psb*A-*trn*H, the forward sequencing reaction gave high quality reads, but the reverse reads were of lower quality and often unreadable. Therefore, just the forward sequences (e.g. the 5' end) were used. The sequences trimmed for the final

analysis were 544 base pairs long; 473 characters were constant, 22 parsimony-uninformative and 49 were parsimony-informative. Thus despite the small fragment size, some phylogenetically informative data was retrieved, among which was a 13 base pair minisatellite motif (CTAAATCTAGACT) which was present in between 0 and 6 copies. A complete matrix for 19 species of *Araucaria*, as well as *Wollemia nobilis* and *Agathis lanceolata* and *Agathis montana* was obtained. In total 35 accessions were represented in the analysis. Due to large differences between *Dacrydium araucarioides* and the rest of the sequences, alignment was not possible. Therefore, the two sister genera, *Agathis* and *Wollemia*, were used as the sole outgroups for analyses involving this region.

The combined sequence dataset (rooted on *Agathis* and *Wollemia*) was 1472 base pairs long; 1339 characters were constant, 40 parsimony-uninformative and 93 were parsimony-informative.

2.3.2 Parsimony

2.3.2.1 *trn*S-*trn*Fm

22 most parsimonious trees were obtained with a tree length of 69 steps. The consistency index (CI) and retention index (RI) were CI=0.96 and RI=0.98. The topology within the ingroup was insensitive to choice or combination of outgroups. The strict consensus tree is shown in Fig. 2.3. The genus *Araucaria* resolved as monophyletic (96% bootstrap support, bs), sister to a clade including *Agathis* and *Wollemia* (82% bs). Section *Araucaria* resolved as monophyletic (69% bs) sister to a well-supported clade (98% bs) containing the two monotypic (based on extant species) sections *Bunya* and *Intermedia*. This group collectively was monophyletic (99% bs) and sister to a strongly monophyletic section *Eutacta* (100% bs). Norfolk Island pine (*A. heterophylla*) and the New Caledonian taxa were resolved as a monophyletic group (87% bs). Within New Caledonia, the majority of the accessions formed a polytomy, but a single clade of the coastal taxa (*A. nemorosa*, *A. luxurians* and *A. columnaris*) was resolved, albeit with relatively weak bootstrap support (74%).

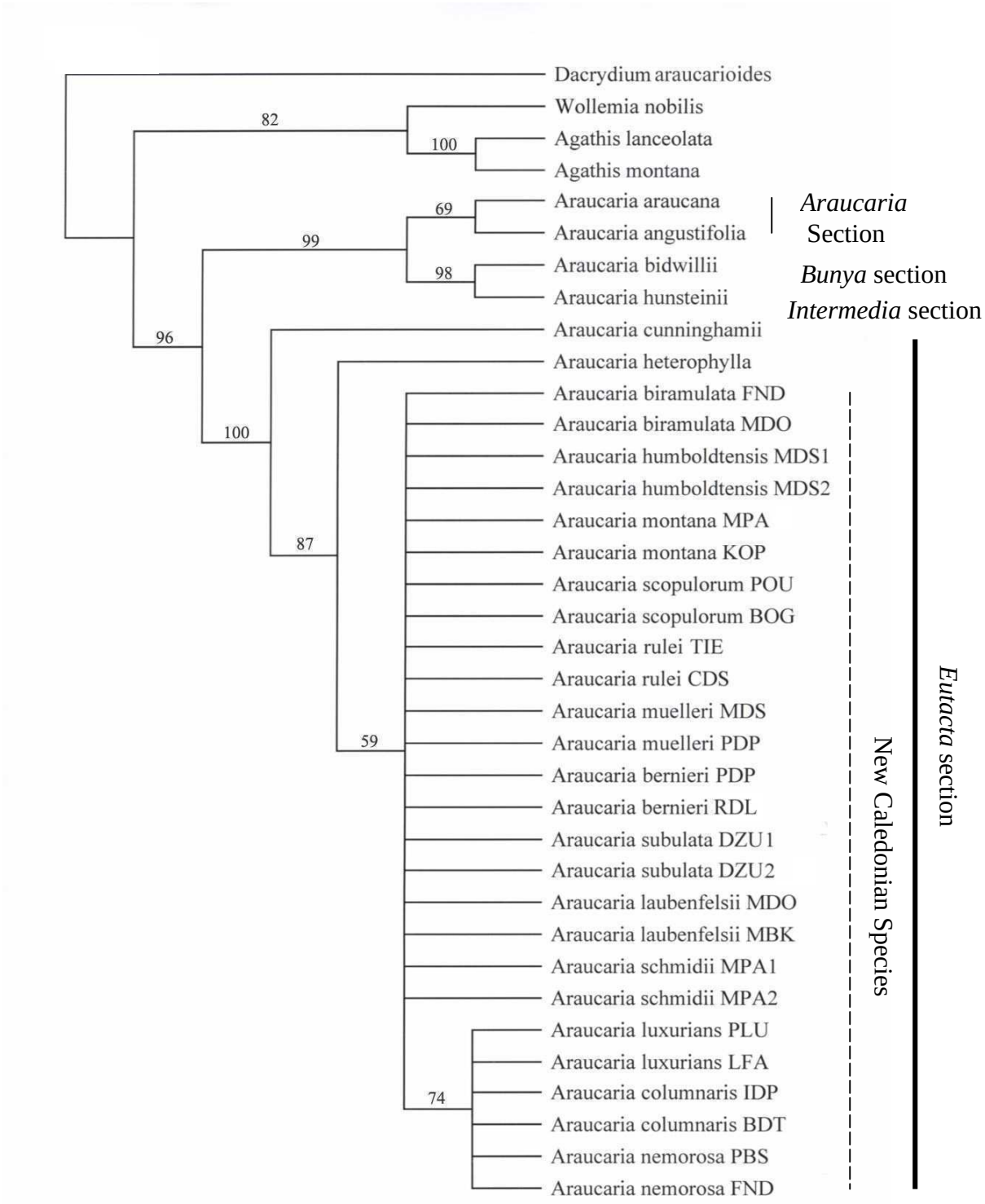


Fig. 2.3: Consensus tree of the 22 equally most parsimonious trees obtained for *Araucaria* based on cpDNA *trn*S-*trn*FM sequences. Length = 69 steps, CI = 0.961 (excluding uninformative characters), RI = 0.984. A percentage of the 1000 bootstrap values (>50%) is given for each node

2.3.2.2 *psb*A-*trn*H

Ten most parsimonious trees were obtained with a tree length of 87 steps, the CI=0.87 and the RI=0.92. When only *Agathis* is specified as the outgroup, *Wollemia* is nested inside *Araucaria* at the base of section *Eutacta* (not shown). When *Wollemia nobilis* is defined as the outgroup (Fig. 2.4), *Agathis* species fall into a clade with non-*Eutacta Araucaria* and again render *Araucaria* paraphyletic. Section *Eutacta* is strongly supported as a monophyletic group with 100% bs. Within section *Eutacta*, Norfolk Island pine (*A. heterophylla*) and the New Caledonian taxa resolved as a monophyletic group (83% bs). However, New Caledonian *Araucaria* does not resolve as monophyletic and instead form an unresolved clade including *A. heterophylla*. Within New Caledonian species, the majority of the accessions formed a polytomy, but a single clade consisting of two "big leaved" species (*A. rulei* and *A. laubenfelsii*) was resolved, albeit with weak bootstrap support (62%). Only accessions of *A. schmidii* and *A. subulata* showed a species-specific synapomorphy that groups the two accessions of each species together. Both of these species are, however, represented by accessions from a single population each.

2.3.2.3 Combined analysis

Two most parsimonious trees were obtained from the combined matrix with a tree length of 149 steps and a CI=0.94 and RI=0.97. The topology within the ingroup was insensitive to choice or combination of outgroups. The strict consensus tree is shown in Fig. 2.5; the two individual phylograms are shown in Fig. 2.6. *Araucaria* resolves as monophyletic with 100% bs. Section *Araucaria* resolved as monophyletic (85% bs) sister to a well-supported clade (98% bs) containing the two monotypic sections *Bunya* and *Intermedia*. This group collectively was monophyletic (100% bs) and sister to a strongly monophyletic *Eutacta* section (100% bs). Norfolk Island pine (*A. heterophylla*) and the New Caledonian taxa resolved as a monophyletic group (97% bs). The New Caledonian species were monophyletic (63% bs), two supported clades were obtained within the New Caledonian species, and a third clade was resolved albeit with no bootstrap support. One clade included all the coastal species (*A. columnaris*, *A. nemorosa*, and *A. luxurians*; 74% bs) and was nested in an

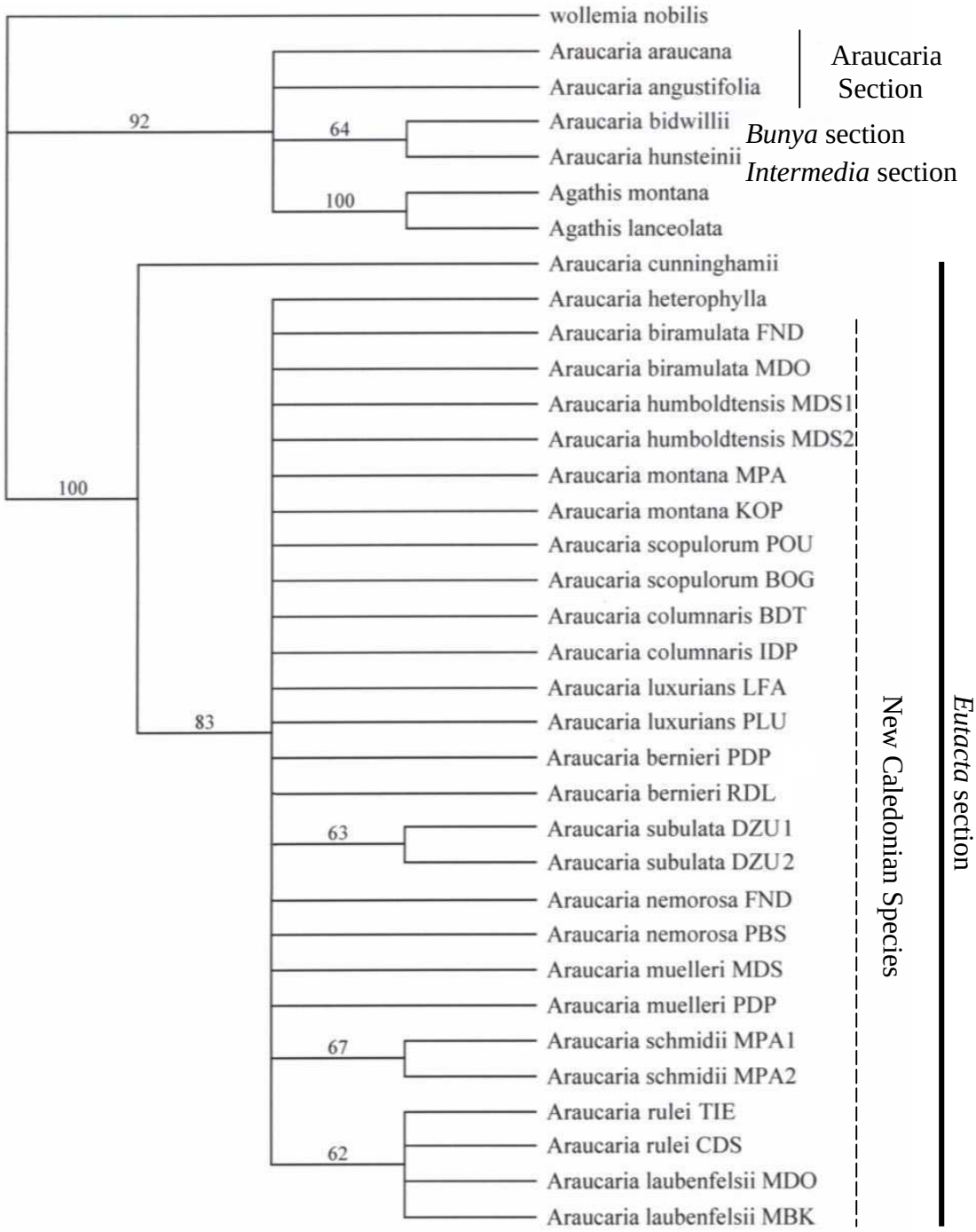


Fig. 2.4: Consensus tree of the 10 equally most parsimonious trees obtained for *Araucaria* based on cpDNA *psb*A-*trn*H sequences. Length = 87 steps, CI = 0.87 (excluding uninformative characters), RI = 0.92. A percentage of the 1000 bootstrap values (>50%) is given for each node

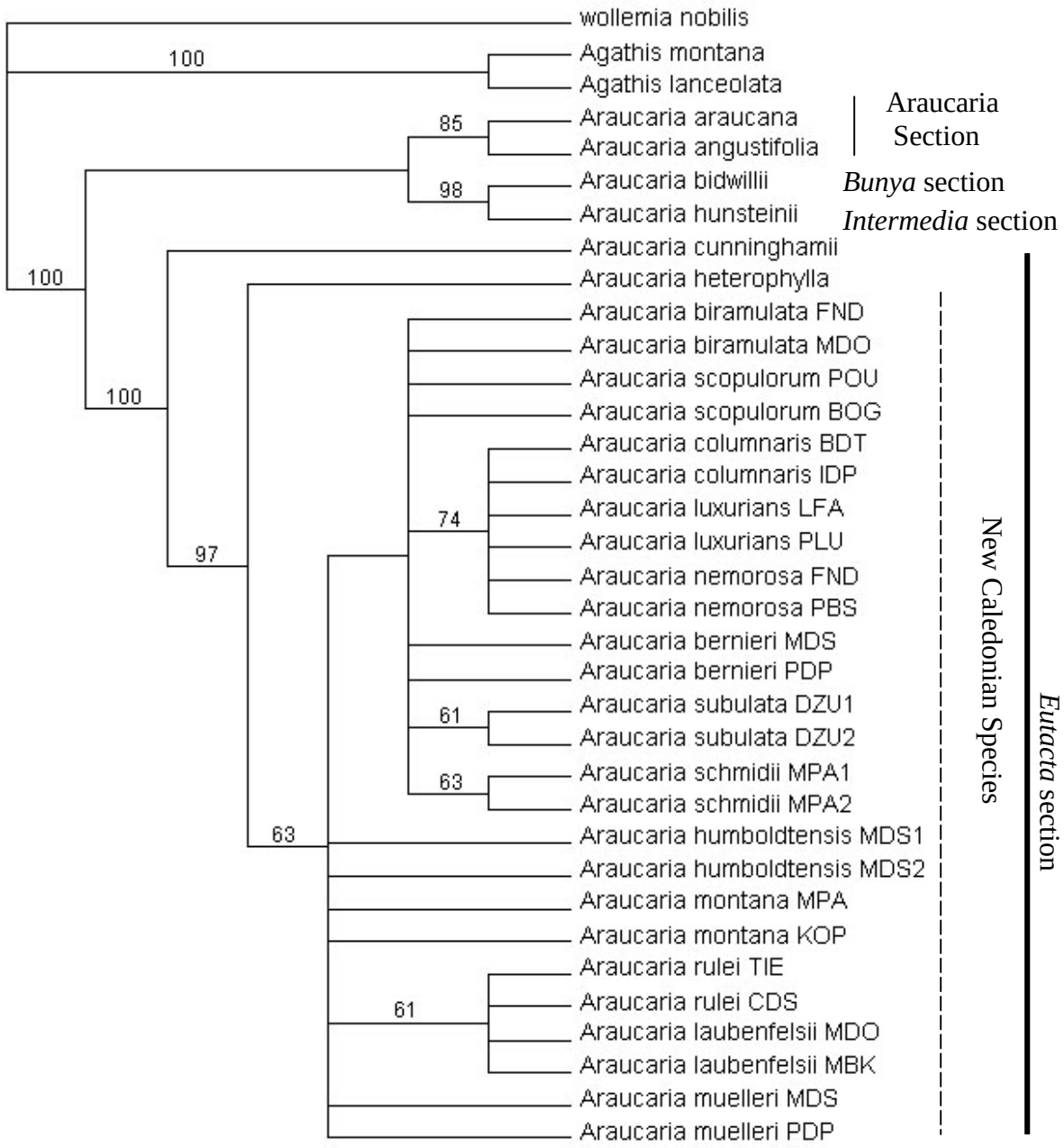


Fig. 2.5: Consensus tree of the 2 equally most parsimonious trees obtained for *Araucaria* based on the combined datasets of *psb*A-*trn*H and *trn*S-*trn*FM. Length = 149 steps, CI = 0.94 (excluding uninformative characters), RI = 0.97. A percentage of the 1000 bootstrap values (>50%) is given for each node

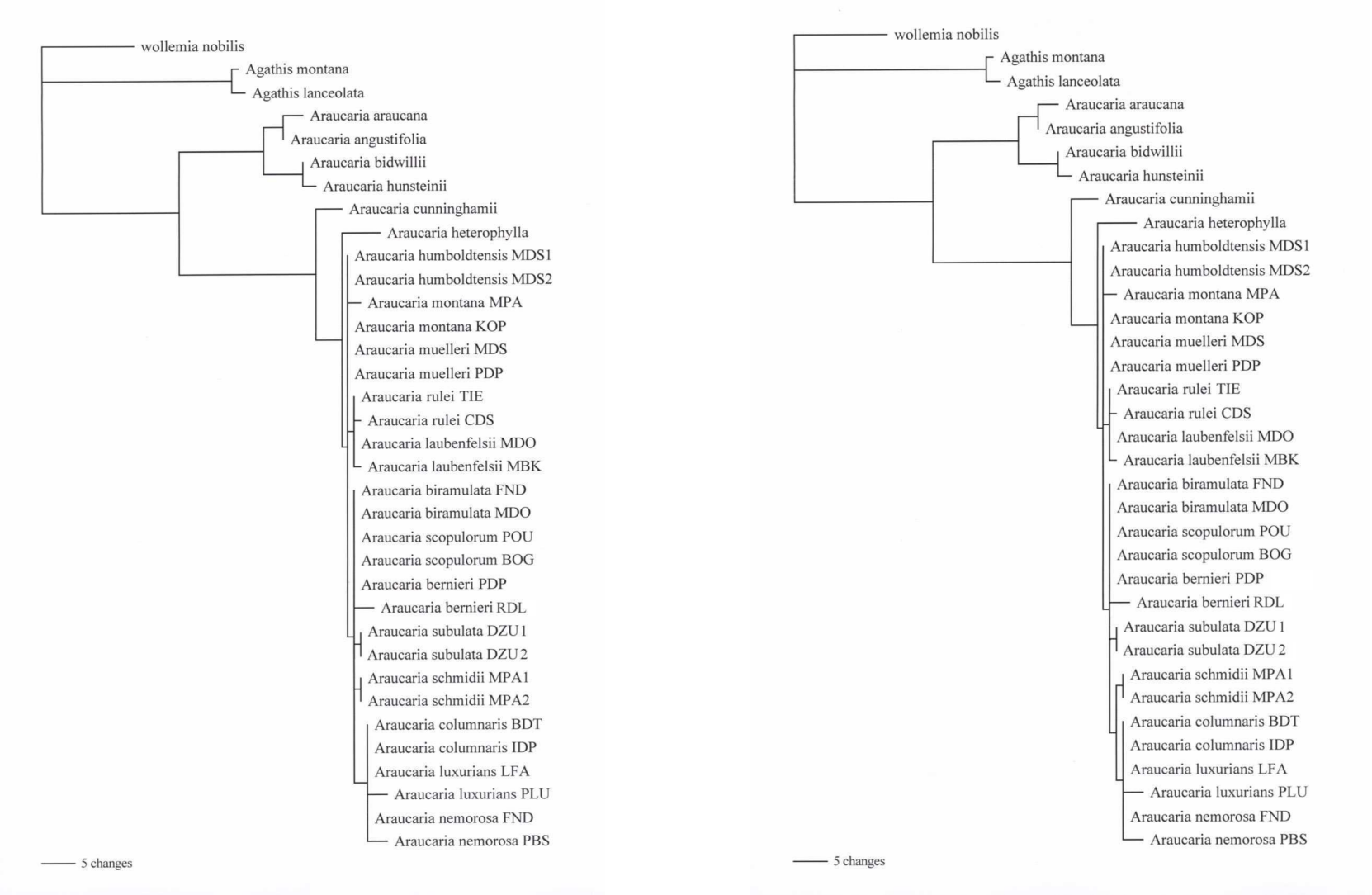


Fig. 2.6: 2 equally parsimonious trees obtained with the combined dataset of *psb*A-*trn*H and *trn*S-*trn*FM using parsimiony criteria. Trees are represented as Phylograms. Trees length is 149 steps long. CI (excluding uniformative characters) = 0.94, RI (excluding uniformative characters) = 0.97

unsupported clade including eight species (the coastal species plus all but one of the other small leaved species: *A. biramulata, A. scopulorum, A. berneri, A. subulata* and *A. schmidii*). The other clade with (albeit weak) bootstrap support included *A. rulei* and *A. laubenfelsii* (64 % bs). The two accessions of *A. schmidii* resolved together (63% bs), as did the two accessions of *A. subulata* (61% bs).

2.3.3 Maximum Likelihood

2.3.3.1 Separate analyses of chloroplast regions

The maximum likelihood method recovered the same tree topology for *trn*S-*trn*Fm as the maximum parsimony method (Fig. 2.7). In the *psb*A-*trn*H analysis, the position of *Agathis* made *Araucaria* paraphyletic (Fig. 2.8). When only *Agathis* was specified as the outgroup, *Wollemia* became basal to section *Eutacta* (analysis not shown). Section *Eutacta* is strongly supported as a monophyletic group with 100% bootstrap support. Within section *Eutacta*, Norfolk Island pine (*A. heterophylla*) and the New Caledonian taxa resolved as a monophyletic group (76% bootstrap support) but the New Caledonian *Araucaria* do not resolved as monophyletic. They instead formed an unresolved clade with *A. heterophylla* (76% bs).

Two main clades were resolved within the New Caledonian clade, albeit with only weak bootstrap support. One clade (58 % bs) included eight species (*A. bernieri, A. biramulata, A. columnaris, A. luxurians, A. nemorosa, A. schmidii, A. scopulorum, A. subulata*). A second clade included *A. rulei* and *A. laubenfelsii* (63 % bs). The two accessions of *A. schmidii* resolved together (68% bs), as do the two accessions of *A. subulata* (66% bs).

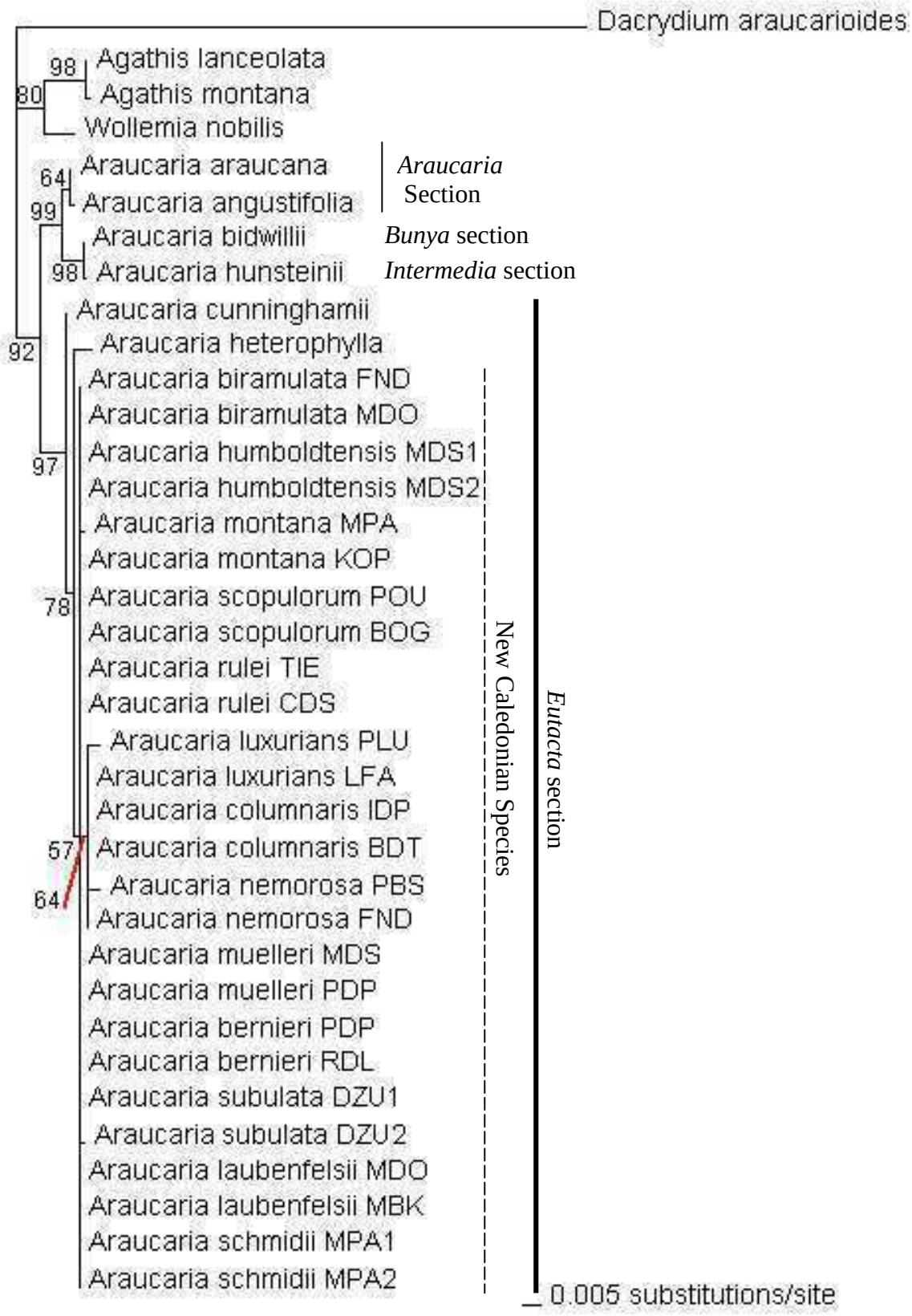


Fig. 2.7: Single tree obtained from the maximum likelihood heuristic search for *Araucaria* based on the datasets of *trn*S-*trn*FM. Model of base substitution used was HKY+G with base frequencies set to (Lset Base) = (0.3080 0.1872 0.1854). A percentage of the 500 bootstrap values (>50%) is given for each node

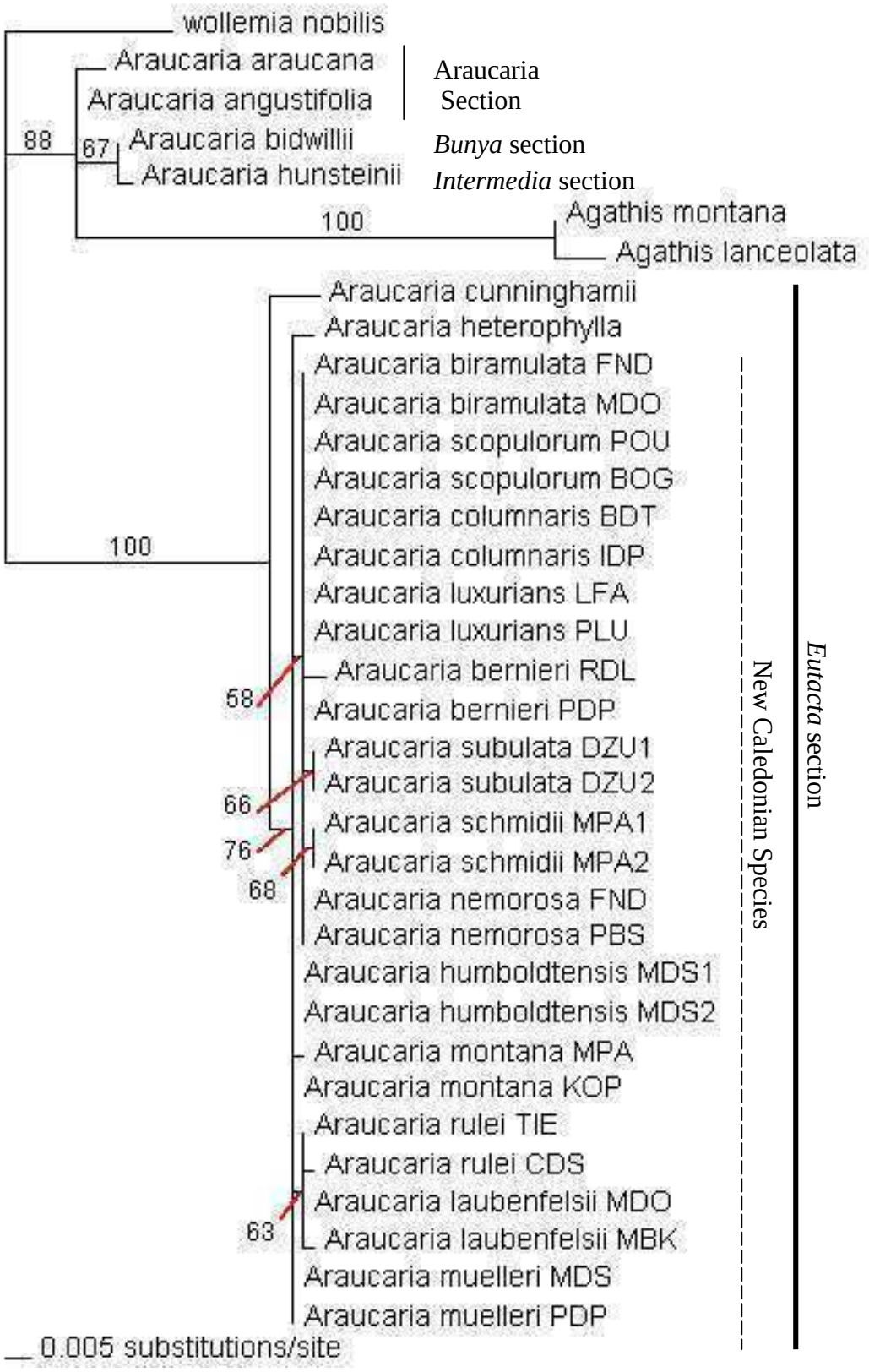


Fig. 2.8: Single tree obtained from the maximum likelihood heuristic search for *Araucaria* based on the datasets of *psb*A-*trn*H. Model of base substitution used was HKY+G with base frequencies set to (Lset Base) = (0.3080 0.1872 0.1854). A percentage of the 500 bootstrap values (>50%) is given for each node

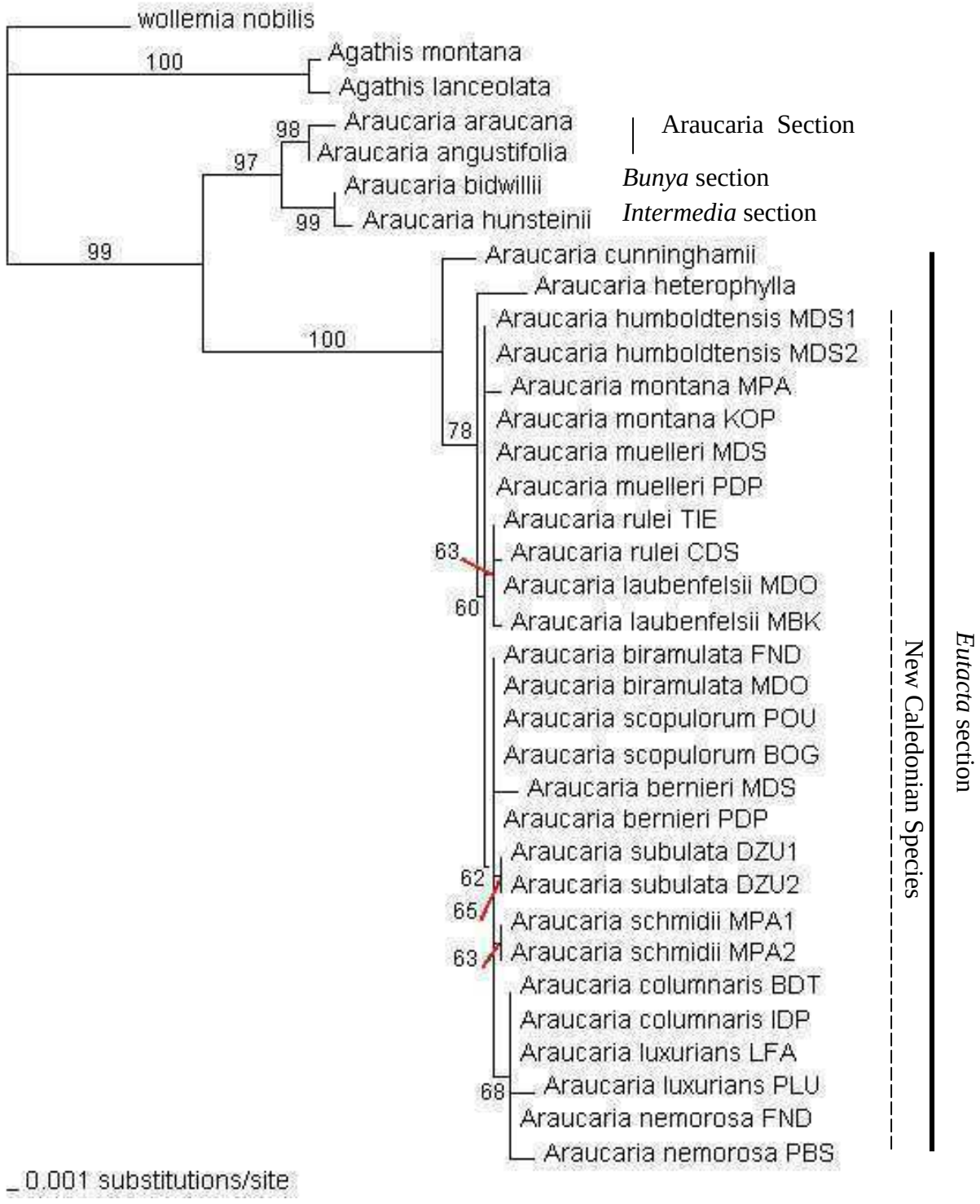


Fig. 2.9: Single tree obtained from the maximum likelihood heuristic search for *Araucaria* based on the combined datasets of *psb*A-*trn*H and *trn*S-*trn*FM. Model of base substitution used was HKY+G with base frequencies set to (Lset Base) = (0.3080 0.1872 0.1854). A percentage of the 500 bootstrap values (>50%) is given for each node

2.3.3.2 Combined analysis

Araucaria is resolved as monophyletic with 99% bootstrap support (Fig. 2.9). Section *Araucaria* resolved as monophyletic (98% bs) sister to a clade containing the two sections *Bunya* and *Intermedia* (99% bs). This group collectively was monophyletic (97% bs) and sister to a monophyletic section *Eutacta* (100% bs). The New Caledonian taxa resolved as a monophyletic group (60% bs) sister to *A. heterophylla* (78% bs). Three clades were obtained, one clade of the three costal species (68% bs) nested in a larger clade which in total included eight small leaved species (62% bs), and finally the third clade included *A. rulei* and *A. laubenfelsii* (63% bs). The two accessions of *A. schmidii* resolved together (63% bs) as did the accessions of *A. subulata* (65% bs). This topology is identical to the topology recovered with maximum parsimony.

2.4 Discussion

Both maximum parsimony and maximum likelihood recovered the same final tree topology from the *trn*S-*trn*Fm region and when the datasets are combined. This is attributable to the clean nature of the data and the low level of homoplasy (high CI and RI), indicating that the topology is insensitive to the method of analysis used for these datasets. The difference in the resolution of the tree resulting from the *psb*A-*trn*H dataset is explained by the higher degree of homoplasy from this region (Fig. 1.4). The highest resolution is obtained when the datasets are combined. The *trn*S-*trn*Fm phylogeny is thus used as a basis for discussing inter-generic relationships, and the phylogenies derived from the combined dataset for discussing relationships within *Araucaria*.

2.4.1 Relationships within the Araucariaceae

Relationships between the three genera of the Araucariaceae were only reliably recovered in *trn*S-*trn*Fm phylogeny, as the *psb*A-*trn*H analyses and the combined analyses lacked an outgroup outside the Araucariaceae. The *trn*S-*trn*Fm analysis resolves *Araucaria* as sister to a clade comprising *Agathis* and *Wollemia*. This result contradicts the relationships obtained by Setoguchi *et al.* (1998) whose *rbc*L data

resolved ((*Wollemia*) (*Agathis, Araucaria*)). However, the topology recovered here agrees with Codrington *et al*. (2002) whose phylogeny was based on the 18S ribosomal gene, Gilmore and Hill (1997) whose phylogeny was based on *rbc*L, and Quinns *et al.* (2002), whose phylogenetic study used both *rbc*L and *mat*K. Quinns *et al.* (2002) attributed the result of Setoguchi *et al.* (1998) to a lack of sufficient sequence divergence in their study, although other methodological issues may also be relevant given that the same gene (*rbc*L) has given different topologies in the studies of Gilmore and Hill (1997) and Setoguchi *et al.* (1998).

The grouping of *Wollemia* and *Agathis* receives partial support from morphological characters (*Agathis* and *Wollemia* both have fully fused bracts, ovuliferous scales, and winged seed; Jones *et al.*, 1995). In addition, the position of *Araucaria* sister to *Agathis* and *Wollemia* matches the fossil evidence, which suggests that the oldest and most widespread Araucarian fossils belong to *Araucaria*, which was present in both hemispheres long before either of the other genera appeared (Hill and Brodribb, 1999). In the light of the topology ((*Araucaria*) (*Agathis, Wollemia*)), the traits shared by *Araucaria* and *Wollemia* should be interpreted as pleisiomorphic (closely crowned sessile and amphistomatic leaves, aristate bract scales).

2.4.2 Relationships within *Araucaria*

Each extant section within *Araucaria* is recovered as monophyletic. Section *Eutacta* is sister to a clade including the three other sections. Section *Araucaria* is sister to the clade containing sections *Intermedia* and *Bunya*. All of these sections share a more recent common ancestor with each other, than any do with section *Eutacta*. This topology was also recovered by Quinns *et al.* (2002), Codrington *et al.* (2002) and Setoguchi *et al.* (1998), but not in the study by Gilmore and Hill (1997) in which the sole representative of section *Araucaria* (*A. angustifolia*) sampled resolved as sister to section *Eutacta*. Given the sparse sampling in the Gilmore and Hill study and the very weak support for the topology difference in their phylogeny, the weight of evidence suggests that the relationship of *Eutacta* as being sister to ‘the rest’ as present here is the best current estimate of the phylogeny.

The tree topology within the genus does not match a simple Gondwanan vicariance model. *A. araucana* and *A. angustifolia*, the two South American species, are recovered as a clade, sister to *A. hunsteinii* and *A. bidwillii* from New Guinea and Australia. There is then a major divergence between this trans-oceanic clade and a west pacific clade (Australia, New Caledonia and Norfolk Island). Therefore, the major split in the data is not (South America (Australasia, New Guinea)) vs. (New Caledonia) (c.f. Sanmartín and Ronquist, 2004). The presence of species in different sections which now occur in Australia being present in phylogenetically divergent positions in the topology matches the observation from fossil evidence that at least some of these sections predate Gondwanan fragmentation (Hill and Brodribb, 1999).

Among the sections of *Araucaria*, *Eutacta* is the most divergent and the distinctness of section *Eutacta* is supported by its long fossil record (Hill and Brodribb, 1999). The monophyly of the section is supported by the fact that it possesses unique features like the presence of four cotyledons (only two are found in the other sections) and the terminal position of male cones (the position is on the axis in the other sections). There are thus morphological and genetic characters supporting the distinct nature of this section. DeLaubenfels (2002) considered it sufficiently distinct to warrant raising the section to generic status. However, while it is clearly a monophyletic unit, the case for generic status does not seem compelling. *Araucaria* as a genus is small and manageable and there are no grounds for splitting it on the basis of overwhelming species numbers. Furthermore, the morphological features uniting species in the genus, separate from other genera in the family, are strong. It seems that taxonomic confusion rather than clarity would be the major outcome of any division of this clearly monophyletic and recognisable small genus into two different genera.

2.4.3 Relationships within section *Eutacta*

Araucaria cunninghamii is sister to the other species of section *Eutacta*, which themselves form a geographically coherent group with the New Caledonian species being monophyletic and sister to *Araucaria heterophylla* from the geographically

proximal Norfolk Island. This placement of *Araucaria heterophylla* as the sister group to the New Caledonian species confirms the result of Setoguchi *et al.* (1998).

A completely resolved phylogeny of the New Caledonian species remains elusive, and only a very small number of genetic differences have been detected amongst these species. However, the datasets presented here has provided more resolution than previous studies. Three groups of species have been retrieved, but a polytomy remains at the base of the New Caledonian clade, and also within the groups within this clade (Fig. 2.5).

2.4.3.1 Clade 1: *A. rulei* and *A. laubenfelsii* (supported by a G->A change at bp 412 and a G->A change at bp 560 of the *psb*A-*trn*H region)

The phylogenetic data give some evidence for a weakly supported relationship between *A. rulei* and *A. laubenfelsii*. Both are high altitude species, growing on top of mountains in rainforests, although *A. rulei* can sometimes be found on maquis minier. The two species have also been recorded from the same localities (although they were not sampled sympatrically in this study). These data suggest shared evolutionary ancestry, although on morphological grounds, *A. laubenfelsii* is more similar to *A. montana* (from which it is very difficult to distinguish). Given the similarity of *A. rulei* and *A. laubenfelsii* in the phylogeny, but not *A. montana* (and the weak bootstrap support), sequencing of additional populations would be prudent before reading too much into this result.

2.4.3.2 Clade 2: *A. columnaris, A. nemorosa, A. luxurians, A. biramulata, A. schmidii, A. subulata, A. scopulorum, A. bernieri* (supported by A->G change at bp 412 and a A->G change at bp 560 of the *psb*A-*trn*H region)

Clade 2 groups species from very diverse habitats and includes taxa which occur at sea level (*A. columnaris*) to those restricted to the highest point on New Caledonia (1628m, *A. schmidii*). The major uniting feature of this group is that it contains the vast majority of the species with small leaves. Of the New Caledonian species *not* included in this clade, *A. muelleri, A. rulei, A. laubenfelsii* and *A. montana* have

markedly larger leaves ranging from 11mm to 35mm in length and 7mm to 18 mm in width, on typical adult foliage. The only small leaved species that is *not* in Clade 2 is *A. humboldtensis.* Thus with the exception of *A. humboldtensis*, Clade 2 contains all of the small leaved species with a Massart model of tree architecture, and the basal polytomy outside of Clade 2 is dominated by species with the Rauh model of tree architecture.

Within Clade 2, species living in rainforest or forest on maquis minier occupy a more basal position (*A. biramulata, A. schmidii, A. subulata, A. scopulorum, A. bernieri*). Only *A. subulata* and *A. schmidii* showed any apparently species specific mutations. However these may be artefacts as both species were sampled from accessions from a single population. Such a result is not as robust as the detection of unique defining base changes from widely separated populations. Further sampling of *A. subulata* should be made to confirm this autapomorphic state; for *A. schmidii* the options for further sampling are limited by the extremely restricted distribution of this species.

2.4.3.3 Clade 3 (nested within clade 2): *A. columnaris, A. nemorosa, A. luxurians* (supported by a C->A change at bp 3 and a C->T change at bp 185 of the *trn*S-*trn*Fm region)

Clade 3, clustered within Clade 2, is the most derived clade among the New Caledonian species. This clade encompasses species with clear cut differences in leaf and cone morphology (see Chapter 5). However, a strong uniting feature of this group is that all three species have a coastal distribution. *Araucaria columnaris* occurs on calcareous soils in the south of the main island of New Caledonia, as well as on smaller island off the south and east coasts. *A. nemorosa* is entirely restricted to a small number of sites on the south of the main island, where it grows slightly inland from *A. columnaris* on ultramafic soils. *A. luxurians* occurs on ultramafic soils in scattered localities on the west, east and south coasts.

2.4.3.4 Comparison of the New Caledonian species with the phylogeny of Setoguchi *et al.* (1998)

The one resolved relationship in the *rbc*L phylogeny of Setoguchi *et al.* (1998) grouped *A. muelleri* and *A. rulei,* should be viewed with caution. This grouping was not recovered in the phylogeny obtained with the use of *trn*S-*trn*Fm nor with *psb*A-*trn*H. To assess the reliability of this putative grouping, I sequenced five individuals of *A. rulei* and *A. muelleri*, and two of *A. bernieri* and *A. columnaris* for *rbc*L. No mutations grouping *A. muelleri* and *A. rulei* were detected. Either sequencing errors, misidentification errors (*A. muelleri* and *A. rulei* have similar gross morphology) or an artefact of chance shared bases which show intra-specific variation in both species may explain the apparently erroneous Setoguchi *et al.* (1998) result.

2.4.4 Overview of the phylogeny of New Caledonian *Araucaria*

The monophyly of the New Caledonian *Araucaria* species, and their sister group relationship with *A. heterophylla* suggest they have originated from a single colonisation event. An alternative explanation would be widespread hybridisation and chloroplast capture. However, if hybridisation were rampant enough to lead to complete homogenisation of c*p*DNA, one would expect species growing in the same geographical areas to have similar *cp*DNA types. This was not recovered and there is at least some taxonomic signal to the data (chloroplast types are not species independent).

The lack of resolution in the phylogeny does not allow an easy identification of the attributes of the ancestral *Araucaria* on New Caledonia. *A. heterophylla,* the sister species to the New Caledonian assemblage occurs on non-ultramafic soils. The only three New Caledonian species of *Araucaria* that are not restricted to ultramafic soils are *A. montana, A. schmidii* and *A. columnaris. A. montana* is one of the most widespread species and occurs on both ultramafic and non-ultramafic soils, and is a member of the basal polytomy in the phylogenetic analyses undertaken here. On edaphic, phylogenetic and distributional grounds it thus has some features, which suggest it could stem from an important lineage in the evolutionary diversification of this group, although this is somewhat speculative.

Nasi (1982) raised the hypothesis that *A. schmidii* was one of the most basally divergent species due to its occurrence on non-ultramafic soil. However the relatively derived position of *A. schmidii* in the phylogenies presented here contradicts this hypothesis, and it is equally plausible that the current edaphic preferences of *A. schmidii* are a secondary adaptation. On the other hand, Nasi (1982) interpreted the presence of *A. columnaris* on calcareous soils associated with recent (<100 k years) coral uplifts as evidence for it being the most recently diverged species. The derived phylogenetic position of *A. columnaris* provides some support to this hypothesis.

The presence of a derived clade containing most of the small leaved species, and a basal polytomy containing the large leaved species (including *A. montana* which occurs on both ultramafic and non-ultramafic soils) makes it tempting to suggest that the ancestral species on New Caledonia was large leaved, and the small leaved species evolved from this. However, the most closely related species to the New Caledonian species (*A. heterophylla* and *A. cunninghamii*) both have small leaves. Given the presence of a single small leaved species (*A. humboldtensis* found only on ultramafic soils) in the basal polytomy, it seems unwise to place too much weight either way as to the morphology of the ancestral New Caledonian *Araucaria*.

One thing that is evident from the phylogeny is that leaf morphology is extremely labile in the genus (Fig. 2.10). *A. araucana* has the largest leaves, with the next largest occurring in *A. muelleri*. However, these two species are separated in the phylogeny by branches and nodes upon which small leaves must be optimised (e.g. near the base of section *Eutacta*). The strong similarity of leaf morphology among juvenile *Araucaria* (all with small leaves) raises the possibility that this transition between leaf sizes could be attributable to recurrent paedomorphogenic mutations, which arrest the development of adult leaf morphologies.

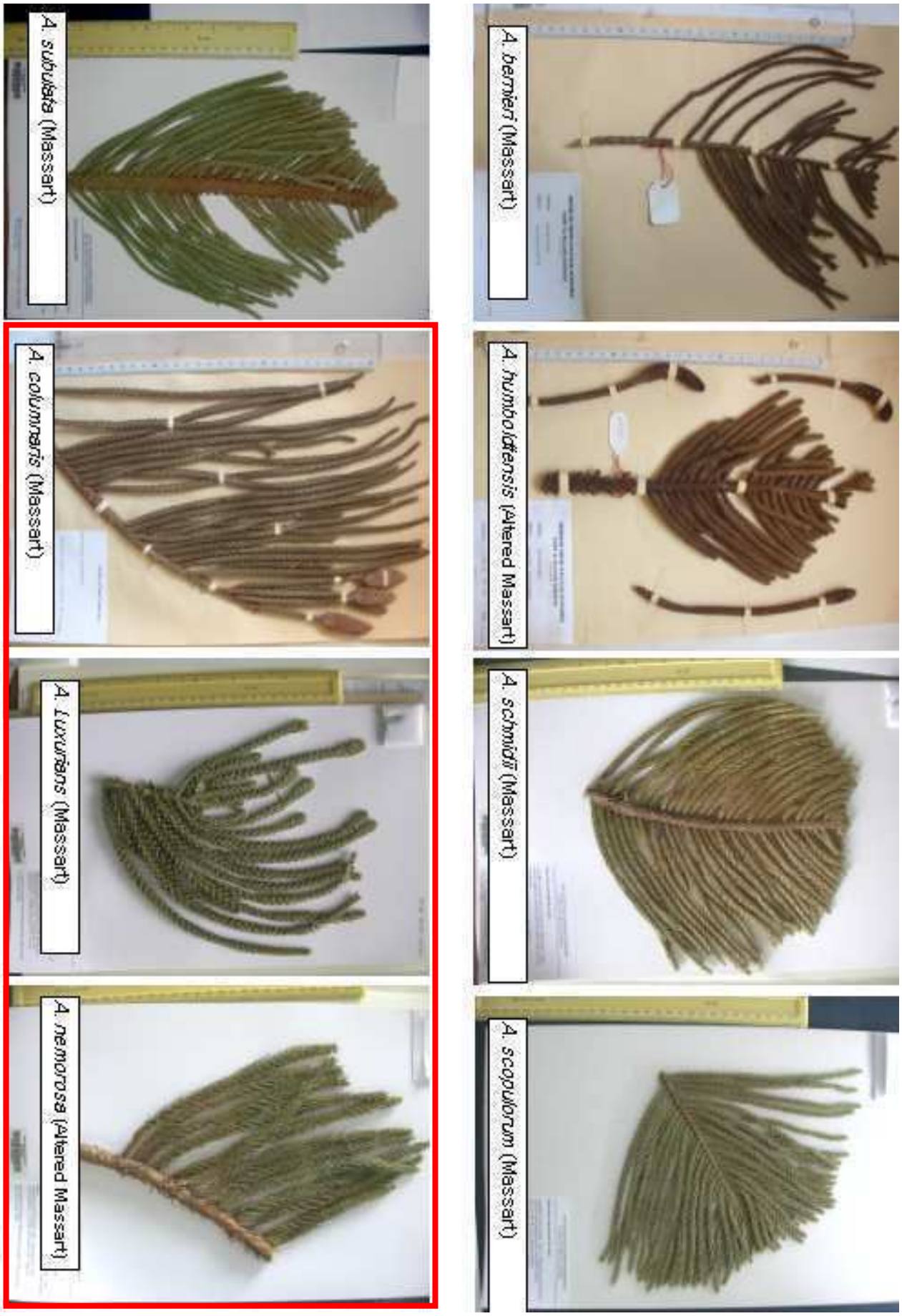


Fig. 2.10: Summary of the leaf morphology of the different species, with indication on their growth habit (Rauh and Massart models). Highlighted in red are the species from the Coastal group. Not highlighted are the 'small leaved not coastal species'.

Fig. 2.10 (continued): Summary of the leaf morphology of the different species, with indication on their growth habit (Rauh and Massart models). Highlighted in blue are the species from the Bigger leaved group. Not highlighted are the 'small leaved not coastal species'.

Another aspect of morphology that seems to be labile is habit. Fig. 2.11 summarises the habit of the different tree species, which have been classified according to the Rauh and Massart models. What is apparent from this figure, and field observations, is that these models represent extremes of a continuum rather than discrete classes. Thus while the candelabra appearance of *A. rulei* and *A. muelleri* are clearly distinct from the other species, there is something of a continuum from the columnar forms of *A. columnaris*, *A. bernieri* and *A. subulata*, through to trees considered as modified forms of the Rauh model like *A. montana* and *A. luxurians.* The presence of species in Clade 2 that are considered Massart (e.g. *A. columnaris*) and modified Rauh (*A. biramulata*), and the presence of species in the basal polytomy that are considered Rauh (e.g. *A. rulei*), modified Rauh (e.g. *A. montana*), and Massart (*A. humboldtensis*) highlights the lack of clear phylogenetic structure within this continuum of habits, even though the extremes *A. rulei, A. muelleri* vs. *A. columnaris, A. subulata, A. bernieri* are phylogenetically separated.

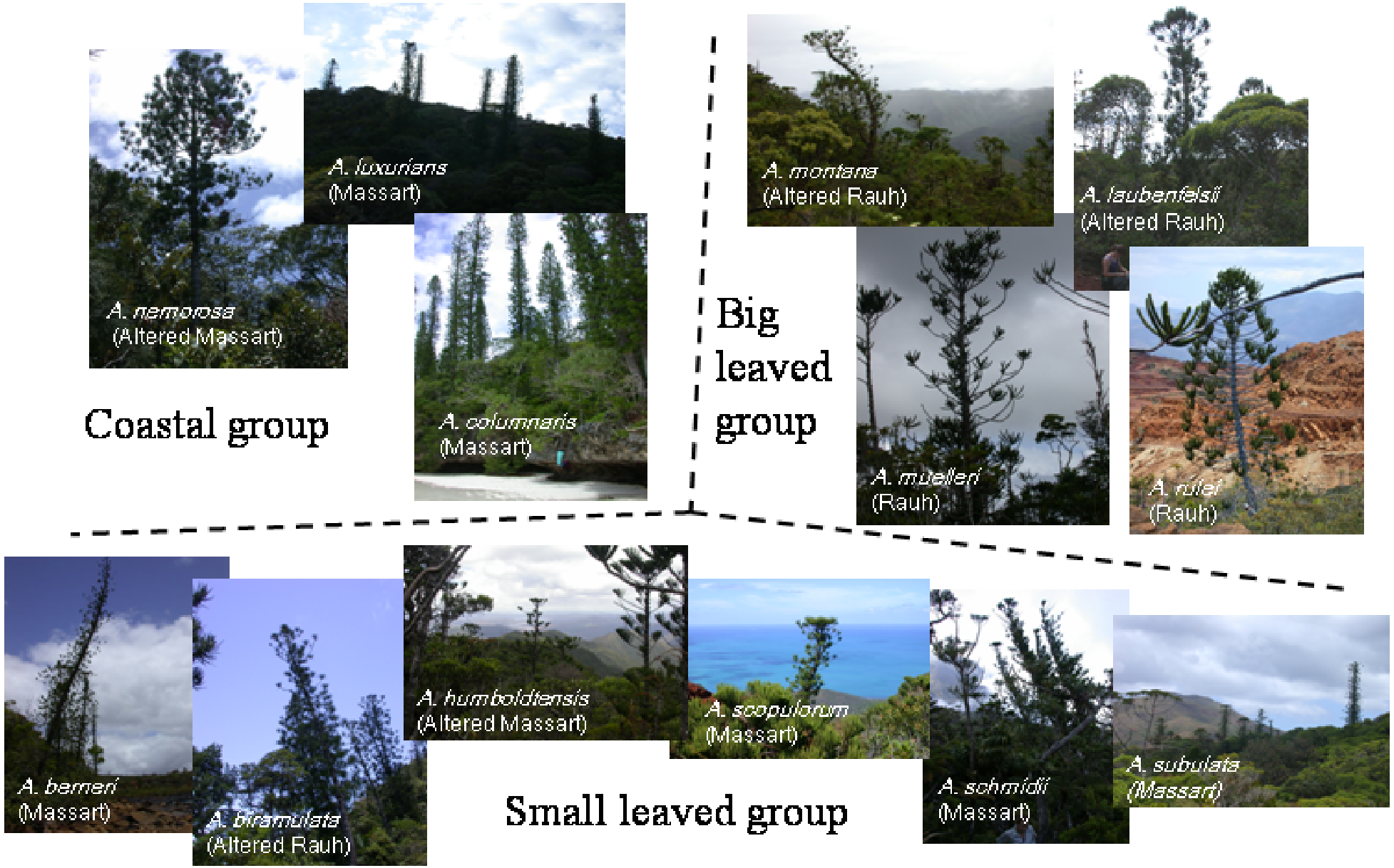


Fig 2.11: Summary of the habit of the different tree species, classified according to the 3 groups defined by the phylogeny with indication on their growth habit (Rauh and Massart models). In this figure *A. humboltensis* is shown with the other small leaved species although in the phylogeny it is placed in the basal polytomy with the large leaved species.

CHAPTER 3 - Dating the radiation of *Araucaria* on New Caledonia.

3.1 Introduction

Araucaria are among the most ancient conifers in the world (Hills and Brodribb, 1999). The island of New Caledonia represents a hot spot for studying this genus, as 13 out of the 19 extant species of *Araucaria* are endemic to this small island in the Pacific Ocean (Watt, 1999). *Araucaria* are found from sea level to 1628 m in maquis or humid rainforest forest and 11 out of 13 grow only on ultramafic soil types (Nasi, 1982; Jaffré, 1995). The taxa usually occupy niches where the competition with other plant species is reduced, such as steep mountain ridges, wind exposed slopes, or soils with low nutrient availability (ultramafic rocks or emergent reefs) (Nasi, 1982). Recent phylogenies (Setoguchi *et al.*, 1998; Chapter1) have suggested that all 13 species on New Caledonia are monophyletic and have therefore radiated in-situ. This raises the question as to what factors have given rise to such diversity and over what time-scales this radiation occurred. Having information on the time of the radiation would enable us to place it in the context of geological and other events that happened in the region at the time. It would also enable us to make comparisons with evolution in other genera on the islands of New Caledonia. Some information is already available for other plant families like Sapindaceae (Balgooy, 1996) and Palms (Pintaud, 1999).

3.1.1 The debated origin of *Araucaria* species

Fossils of *Araucaria* are among the oldest fossils of extant coniferous genera that have been found since the Triassic or Jurassic eras (Setoguchi *et al.*, 1998; Hill and Brodribb, 1999; Kershaw and Wagstaff, 2001) and the oldest fossils recorded for all sections predate the Late Cretaceous. It seems likely, therefore, that all sections of the genus have evolved before Gondwanan fragmentation (Setoguchi *et al.*, 1998). Moreover, the fossil record of *Araucaria* suggests a more widespread distribution than that of the extant species (Table 3.1). Once present in both hemispheres, it is

Section	Extant species		Extinct species		
	Species	Location	Species	Location	Age (MY)
Araucaria	*A.angustifolia (Bertol.) Kuntze*	Brazil	*A. lanceolata* Cantrill	Australia (Victoria)	*105-97*
	A. araucana (Molina) K. Koch	Chile	*A. seorsum* Cantrill	Australia (Victoria)	*105-97*
			A. balcombensis Selling	Australia (Victoria: Balcombe Bay deposit, Mornington Peninsula)	*065-54*
			A. hastiensis R. S. Hill & Bigwood	Tasmania	42.1-35.4
			A. nathorstii Dusén	South America (Argentina)	[Cenozoic]
Eutacta(Link) Endl	*A. bernieri* Bucholz	New Caledonia	*A. acutifoliata* Cantrill	Australia (Victoria)	105-97
	A. biramulata Bucholz	New Caledonia	*A. carinata* Cantrill	Australia (Victoria)	105-97
	A. columnaris (Forster) Hooker	New Caledonia	*A. falcata* Cantrill	Australia (Victoria, several sites)	105-97
	A. humboltensis Bucholz	New Caledonia	*A. otwayensis* Cantrill	Australia (Victoria: Moonlight Head)	105-97
	A. laubenfelsii Corbesson	New Caledonia	*A. imbricatiformis* R. M. Johnston	Tasmania	070-65
	A. luxurians (Brongn. & Gris) Laubenfels	New Caledonia	*A. annulata* (Bigwood & R. S. Hill) Pole	Tasmania	050-35
	A. montana Brongn. & Gris	New Caledonia	*A. lignitici* Cookson & Duigan	Australia (Victoria)	35.4-23.3
	A. muelleri (Carr.) Brongn. & Gris	New Caledonia	*A. readiae* R. S. Hill & Bigwood	Tasmania	35.4-23.3
	A. nemorosa Laubenfels	New Caledonia	*A. plana* R. S. Hill	Tasmania	029-20
	A. rulei Müll.	New Caledonia	*A. prominens* R. S. Hill	Tasmania	029-20
	A. schmidii Laubenfels	New Caledonia	*A. derwentensis* Selling	Tasmania	025-022
	A. scopulorum Laubenfels	New Caledonia	*A. uncinata* R. S. Hill	Tasmania	024-21
	A. subulata Vieill.	New Caledonia	*A. fletcheri* Selling	Australia (New South Wales)	[Cenozoic]
	A.cunninghamii Aiton ex D. Don in Lambert	Australia	*A. crassa* (Tenison-Woods) Townrow	Australia (Queensland)	[Cenozoic]
	A.cunninghamii Aiton ex D. Don var. *papuana* Lauterb.	New Guinea			
	A.heterophylla (Salisb.) Franco	Norfolk Island			
Bunya Wilde & Eames	*A. bidwillii* Hook	Australia	*A. brownii* R. A. Stockey	England (Dorset)	173.5-166.1
			A. sphaerocarpa Carruthers	England (Somerset)	173.5-166.1
			A. mirabilis (Speg.) Windhausen	Argentina (Cerro Cuadrado Petrified Forest)	165-160
Intermedia C. T. White	*A. hunsteinii* K. Schum.	New Guinea	*A. haastii* Ettingsh	New Zeland	074 to 65
Perpendicula M. Pole			*A. desmondii* M. Pole	New Zeland	095-70

Table 3.1: List of extant and extinct *Araucaria* species and their known locations

Section	Extant species		Extinct species		
	Species	Location	Species	Location	Age (MY)
Yezonia(Stopes & Fujii) T. Ohsawa et al.			A. vulgaris (Stopes & Fujii) Ohsawa et al. .	Japan (Hokkaido)	090.4-83.0
Non determined			*A. cleminshawii* Mansell-Pleydell	England (Dorset)	173.5-166.1
			A. indica (Sahni) Sukh-Dev & Zeba-Bano	India	150-140?
			A. africana V. A. Krassilov	Africa (Sahara: Mali-Nigerian depression)	150
			A. cutchensis (Feistm.) Pant & Srivast.	India (near Chandia)	140-110
			A. grandifolia Feruglio	Paraguay (Baqueró Formation)	119 to 113
			A. alexandrensis Cantrill & Falcon-Lang	Antarctica (Alexander Island)	112-97
			A. chambersii Cantrill & Falcon-Lang	Antarctica (Alexander Island)	112-97
			A. clarkii E. W. Berry	USA (North Carolina coastal plain: Cape Fear River)	100-90 ?
			A. darlingtonensis E.W. Berry	USA (North Carolina)	100-90 ?
			A. nihongii Stockey et al.	Japan, Hokkaido	090.4-83.0
			A. nipponensis Stockey *et al.* .	Japan (Hokkaido)	090.4-83.0
			A. jeffreyi E.W. Berry	USA (North Carolina, Black River outcrops)	083-70
			A. danae Ettingsh.	New Zeland	074 to 65
			A. owenii (Ettingsh.) Pole	New Zeland	074 to 65
			A. taieriensis M. Pole	New Zealand (Otago: Kai Point Mine, Taieri River)	074-65
			A. fimbriata R. S. Hill	Tasmania	035.4-23.3
			A. lignitica Cookson & Duigan emend. R. S. Hill	Australia (Victoria)	035.4-23.3-
			A. macrophylla Bozzi	Europe	

Table 3.1 (continued): List of extant and extinct *Araucaria* species and their known locations

now only distributed throughout the southern hemisphere (New Caledonia (13 species), Chile (*A. angustifolia*), Brazil (*A. araucana*), Norfolk Island (*A. heterophylla*), Australia (*A. bidwillii, A cunninghamii*), and New Guinea (*A. hunsteinii, A. cunninghamii* var. *papuana*)) in countries that once belonged to the super continent Gondwana 100 million years ago. The distribution of *Araucaria* is thus considered to be a relic of the Gondwana flora. This has led to the hypothesis of a possible Gondwanan origin of the New Caledonian species (Nasi, 1982; Jaffré, 1995; Hill and Brodribb., 1999). However, recent phylogenies (Setoguchi *et al.*, 1998; Chapter 1) have raised questions about the age of New Caledonian species as very few nucleotide substitutions were detected within this monophyletic group. This indicates either a far more recent emergence of the species or an extremely slow substitution rate.

Several hypotheses concerning the diversification of *Araucaria* on New Caledonia have also been proposed in order to explain the large number of endemic species present on the island (13 out of 19 in the world). The high rates of endemism (93%) in the flora in general on ultramafic soils as well as the presence of 11 out of 13 *Araucaria* species on these types of substrate have lead to the conclusion that ultramafic soils might have been a major factor driving speciation. A layer of ophiolite (oceanic crust) was laid down on New Caledonia 37 *mya* (million years ago). Due to the toxic characteristics of these types of soils, which are poor in vital elements like potassium or calcium, and relatively rich in heavy metal (e.g. nickel, magnesium, and iron), both Nasi (1982) and Jaffré (1995) have suggested an enhanced competitiveness of gymnosperms against angiosperms on this substrate since angiosperms tends to be more sensitive to the toxic conditions. In such an environment, with reduced competitive ability of the angiosperms, the range and abundance of *Araucaria* may have been enhanced. Indeed, the study of Riggs *et al.* (1998) on *A. laubenfelsii* populations revealed that *Araucaria* are pioneer species and have light demanding seedlings. Aside from the evolutionary opportunities offered by a reduction in competition from angiosperms, isostatic movement (following the erosion of the ultramafic soils) and successive glaciations events have resulted in changes in the sea level (30 *mya* to present). The subsequent formation of calcareous

substrates (emergence of coral reefs), may have also led to conditions, which promoted isolation of populations and the potential for speciation (Nasi, 1982).

In order to check whether any of these hypotheses are valid, assessing time estimates for the nodes of the phylogeny of the genus will give some insight into the timing of the speciation events and allow us to choose between each of the possible scenarios. Before any time estimate can be obtained, it is necessary to check whether the nucleotide substitution rate in our phylogeny follows a molecular clock.

3.1.2 The theory behind the molecular clock

3.1.2.1 The neutral theory

The molecular clock is a concept based on the assumption that spontaneous mutations accumulate at a constant rate within a particular gene over evolutionary time. The clock is assumed to be constant for a particular gene but different for different genes, some genes mutating more rapidly over time since the selection processes operating on them might be less stringent than on others (Gaut *et al.*, 1996). In addition mutation rates can vary over time. Birky and Walsh (1992) also suggest that there is a difference in the mode of fixation of mutation between organelles and nuclear DNA as organelles are present in multiple copies in the cells. This means that the observed mutation rates are determined not only by mutational processes at the molecular level but also by intracellular population dynamics that control fixation or loss of the mutant allele. Furthermore, there are some limitations associated with the calibration of a molecular clock and caution should be taken when using calibration point (Soltis *et al*., 2002, Sanderson, 2002). A Likelihood Ratio Test (LRT) can test the congruence of a dataset with a molecular clock (Huelsenbeck, and Bull, 1996). The LRT will assess whether the nucleotide substitution rates in a dataset follows a clock-like rate.

Theory of the LRT: two maximum likelihood analyses are run, one with a constrained clock-like change, and one without. If the two likelihoods are significantly different, the hypothesis of the molecular clock is rejected. In order to run the test, the molecular clock is set as the null hypothesis. The variable 2Δ is given by twice the difference in log likelihood of branch lengths between a rate-

constrained tree and a tree that has no constraint on branches. Here Δ = -ln constrained - (-ln unconstrained). The degrees of freedom are calculated as (n-2), where n is the number of taxa. The result is then compared to a Chi-squared distribution. The null hypothesis (molecular clock) is rejected if the probability of having such a high difference between the two likelihoods by chance is less than 5%. If the null hypothesis is rejected this implies that the nucleotide substitution rate is not constant over time.

3.1.2.2 Dating the phylogeny

If the molecular clock hypothesis is accepted, it is then possible, by knowing the time at which a split occurs in the phylogeny, to deduce the time of other splits (the rate of accumulation of substitution being constant) and therefore dating other events.

When the molecular clock is rejected, an ultrametric tree can be used to estimate divergence times on the basis of parsimony branch lengths, using a non-parametric rate smoothing (NPRS) function. NPRS does not assume equal substitution rates across the tree, which should be the case in the hypothesis of a molecular clock. The function attempts to minimise the ancestor-descendant rate difference for every node of the tree. It will therefore smooth all local transformation in rate as it changes across the tree. It is driven by the likelihood that evolutionary rates are auto correlated in time, and therefore that there is a degree of rate heritability although the degree of correlation is not fixed to any value. This means that in a case where, for example, terminal species have an accelerated substitution rate, reflected as a long branch, the length of the branch would be corrected according to other branch lengths in the same clade. Local calibration can then be applied to a node in the tree and other time estimates deduced using NPRS.

Using independent dates for two splits in the phylogeny allow us to test the consistency of the results obtained. If the different time estimates are congruent, then the confidence in the result is greater. If the estimates are different then it is important to look at the bias in the calibration point, and then decide which scenario is the most probable by comparing it with other studies or other events.

3.1.2.3 Time constraint and calibration point: the tools for dating the phylogeny

- **The fossil record**

Knowing the date of a fossil can help estimate the age of a group of individuals derived from it, or related to it, by using the fossil's age as a reference point for the appearance of the group to which it belongs. However, the use of fossil record as a calibration point requires caution. Soltis *et al.* (2002) reviewed the different problem encountered while using fossils. The first potential problem comes from the age estimation of the fossil, as accurate dating is difficult to achieve and estimations are usually used. The second point concerns the placement of the fossil along the cladogram. It is important to decide whether the fossil will be place in the stem lineage, in which case it will represent the time the lineage has diverged from its sister species, or whether it will be place in the crown group, in which case it will represent the most recent ancestor of the extant group. Finally, the age retrieved will only be a minimal age estimation. Indeed, it represents the first known record of its group and not the first occurrence. Therefore the time estimates based on fossils are an underestimation of the real age.

In this study, fossils without a sectional assignment will only be used to provide general information on the age of the genus. The first fossil for sections *Araucaria*, *Eutacta* and *Intermedia* have been found in Australia and Tasmania, and date back to 105 *mya* for the first two sections and 74 *mya* for the *Intermedia* section. The date of 105 *my* will be used as an estimate for the divergence between section *Eutacta* and the 3 other sections. The age of section *Bunya* will be used with extra caution in this study, as there are possibilities that the extant *Bunya* species (*A. bidwilli*) is not related to the extinct section *Bunya* (Setoguchi *et al.*, 1998).

- **De novo creation of virgin land to colonize: Volcanic Islands**

Using the age of an oceanic island on which an endemic species occurs can give an estimate on the appearance of the species by suggesting that the age of the species cannot be more than the island on which it lives. However, by doing so, the age of

the species can be overestimated, as this speciation event may long postdate the island origin. Another scenario could be that the species evolved long before its arrival on the island and dispersed there, going extinct elsewhere. The age of the island would therefore underestimate the species age.

- **Allopatric speciation: modification of the habitat**

Geological events like the split of a continent into two by the opening of a water channel or change in the environment by any tectonic event can also be used as calibration point to define the age of the separation between two populations of the same species and subsequent speciation. However, the time estimates using geological changes in the species environment are also slightly biased. In the case of two species living on separated continents, using the time when the two landmasses separated overlooks the possibility of long distance dispersal occurring after the break-up. The time estimates from such dating would result in an overestimation of the age of the split. In contrast, if speciation occurred before fragmentation, then the age of the species would be underestimated.

3.1.3 Substitution rates in plants

Bearing in minds all the bias that can result from the use of calibration points, it is still possible to obtain estimates of nucleotide substitution rates when an appropriate dataset is available. Several studies have then raised the issue of the heterogeneity of rates among plant lineages (e.g. Soltis *et al.*, 2002; Kasuga *et al.*, 2002; Muse, 2000). Wolfe *et al*. (1987) estimated an average range of chloroplast substitution rates for synonymous substitutions ($1.0\text{-}3.0 \times 10^{-9}$ substitution per site per year) and observed that the two IR (inverted region) had slower rates than the two single copies region. Clegg *et al.* (1994) demonstrated that the LSC (large single copy region) had even faster mutation rate than the SSC (small single copy region). Since then, numerous authors (e.g. Birky and Walsh, 1992; Bousquet *et al.*, 1992; Muse and Gaut, 1994; Provan *et al.*, 1999) have investigated the variability of rates of particular regions within specific genera or families. Bousquet *et al.* (1992) underlines the fact that annual plants have faster rates of synonymous nucleotide substitution than

perennials. However, rates for non synonymous substitutions are on average identical in both, which lead the authors (Bousquet *et al.*, 1992) to suggest that more than the generation time, factors such as population size (assuming perennials maintain larger effective population size) may be a major factor influencing the observed rate (mutations are more easily fixed in small populations).

Shifting the emphasis to non-coding regions, Gielly and Taberlet (1994) compared the evolution rate of coding regions (*rbc*L) against non-coding regions (*trn*L-*trn*F) and observed that the intergenic spacer evolved from 4 to 11 times faster than *rbc*L. Provan *et al.* (1999) also found faster evolutionary rates when studying simple sequences repeats (cpSSR) in *Pinus torreya* Parry ex Carriere. The rates they obtained for 17 loci were $3.2\text{-}7.9 \times 10^{-5}$ substitutions per site per year. Still this high mutation rate appears to the exception rather than the rule (Parfitt and Badanes, 1997; Hamilton *et al.*, 2003), and of course microsatellite regions are expected to evolve more rapidly than nucleotide substitutions.

The current study focuses on non-coding chloroplast regions in conifers. Following Gielly and Taberlet (1994) the expected values would be around four times the estimated rates for synonymous nucleotides substitution in coding regions, i.e. around $4.0\text{-}12.0 \times 10^{-9}$ substitutions per site per year.

Aims of this study

To understand the *Araucaria* radiation on New Caledonia, data on the geological and climatic history of the region will be examined and placed into the context of a molecular phylogeny in order to examine potential rates of nucleotide substitutions and estimates of divergence times of *Araucaria* species.

The phylogeny obtained in the previous chapter serves as the baseline dataset. The questions raised are: What is the time of divergence for New Caledonian species? What are the rates of evolution in *Araucaria* species? Do New Caledonian species have a Gondwanan origin?

In order to answer these questions, an insight into the geological events occurring in the area is necessary.

3.2 The geological and climatic history of New Caledonia (Table 3.2).

3.2.1 Geodynamics of the southwest Pacific (Kroenke, 1996; Pintaud, 1999; Picard, 1999; McLoughlin, 2001).

Understanding the different events involved in the radiation of *Araucaria* requires knowledge of paleogeographic and paleoclimatic events of the South Pacific area. The present structure of the region surrounding New Caledonia is complex as it is the result of several successive tectonic events.

Plate tectonic movement started 2.5 billion years ago. Since then, convergence and break up of super continents have followed each other during cycles of 400 to 500 my. The current cycle started during the middle Cretaceous, 320 *mya*, when all landmasses started to regroup as the super continent Pangea, which was completed 230 *mya*. The break-up of Pangea (due to the accumulation of heat from the mantle under the super continent) started during the Jurassic, 160 *mya*. The break-up resulted in two main landmasses, the super continent Laurasia in the north and the super continent Gondwana in the south.

3.2.1.1 Precambrian to Early Cretaceous (700-85 *mya*)

The eastern margin of Gondwana comprised Tasmantis, a piece of continent protruding from the east of present-day Australia. New Caledonia and New Zealand were located at the margin of the Tasmantis, right where the Pacific plate was in collision with Gondwana. An important collision event occurred at the end of the Jurassic (140 *mya*), when the continental and oceanic sediments accumulated in the forearc basin (a depression of the sea floor located between the Gondwanan margin and the Pacific plate) were compressed into a chain of mountains. This marked the

Period	Timeline	Epochs	Geological events		General Climate changes	Araucaria fossil records						
			Events on Gondwana	**Events on New Caledonia**		**New Zealand**	**Tasmania**	**Australia**	**Europe**	**Asia**	**America**	**Others**
Secondary Period - MEZOZOIC	175 MYA	JURASSIC	150 MYA: Gondwana and Laurasia start stretching, Australia start stretching		Wet and warm. No polar ice				173.5-166.1 MYA: - *Araucaria brownii* [*Bunya*] - *Araucaria cleminshawii* - *Araucaria sphaerocarpa* [*Bunya*]	150-140 MYA: - *Araucaria indica*	165-160MYA - *Araucaria mirabilis* [*Bunya*]	150 MYA: *Araucaria africana*
	135 MYA	CRETACEOUS	125 MYA: Africa leaves Antarctica, India separates from Antarctica and Australia, India go north 100 MYA: Africa separates from south America 80 MYA: New Zealand separates from Australia, Australia stay attached to Antarctica	80 MYA: New Caledonia separates from Gondwana	Sudden cooling, many extinction, volcanic activity, possibly cause by asteroid/comet impact?	95-70 MYA: -*Araucaia desmondii* [*Perpendiculata*-extinct] 74-65 MYA: - *Araucaria danae* [*A. heterophylla*-leaves arrangment/*A. angustifolia*] - *Araucaria haastii* [*Intermedia*] - *Araucaria owenii* - *Araucaria taieriensis*	74-65 MYA: - *Araucaria imbricatiformis* [*Eutacta* - *A. muelleri*]	105-97 MYA: -*Araucaria otwayensis* [*Eutacta*] -*Araucaria acutifoliata* [*Eutacta*] -*Araucaria falcata* [*Eutacta*] -*Araucaria lanceolata* [*Araucaria*] -*Araucaria carinata* [*Eutacta*? *A. bernieri*?] -*Araucaria seorsum* [*Araucaria*]		140-110 MYA: - *Araucaria cutchensis* 90.4-83 MYA: - *Araucaria vulgaris* [*Yezonia*] - *Araucaria nihongii* - *Araucaria nipponensis*	119-113 MYA: - *Araucaria grandifolia* 100-91 MYA: - *Araucaria clarkii* - *Araucaria darlingtonensis* - *Araucaria jeffreyi*	112-97 MYA: - *A. alexandrensis* - *A. chambersii*

Table 3.2: Summary of the geological and climatic events of the southwest Pacific and fossil information for *Araucaria*

Period	Timeline	Epochs	Geological events		General Climate changes	Araucaria fossil records						
			Events on Gondwana	**Events on New Caledonia**		**New Zealand**	**Tasmania**	**Australia**	**Europe**	**Asia**	**America**	**Others**
Tertiary Period - CENOZOIC	65 MYA	PALEOCENE	50 MYA: India collides with Asia, Australia separates from Antarctica (45 MYA).	55 MYA: Calcareous substrata from Koumac, Hienghene, Nouville go onto the Norfolk ridge	Wet and warm. No polar ice			65-54 MYA: *-Araucaria balcombensis* [*Eutacta -A. muelleri-A. araucana*] *- Araucaria lignitici* [*Eutacta* -NC species- *A. nemorosa*] *- Araucaria crassa* [*Eutacta*] *- Araucaria fletcheri* [*Eutacta -A. bitamulata*]				
	53 MYA	EOCENE		37 MYA: The Poya layer and the peridotites get in position			50-35 MYA: *- Araucaria annulata* [*Eutacta -A. columnaris*] 42.1-35.4 MYA: *- Araucaria hastiensis* [*Araucaria- A. araucana*]					
	36 MYA	OLIGOCENE	30MYA: Opening of the drake passage (circum polar current forms), Australia collide with New Guinea. Separation of Australia and New Guinea		First glacier on Antarctica. Cooler and drier.		35.4-23.3 MYA: *- Araucaria fimbriata - Araucaria readiae* [*Eutacta - A. cunninghmii*, NC species] 29-20 MYA: *- Araucaria plana* [*Eutacta* - new caledonian species] *- Araucaria prominens* [*Eutacta* -new caledonian species]					

Table 3.2 (continued): Summary of the geological and climatic events of the southwest Pacific and fossil information for *Araucaria*

Period	Timeline	Epochs	Geological events		General Climate changes	Araucaria fossil records						
			Events on Gondwana	**Events on New Caledonia**		**New Zealand**	**Tasmania**	**Australia**	**Europe**	**Asia**	**America**	**Others**
Tertiary Period - CENOZOIC	23 MYA	MIOCENE	20 MYA: Isolation and cooling of Antarctica 15 MYA: Southern Ice Cap forms 10 MYA: Australia drying out and arriving in sub-tropics	15 MYA: The rodholite calcareous soils get in position.	Warm and wet for a short period initially		25-22 MYA: - *Araucaria derwentensis* [*Eutacta -A. columnaris*] 24-21 MYA: - *Araucaria unicinata* [*Eutacta*]					
	5.3 MYA	PLIOCENE	3.7 MYA: formation of the volcanic island of Norfolk		Antarctica freezes over, brief warm period, colder again, formation of northern ice cap							
Quaternary period	1.75 MYA	PLEISTOCENE	Climate drying (ice age-sea level variation)	125 000YRS: Major reef starts building.	Ice age. Fall and rise of sea level several times							
	100 000 YRS	HOLOCENE			Current							

Table 3.2 (continued): Summary of the geological and climatic events of the southwest Pacific and fossil information for *Araucaria*

paroxysm of the Rangitata Orogeny (climax of the compressional deformation). This orogenesis resulted in the emergence of New Caledonia and New Zealand. 125 *mya*, Africa separated from Antarctica, India separated from Antarctica and Australia and migrated northward. 100 *mya*, Africa separated from South America. A rift appeared between Australia and Antarctica during the early Jurassic (96 *mya*) and started to slowly separate the two continents (Fig. 3.1).

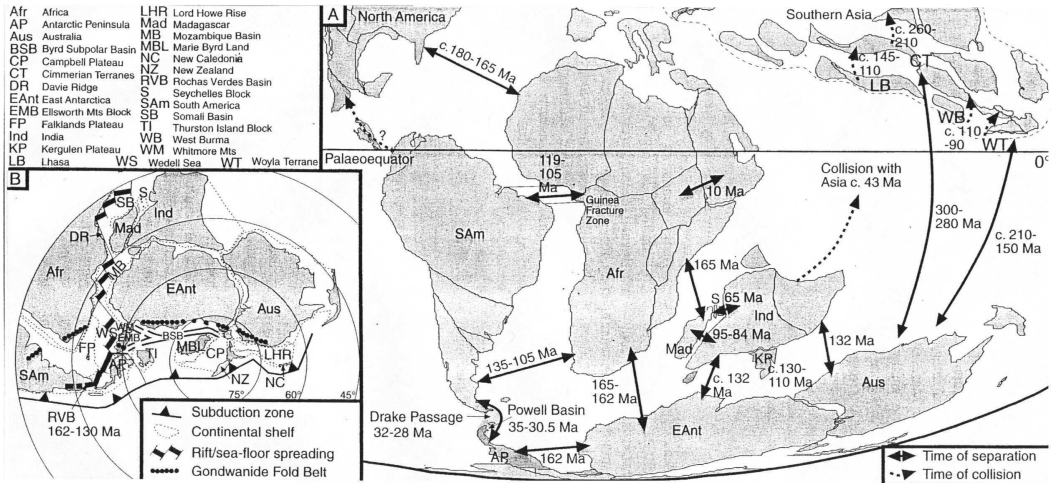


Fig. 3.1: The Gondwana break-up episodes. A: reconstruction showing the timing of separation and collision of the Gondwanan fragments. B: Polar projection of southern Gondwana (150 million years ago (from McLoughlin, 2001)

3.2.1.2 Early Cretaceous to Lower Eocene (85-55 *mya*)

The Tasman Sea started to open, which resulted in the separation of the Tasmantis from the rest of the Gondwana (Antarctic-Australia). As Tasmatis moved further from Gondwana, it started to break apart and to subside. Most of the lands were submerged except for a few landmasses on New Zealand and on the Norfolk Ridge, particularly at the level of New Caledonia. The New Caledonian Basin started to open 74 *mya*, separating the Norfolk Ridge from the Lord Howe Ridge.

The expansion of the New Caledonian Basin stopped 65 *mya* together with the expansion of the Tasman Sea 55 *mya*. To the north of Gondwana, the Coral Sea opened, separating New Guinea from the North of Australia.

While New Caledonia separated from Australia, a partial transgression occurred (increase of the sea level resulting in the partial immersion of lands). At that time, the emerged land was located west of the current one, as well as a chain of mountains located at the current site of the Central chain of New Caledonia. Due to oceanic expansion surrounding New Caledonia an abundant basaltic layer as well as basic soils covered part of New Caledonia. From Palaeocene to lower Eocene, a major transgression left very little emergent land but siliceous sedimentation indicates the presence of an emerged land in the near proximity. Though an emergence of land occurred in the lower Eocene, a new transgression partially invaded New Caledonia during the middle and high Eocene. A few coral reefs have been recorded from this period, a witness to the presence of a tropical climate.

3.2.1.3 Eocene to present (55-0 *mya*)

The Loyalty Basin started to open and final separation of Australia and Antarctica started 50 *mya* with the Australian plate moving north.

About 43 *mya*, a major change occurred in the movement of the Indo-Australian plate and the Pacific plate, due to the expansion of oceanic crust between Antarctica and Australia. This gave rise to constraints that led to accretion of a part of the oceanic crust of the Loyalty Basin onto New Caledonia. A southward subduction of the pacific plate below the Indo-Australian plate began along the Melanesian Trench. As a result a chain of oceanic islands was formed which gave birth to the Vanuatu archipelago. The subduction stopped around 25-15 *mya*. During the lower Oligocene/late Eocene all of New Caledonia was covered by peridotite (37*mya*). The accretion induced a metamorphism of high pressure and low temperatures in the south of the island. The northern massif of New Caledonia is composed of schist resulting from the metamorphism of the Eocene-Oligocene period. During the Oligocene, an important uplift of the whole island occurred and erosion became important. A new transgression occurred during the lower Miocene. The uplift started again during the middle Miocene until the Pleistocene. The barrier reef developed during the Pleistocene and Holocene. Due to major erosion, the ultrabasic crust only occupies one third of the territory nowadays. The loss of an

estimated 6000m of earth due to erosion and eustatic readjustment resulted in an uplift of the island.

Some 15 *mya*, the southern part of Tasmantis (including the southern island of New Zealand, the Campbell Plateau and the Chatman ridge) moved north and collided with the Challenger plate and the north island of New Zealand. 12 *mya* a subduction gave birth to the islands of the extern Melanesian arc. New Caledonia reached the tropic of the Capricorn 10 *mya*.

At 6 *mya*, a hotspot under the Lord Howe ridge formed Lord Howe Island. This hotspot had already given rise to a succession of submarine volcanic island except for the atoll of Chesterfield Islands and the reefs of Bellona. 3 *mya* another hotspot located under the Norfolk ridge led to the formation of Norfolk Island. Previous seamounts resulting from the activity of this hotspot are found north of the island and south of New Caledonia at less than 100 m depth.

3.2.2 Paleoclimate of the southwest Pacific (Jurassic to present).

Global climate during the Jurassic and the beginning of the Cretaceous (200-130 *mya*) was warm and humid. There was no icecap. Forests were dominated by gymnosperms. During the lower Cretaceous (120 *mya*), the climate started cooling and an icecap began to form on the South Pole, which was located on Tasmantis, between New Caledonia and New Zealand. Around 100 *mya*, the climate started to warm and the icecap melted. The climate stayed warm and humid during the Cretaceous, Palaeocene and middle Eocene. The vegetation was composed of mixed forests of gymnosperms and primitive angiosperms. Starting in the middle Eocene (50-45 *mya*), the opening of the Drake Passage resulted in the formation of the circumpolar current. A marine circulation started between Antarctica and Australia. Antarctica started to cool down and the first ice appeared on its summits (52 *mya*) .The climate in Australia started to split in distinct zones and a dry climate developed in some places. While the continental continuity persisted among the continents of Gondwana, the climate remained warm, as warm equatorial streams still reached the Campbell Plateau. Starting 40 *mya*, circulation of the deep cold stream started and

Antarctica started cooling rapidly. The flora was very poor during the Eocene though the *Nothofagus* forests persisted. At the beginning of the Oligocene, Antarctica was totally isolated and some of the ice reached the margin of the continent. Its *Nothofagus* forests were almost totally extinct at the end of Oligocene. During this time, New Caledonia and New Zealand kept a warm and humid climate. With the widening of the gap between Antarctica and Australia, the cold stream became more intense and Antarctica was slowly totally covered by ice. In the mean time, some places in Australia became drier. The climate became cooler around 10-5 *mya* and many elements of the tropical flora disappeared from New Zealand. Around 4 *mya* a warming of the temperature resulted in the partial melting of the ice on Antarctica and the sea level rose. At 3 *mya* the first glaciations started. Variation in the Pleistocene climate due to the glacial and interglacial cycles resulted in variation in the sea level by up to 140 m. When the sea level was at its lowest, land connection between New Guinea and Australia were partially re-established. New Caledonia's surface area was doubled and the Main Island was connected to the Isle of Pines in the south as well as the Belep Island in the north (Fig. 3.2 shows the actual surface of New Caledonia).

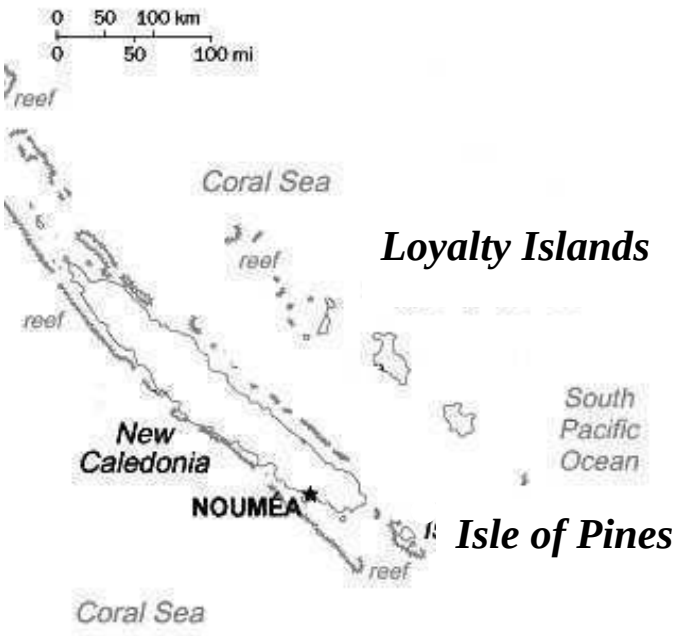


Fig. 3.2: Map of New Caledonia.

Evidence shows that many parts of New Caledonia were been immersed before the upper Eocene. However, a part of it, or at least a part of the Norfolk ridge seems to

have always remained emergent since the fragmentation of New Caledonia from Gondwana (Balgooy, 1996; Pintaud, 1999).

3.2.3 Possible land bridges to New Caledonia.

Following on the geological and climatic history of the region, three main hypotheses can be proposed to explain the arrival of *Araucaria* on New Caledonia, based on the floristic affinities of the region.

- Comparative studies of floristic affinities have highlighted the fact that the New Caledonian flora and the Australian flora are strongly related with 26.14 % of affinities (percentage of species found in both localities) (Morat *et al*, 1994). This high percentage of affinities supports a possible vicariant explanation for the origin of the New Caledonian flora (e.g. the flora predates the break-up of Gondwana). However, hypotheses of long distance dispersal across the Tasman Sea should not be discarded.

- Floristic affinities between the New Caledonian flora and the New Guinean flora are around 18% (Morat *et al*, 1994). The existence of stepping-stone dispersal via New Guinea is another hypothesis as patterns of dispersal and speciation from New Guinea to New Caledonia (via the Solomons and Vanuatu) have been argued to explain the distribution of the Sapindaceae (Balgooy, 1996).

- New Zealand and New Caledonia were linked by the continental crust along the Norfolk Ridge from the Permian (McLoughlin, 2001) until the subsidence of the Norfolk Ridge and the opening of the New Caledonian Basin 30 *mya*. The lack of high similarity between the New Zealand flora and the New Caledonian flora (Morat *et al.*, 1994; Sanmartin and Ronquist, 2004) may be due to the extinction of the tropical flora in New Zealand after the cooling of the Archipelago. Another possibility is that dispersal took place via stepping-stones across the Norfolk Ridge.

Assessing a relative time of divergence of *Araucaria* species will help in choosing between those different hypotheses. If the divergence time between New

Caledonian species and the Australian species (essentially *A. cunninghamii*) is superior or equal to 80 *mya*, then the hypothesis of a Gondwanan vicariance can be considered. On the other hand, if time estimates of the divergence of New Caledonian species are much more recent, then other scenario should be considered, involving either dispersal vicariance via another land bridge, or both.

3.3 Material and methods:

3.3.1 Materials: field sampling

Plant material of New Caledonian species was collected during three successive field seasons in December 2001, 2002 and 2003 (Chapter 1). The sampling season was chosen to match the coning season in order to ensure that cones were present to confirm species identification. Populations were located using the flora (Delaubenfels, 1972), and local knowledge. Species were determined from herbarium and field observations using both the key in the flora (Delaubenfels, 1972) and comparison to other herbarium material. A total of 23 *Araucaria* populations were sampled, including all 13 species and two or more populations per species when possible (only one population of *A. humboldtensis*, *A. subulata*, and *A. schmidii* were obtained). From each population, two individuals were sampled. For each individual 6 to 10 leaves were collected and pictures of tree shape, bark and leaves were taken. The material was dried and preserved in silica gel. Herbarium specimens, including adult foliage and juvenile foliage (when possible) were made for most of the populations.

To facilitate discussion of the results, the node separating the coastal species (*A. columnaris, A. nemorosa*, and *A. luxurians;* Clade 3 in Chapter 2) will be referred to as the coastal group. The node separating the small leaved species (Clade 2) in Chapter 2 will be referred to as the small leaved species. Accessions of *A. rulei* and *A. laubenfelsii* were grouped together in the phylogenetic analysis. This grouping is

interpreted with some caution and will be referred to here as the *A. rulei*/*A. laubenfelsii* clade, but this will not feature heavily in biological interpretation.

3.3.2 Methods

3.3.2.1 The phylogeny

The DNA regions chosen to run the analysis were the chloroplast regions *trn*S-*trn*fM as well as *psb*A-*trn*H. Considering the fact that the chloroplast genome is uniparentally inherited as a unit and not subject to recombination (Soltis and Soltis, 2000), the two regions were combined for the analysis.

Two most parsimonious trees were obtained by running a maximum parsimony analyses performed with Paup (Swofford, 2000) with heuristic searches. The tree bisection reconnection (TBR) branch-swapping algorithm was used alongside MULPARS and COLLAPSE options (collapse branch if minimum length is 0). Optimisation in the analysis was performed using Accelerated transformation (ACCTRAN) (see details in first chapter). Pairwise distances among between taxa were obtained using the option “distance” in PAUP under the setting of maximum likelihood. The model retained was HKY+G.

3.3.2.2 Molecular Clock

In order to run the maximum likelihood analysis, Modeltest 3.06 (Posada and Krandall, 1998) was run on the nexus file containing the sequences used in the phylogeny. The HKY+G model was selected. In this model, base frequencies were set to (Lset Base) = (0.3080 0.1872 0.1854) and the Ti/Tv ratio set to 0.8610.

Different calibration points were used to allow the differences in times to be compare. The calibration points chosen are (Stockey, 1982):

Fossils record (used as a minimum estimate):

- Age of the oldest fossil for each section:
  - *Bunya*: *Araucaria brownii* Stockey 173 -166 *my* (node **5**)
  - *Araucaria: A. lanceolata* Cantrill. 105-97 *my* (node **2**)
  - *Intermedia*: *Araucaria haastii* Ettingsh 074 to 65 *my* (node **5**)
  - *Eutacta*: *Araucaria acutifoliata* Cantrill. 105-97 *my* (node **1**)

Geographic events (used as a maximum estimate):

- Age of Norfolk Island: 3.7 *my* (Pintaud, 1999) as *A. heterophylla* is endemic to the island (node **6**).

- Separation of South America from Australia: 50 *my* (McLoughlin, 2001; San Martin *et al.*, 2004). This date was chosen as the section *Araucaria* only occurs in South America (node **2**).

- Separation of New Caledonia from Australia: 80 *my* (McLoughlin, 2001; San Martin *et al*., 2004). This will date the split within the *Eutacta* section between *A. cunninghamii* and the rest of the *Eutacta* species (node **3**).

Upper time constraint: The earliest record of Araucariaceae pollen is in the Triassic.

3.3.2.3 Estimation of rates using time estimations from NPRS

In order to get an estimation of the number of substitution per site per year, nucleotide substitution pairwise distances were calculated using PAUP 4.0 (Swofford, 2000). The number of substitution per site (i.e. branch length) is obtained by dividing the distance by 2. Rates are obtained by dividing the result by the time

estimated for a node. The average branch length is the mean of all the branch length from a given node to the terminals.

3.4 Results

Two most parsimonious trees were obtained from the combined analysis with a tree length of 149 steps (Chapter1), with a CI=0.94 and RI=0.97 (Fig 3.3 shows one of the two most parsimonious tree).

3.4.1 Molecular clock hypothesis rejected by modeltest

A likelihood ratio test was run on one of the most parsimonious tree obtained in the combined analysis. The log likelihood of the constrained model was 3005.01 and that of the unconstrained model was 2968.65. The chi-squared value was $2\Delta = 72.72$ with 33 degrees of freedom. The molecular clock hypothesis was rejected on the basis of this test ($p<0.001$).

3.4.2 Divergence time estimations

This study does not aim to get an exact age estimate for each node, but rather to answer broader question like the estimation of whether an *Araucaria* ancestor arrived from Gondwana, or whether it was a dispersal event. The other aim is the estimation of the age of the radiation of *Araucaria* species in New Caledonia, as to whether it has occurred during the last 3 *my* or more during the last 30 my.

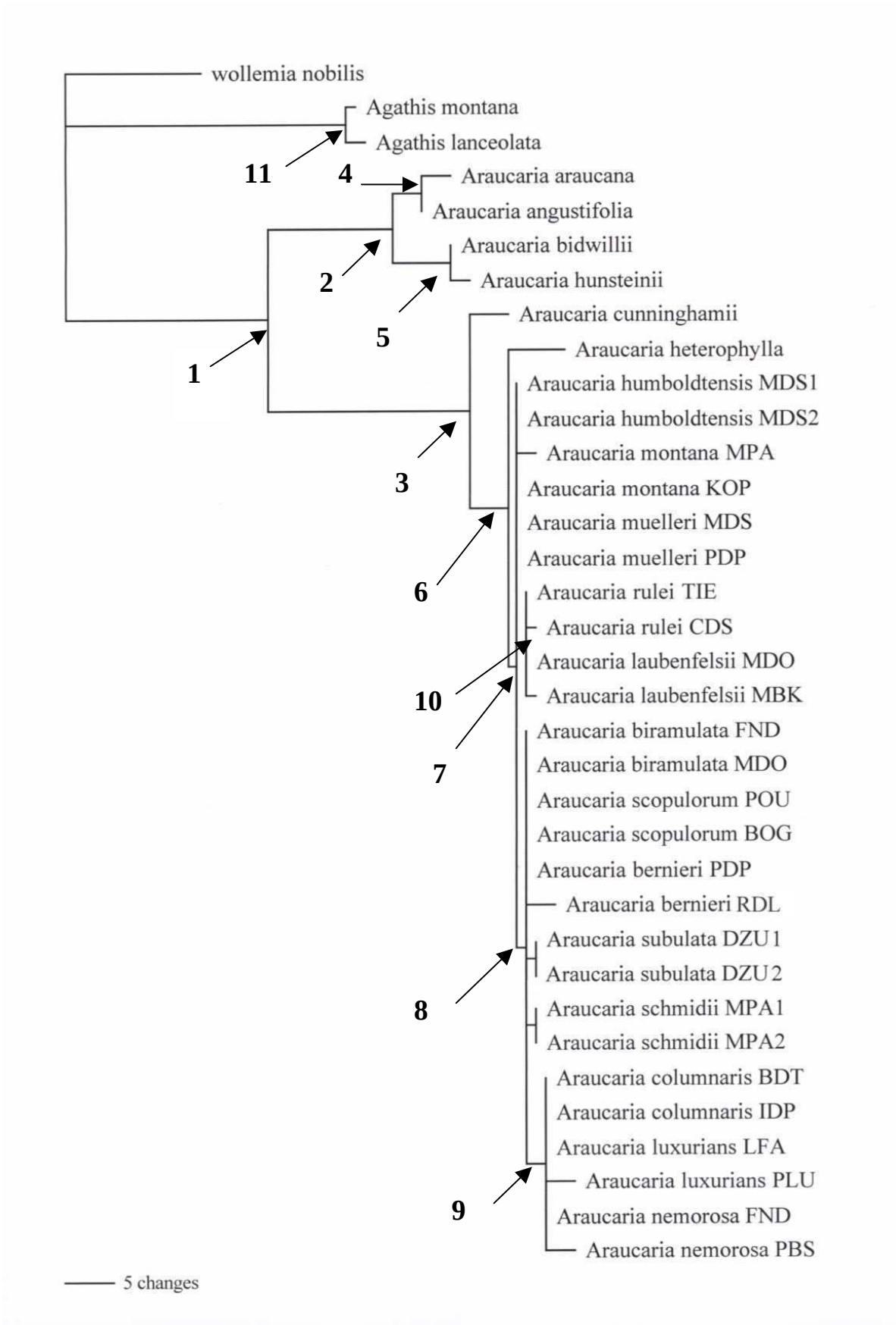


Fig. 3.3: One of the two equally most parsimonious trees obtained with the combined dataset of *psb*A-*trn*H and *trn*S-*trn*FM using parsimiony criteria. The tree is represented as a Phylogram. Tree length is 149 steps long. CI (excluding uniformative characters) = 0.94, RI (excluding uniformative characters) = 0.97; the node are numbered from 1 to 13

3.4.2.1 Estimation using fossils as calibration points (Table 3.3)

Node	Partition	Data of node according to fossil calibration		Mean	Stdev (+/-)
		Intermedia section (74*mya*)	*Eutacta*/*Araucaria* section (105*mya*)		
1	*Eutacta* vs. rest of the *Araucaria* genus	1184	**105**	644.50	762.97
2	*Araucaria* section vs. *Intermedia* and *Bunya*	296	26.25	161.12	190.74
3	Australian *Eutacta* vs. Non-Australian *Eutacta*	407	36.09	221.54	262.27
4	*A. angustifolia* vs *A. araucana*	111	9.84	60.42	71.53
5	*Intermedia* vs. *Bunya*	**74**	6.56	40.28	47.69
6	New Caledonian *Araucaria* vs. *A. heterophylla*	259	22.96	140.98	166.91
7	New Caledonian *Araucaria* clade	222	19.68	120.84	143.06
8	Small leaved species clade	135	16.4	75.70	83.86
9	Coastal species clade	111	9.8	60.40	71.56
10	*A. rulei*/*A laubenfelsii* clade	37	3.2	20.10	23.90
11	*Agathis* clade	74	6.5	40.25	47.73

Table 3.3: Time estimations (in my) from the fossil record (Times in bold are the calibration point)

Table 3.3 shows the dates estimated for each node. Only results for the *Intermedia* and *Araucaria*/*Eutacta* sections are shown as calibration points, as time estimates from the calibration of the *Bunya* section are even older than the one from the *Intermedia* section and were rejected as completely implausible.

Divergence times estimated differ greatly depending on the calibration point used. When using 74 *my* as the age of the oldest ancestor of the *Intermedia* section, the age of the genus *Araucaria* goes back to nearly 2000 *mya*. The divergence between section *Eutacta* and the three other sections is placed at 1000 *mya* (n. 1) and the radiation of the New Caledonian species is estimated at 222 *mya* (n. 7). Divergence of the small leaved species group, coastal group and the *A. rulei*/*A laubenfelsii* clade fall respectively around 135 (n. 8), 111 (n. 9) and 37 (n.10) *mya*.

Time estimates obtain from the calibration with the fossil record of the two other sections (105 my) set the age of the genus at 173 *mya*. The separation between South American species and Australasian species is placed around 26 *mya* (n. 2). Divergence of *A. cunninghamii* from the rest of the *Eutacta* section is estimated to 36 *mya* (n. 3), and the emergence of the New Caledonian species ancestor is placed around 22 *mya* (n. 6). Within New Caledonian species, divergence of the small

leaved species group, coastal group and the *A. rulei*/*A laubenfelsii* clade fall respectively around 16.4 (n. 8), 9.8 (n. 9) and 3.2 (n.10) *mya*.

3.4.2.2 Estimation using a volcanic island as a calibration point (Table 3.4)

Node	**Partition**	**Date of node based on the emergence of Norfolk island (3.7 *mya*) as a calibration point**
1	*Eutacta* vs. rest of the *Araucaria* genus	8.98
2	*Araucaria* section vs. *Intermedia* and *Bunya*	4.39
3	Australian *Eutacta* vs. Non-Australian *Eutacta*	4.83
4	*A. angustifolia* vs *A. araucana*	2.22
5	*Intermedia* vs. *Bunya*	1.02
6	New Caledonian *Araucaria* vs. *A. heterophylla*	3.7
7	New Caledonian *Araucaria* clade	2.91
8	Small leaved species clade	2.06
9	Coastal species clade	1.32
10	*A. rulei*/*A laubenfelsii* clade	0.96
11	*Agathis* clade	0.42

Table 3.4: Time estimations (in my) using the age of Norfolk Island as a calibration point

When the age of Norfolk Island (3.7 my) is used as a calibration point, the divergence between section *Eutacta* and the three other sections is estimated around 9 *mya* (n. 1). The divergence of *A. cunninghamii* from the rest of the *Eutacta* section is set around 5 *mya* and the radiation of the New Caledonian species around 3 *mya* (n. 7). From this dataset, the small leaved species group emerged 2 *mya* (n. 8 and the three coastal species evolved around 1.3 *mya* (n. 9). The divergence of the *A. rulei*/*A laubenfelsii* clade is estimated around 1 *mya*.

3.4.2.3 Estimation using geological events as calibration points (Table 3.5)

Node	**Partition**	**Date of node according to the geological calibration**		**Mean**	**Stdev (+/-)**
		New Caledonia's separation from Australia (80*mya*)	South America's separation from Australia (50*mya*)		
1	*Eutacta* vs. rest of the *Araucaria* genus	232.72	240	236.36	5.15
2	*Araucaria* section vs. *Intermedia* and *Bunya*	58.18	**50**	54.09	5.78
3	Australian *Eutacta* vs. Non-Australian *Eutacta*	**80**	72.5	76.25	5.30
4	*A. angustifolia* vs *A. araucana*	21.81	12.5	17.16	6.58
5	*Intermedia* vs. *Bunya*	14.54	11	12.77	2.50
6	New Caledonian *Araucaria* vs. *A. heterophylla*	50.9	43	46.95	5.59
7	New Caledonian *Araucaria* clade	43.63	35.9	39.77	5.47
8	Small leaved species clade	36.36	32.1	34.23	3.01
9	Coastal species clade	21.81	22.5	22.16	0.49
10	*A. rulei*/*A laubenfelsii* clade	7.2	7.5	7.35	0.21
11	*Agathis* clade	14.5	15	14.75	0.35

Table 3.5: Time estimations (in my) from geological events (Time in bold represent the calibration point)

The results obtained when geological events are used as calibration point (50 *my* for the separation of South America from Australia and 80 *my* for the isolation of New Caledonia) agree roughly on the figures. The age of the section *Eutacta* is set around 230 *mya* (n. 1). Both datasets are consistent with the vicariance dates inferred from one node specified by another (e.g. the separation between South American and Australasian species around 50 *mya* (n. 2) and a separation between *A. cunninghamii* and the rest of the *Eutacta* section around 70-80 *mya* (n. 3)). The divergence of the New Caledonian species ancestor is estimated at 45-50 *mya* (n. 6). Within New Caledonian species, divergence of the small leaved species group, coastal group and the *A. rulei*/*A laubenfelsii* clade fall respectively around 30-36 (n. 8), 22 (n. 9) and 7 (n.10) *mya*.

3.4.3 Estimations of rates using the time estimate obtained in 4.2 and 4.3 as time estimation for the nodes (Table 3.6)

The rates obtained vary depending on the calibration point used, as well as within each calibration point's dataset. The fastest rates are estimated when using the age of Norfolk Island as a calibration point (8.81×10^{-9} to 2.72×10^{-10} s/s/y). The slowest rates are found when using New Caledonia separation's date ($9.90\text{-}2.39 \times 10^{-11}$ s/s/y).

The highest rate heterogeneity is within the Norfolk Island's dataset, with the highest rate being 26 faster than the slowest. The other datasets have rate differences of 3-4 fold.

Node	Partition	Average Branch length	Age (my) based on *Eutacta*/ *Araucaria* divergence	Rate estimated s/s/y	Age (my) based on Norfolk Island age	Rate estimated s/s/y	Age (my) based on New Caledonia's separation	Rate estimated s/s/y	Age based on South America's separation (my)	Rate estimated s/s/y
1	*Eutacta* vs. rest of the *Araucaria* genus	0.019197	105	1.83×10^{-10}	8.98	2.14×10^{-9}	232.72	8.25×10^{-11}	240	8.00×10^{-11}
2	*Araucaria* section vs. *Intermedia* and *Bunya*	0.00459	26.25	1.75×10^{-10}	4.39	1.05×10^{-9}	58.18	7.89×10^{-11}	50	7.65×10^{-11}
3	Australian *Eutacta* vs. Non-Australian *Eutacta*	0.004256	36.09	1.18×10^{-10}	4.83	8.81×10^{-9}	80	5.32×10^{-11}	82.5	5.16×10^{-11}
4	*A. angustifolia* vs *A. araucana*	0.00117	9.84	1.19×10^{-10}	2.22	5.27×10^{-10}	21.81	5.36×10^{-11}	22.5	5.20×10^{-11}
5	*Intermedia* vs. *Bunya*	0.00078	6.56	1.19×10^{-10}	1.02	7.65×10^{-10}	14.54	5.36×10^{-11}	15	5.20×10^{-11}
6	New Caledonian *Araucaria* vs. *A. heterophylla*	0.003294	22.96	1.43×10^{-10}	3.7	8.90×10^{-10}	50.9	6.47×10^{-11}	52.5	6.27×10^{-11}
7	New Caledonian *Araucaria* clade	0.001125	19.68	5.71×10^{-11}	2.91	3.86×10^{-10}	43.63	2.58×10^{-11}	45.1	2.49×10^{-11}
8	Small leaved species clade	0.000871	16.4	5.31×10^{-11}	2.06	4.23×10^{-10}	36.36	2.39×10^{-11}	37.5	2.32×10^{-11}
9	Coastal species clade	0.000731	9.8	7.46×10^{-11}	1.32	2.72×10^{-10}	21.81	3.35×10^{-11}	22.5	3.25×10^{-11}
10	*A. rulei*/*A laubenfelsii* clade	0.000359	3.2	1.12×10^{-10}	0.96	7.61×10^{-10}	7.2	4.99×10^{-11}	7.5	4.79×10^{-11}
11	*Agathis* clade	0.001435	6.5	2.21×10^{-10}	0.42	3.42×10^{-9}	14.5	9.90×10^{-11}	15	9.57×10^{-11}

Table 3.6: Nucleotide substitution rates (in substitution per site per year) estimated from the different calibration points

3.5 Discussion:

3.5.1 Variations in time and rate estimates from different partitions

Several authors (e.g. Sanderson, 1997; Soltis *et al.*, 2002; Yoon *et al.*, 2004) have stressed the importance of reliable calibration points and this study is a good example of how much variation can be obtained depending of the source of information used.

3.5.1.1 Time estimations from the fossil record

In the present study, when the age of the first appearance of a fossil record assigned to either section *Intermedia* or *Bunya* were used for calibration, the divergence estimated for New Caledonian species fell back to 220 *mya*, and the age of the genus to a remarkably implausible 1,900 *mya* (the age of the split between green algae and red algae is estimated at 1400 *my* (Yoon *et al.*, 2004)). Such incoherence might be explained by the synapomorphies used to assign the fossil to each section. It is likely that the current *Bunya* and *Intermedia* section are not directly related to the extinct sections. Therefore, the dates for the divergence of sections *Bunya* and *Intermedia* might be more recent than the fossil evidence suggest.

Fossil dating using the dates of the divergence of section *Eutacta* and *Araucaria* were more reasonable, and these gave an estimated age for the genus at 173 million years, and the divergence of the New Caledonian species at 20 million years ago.

3.5.1.2 Time estimations from a volcanic island dating

When calibrating the tree with the emergence of Norfolk Island, the overall age for *Araucaria* genus was equally implausible, being placed at only 11 *my* old, and at great conflict with its 150 million year+ fossil record. Thus again the dates of the origin of Norfolk Island need utilising as a calibration point with great caution. The presence of an endemic species on a volcanic island can be problematic. The age of

an island is not the date of the arrival of the species on the island, which could be more recent, or the date of the birth of the species that can have arisen elsewhere and become extinct in other places could be far older than the island (e.g. Hawaiian-Emperor volcanic chain (Clague, 1996)).

3.5.1.3 Time estimations from the geological events

The geological events (separation of South America and the isolation of New Caledonia) gave dates that were roughly twice as old as those from the fossils of sections *Araucaria* and *Eutacta*. Thus when geological events were used to calibrate the clock, time estimates for the New Caledonian *Araucaria* divergence were around 35-44 *mya*. Of course, there is no reason to assume that lineage divergence necessarily correlates with vicariance events, and to assume that this was the case would effectively be invoking an evolutionary model which did not accommodate long distance dispersal.

3.5.1.4 Rate variation in the context of different calibration points

Depending on the calibration type used (fossil record/volcanic island/geological events) a large amount of substitution rate variation is observed. Rates vary with the calibration point used but within the data of each calibration point as well. The trend is to a slow down of the mutation rates toward the end of the branches. However, other than when Norfolk Island is used as the calibration point, rates are typically 10 or 100 fold slower than published rate estimates for other plant groups (Wolfe *et al.*, 1987; Gielly and Taberlet, 1994). Either the rates of nucleotide substitution in *Araucaria* have varied greatly during time, or the genus has one of the slowest rates of chloroplast mutation known for any plant group. One contributing factor to this may be attributed to the longevity of these species, and a long generation time could potential contribute towards slower rates of change. Another study on conifers from Krupin *et al.* (1996) reported a similar slow estimated rate of substitution in the subgenus *Pinus*.

Wright *et al.* (2003) have compared the evolution of the genus *Metrosideiros* alongside a climate gradient, going from colder latitude (New Zealand) to species living in a warmer climate (New Guinea). Their study suggests that tropical climate and warm temperature favour the metabolism of plants and increases the rate of substitution. New Caledonian species comes out as having an average rate in between New Zealand species and New Guinea species. Though such a result is only based on the observation of one genus and doesn't take into account the whole history of the genus itself or the age of the species, it raises an interesting argument regarding evolutionary change under different external environmental conditions.

3.5.2 Interpretation: Time estimate and Biogeography

The intervals of time obtained here are very broad. The following interpretations of some points in the history of the genus try to accommodate some of these wide ranges, even though this results in somewhat vague statements or multiple scenarios.

The first controversial point is the emergence of the section *Bunya* and *Intermedia*. The existence of European fossil sharing leaf synapomorphies with *A. bidwillii* has resulted in the assignment of the fossil to section *Bunya*. The same situation occurred with *A. haastii*, the *Araucaria* fossil of section *Intermedia* from New Zealand. If fossils' ages are taken into account, the two sections are rather old and diverged a long time ago, with section *Bunya* being the oldest *Araucaria* section. However, regarding the position of the two sections in several chloroplast phylogenies (Setoguchi *et al*., 1998; Chapter 1), the two extant species are not placed basally in any of the trees. Moreover, the time estimate give the divergence between the two species at no more than 15 my. If we consider their current distribution, *A. hunsteinii* occurring only in New Guinea and *A. bidwillii* being an Australian species, an allopatric speciation event related to the opening of the Coral Sea Basin would seem plausible (San Martin and Ronquist, 2004). This barrier developed 30 mya (Table 3.2).

The divergence between the section *Intermedia, Bunya* and *Araucaria* seem to be attributable to continental fragmentation as the two first sections have an

Australasian distribution, when section *Araucaria* only occurs in South America. Using the age of the section *Araucaria* and *Eutacta* as calibration point makes the age of the divergence 26 *my* old, and makes the age of the genus 173 *my* old, which fits with the age of the oldest fossils of *Araucaria* found in Europe (Stockey, 1984). However, estimating the age of the divergence at 50 *my* old (separation between Australia and South America) would make the genus 380 *my* old, which is probably too old given that the oldest conifer fossil, *Swillingtonia denticulate,* dates from the Carboniferous, and is around 310 *my* old (Adam Dimech, Website). A divergence around 26 *mya* would fit with the opening of the Drake Passage, 30 *mya*, between Antarctica and South America (McLoughlin, 2001). More information on Antarctica *Araucaria* fossil should provide a better understanding of the events that led to the emergence of the three sections.

One interesting point in this study is the age of the sister species of the New Caledonian species. *A. heterophylla* is endemic to Norfolk Island. The divergence of the species seems to be older than the age of the island. When 3.7 *my* is used as a calibration point, the age of the genus is no more than 11 *my* old, which is far too young, even considering that some of the fossils might be wrongly assigned. The other time estimates obtained place the divergence of the species back to 22 to 52 my. In between these two dates, a whole list of fossils has been found in Tasmania and Australia fitting the characteristic of the section *Eutacta* (Hills and Brodribb, 1999). It seems possible for *A. heterophylla* to have arisen outside Norfolk Island and that the island has since become a refuge area for the species.

Following on, New Caledonian species seem to have emerged sometime in the last 45 my. Depending on the calibration point used, the ancestor might have colonised the island 10 *my* before or after the layering of the ultramafic soils. Whatever the answer is, the divergence of the group including the species with smaller size of leaves is estimated to have occurred between 45 and 16 my, which would fit with the hypothesis that the ultramafic layer might have played an important role in the present diversity of the genus. The erosion of the New Caledonian relief which resulted in the uplift of the island and therefore variation in the topology and humidity level might have been one of the factors driving speciation. What is very clear is that the small number of substitutions separating the

New Caledonian species does not necessarily seem to equate with very recent speciation, as ‘conventional’ plant cpDNA rates would translate to an overall very young genus in conflict with the fossil record. Thus a vague approximation of the diversification of the New Caledonian *Araucaria* species is favoured as being between 10 and 43 *my* before present.

This uncertainty in timing precludes a confident assessment of whether the species presence on New Caledonia is attributable to vicariance or dispersal. The most realistic fossil dating (section *Eutacta* and section *Araucaria*) dates the New Caledonian lineage as diverging from the Australian lineage some 45 *my* after the land masses separated, which could indicate long distance dispersal. However, given that fossils do not represent the maximum age of a lineage, this should be interpreted with caution. Certainly the date estimates obtained from using vicariance events are not wildly incongruent with the fossil record, and for now, the most conservative option is to rule neither possibility out. However, it is worth noting that the New Caledonian *Araucaria* might not have persisted on New Caledonia since Gondwanan fragmentation.

4.1 Introduction

More than one third of the world's conifers are listed on the IUCN red list for conservation (Farjon and Page, 1999). The urgency to protect those species is rising in the context of more awareness of the importance of Biodiversity (International Conference on "Biodiversity: Science and Governance", 2004). New Caledonia is one of the hotspots of conifer biodiversity with more than 6% of the world conifers. An important focus is currently being placed on the genus *Araucaria* of which 70% of the species are endemic to the island and 11 are 'red listed' (Manauté *et al*., 2003). Indeed, 11 out of the 13 *Araucaria* species are found on ultramafic soils, which are exploited for their minerals, particularly for nickel extraction. Three species are particularly affected by mining: *A. rulei*, *A. montana* and *A.nemorosa*. With such a high degree of threat, it has become important for each population of *Araucaria* to be clearly located and determined. Having confidence in the identity of each conifer population present on the island is essential for producing effective conservation measures, as well as contributing to better knowledge of conifer biodiversity in New Caledonia.

4.1.1 Weakness of the Flora.

Following the publication of the conifer account in the Flora of New Caledonia (DeLaubenfels, 1972), researchers in the field and the herbarium have experienced some difficulties in distinguishing some of the taxa (Jaffré, pers. comm., 2002; Manauté, pers. comm., 2002; Chauvin, pers. comm., 2002). There are several types of problem.

- The flora was produced in 1972. Many more records and herbarium specimens have come to light since then.

- Following on from this, some species do not appear to be morphologically distinct. This perhaps relates to the original descriptions being based on limited material.
- Some locations listed for a given species in the Flora are doubtful, such that the species present at a given site sometime do not appear to correspond to the species listed in the Flora for that site. This can lead to confusion as to what the range limits of a species are. For instance, if all of the localities for a given species in the Flora are considered to contain members of that species, but actually include populations of other species, the defining characters for species have effectively been blurred.
- The Flora makes extensive use of reproductive characters. These are rarely scorable in the field making identifications difficult. On of the main characters used in the Flora to tell the species apart is the shape of the microsporophylle of the male cone. However, during the coning season (November to February), very few cones are available and most of the time these are out of reach. It is therefore very difficult to use this character to determine species identity when a doubtful individual is sampled.
- All juvenile *Araucaria* have a similar gross morphology and leaf shape. It is almost impossible to tell species apart at this stage except in the nursery, where, by observing individuals growing side by side and from the same age, differences in height can be observed. This problem occurs in sampling too, as the lower and more accessible branches of adult trees (2-3m above ground) often show juvenile characters.

4.1.2 Introduction to the problem of species determination

Following field observations, personal communications (Jaffré, pers. comm., 2002; McCoy, pers. comm., 2002; Chauvin, pers. comm., 2002) and previous work (DeLaubenfels, 1972; Veillon, 1980; Nasi, 1982; Manauté, 2003), conclusions have been drawn on possible cases where confusion might arise.

The following diagram (Fig. 4.1) summarises the possible ambiguity existing between each pair of species (see also Chapter 5). Some species are easily identified, like *A. humboldtensis* or *A. schmidii*, two species occupying the highest localities of New Caledonia, Mont Humboldt (1618m) and Mont Panie (1628) respectively. Both have very robust branches. Aside from this, and their very distinctive foliage (see Chapter 5), *A. humboldtensis* has a tendency to be covered by white exudates and *A. schmidii* to naturally generate multi-stemmed trees. Among the coastal species group, *A. nemorosa* is quite easy to identify. For one, it only grows in a very restricted area on the island, and has very distinct leaves, spear-like and straight, a feature that is observable on juveniles of other species but disappears in these once the foliage gets older. The very long bracts at the base of the male cone are also very characteristic. *A. columnaris* is also very distinctive. Though its adult shape might resemble those of other species (*A. luxurians* or *A. biramulata*), the leaf characteristics (see Chapter 5) as well as its male cones (particularly the blade of the microsporophyll) are very distinctive. Also, it is the only New Caledonian *Araucaria* that develops well on calcareous soil.

The major problems seem to lie within the big-leaved group where the separation between each species is less clear. The difficulty in distingushing *A. montana* and *A. laubenfelsii* has been raised on several occasions, as the separation between the two species seems more altitudinal than morphological (Jaffré, pers. comm., 2002). Though typical samples (like those described in the Flora) can be found, lots of intermediate states exist. Another problem, which has resulted in confusion between *A. rulei* and *A. montana*, is the difference between the living samples and the dried specimens. Leaves have a tendency to curl upon drying,

resulting in a change in the leaf shape. The shape of dried leaves of young *A. rulei* can be confused with those of adult *A. montana*. The distinction between *A. muelleri* and *A. rulei* can also be difficult, particularly in some populations where the leaf size and morphology is intermediate between the two species. Further problems are encountered when considering the pair *A. bernieri* and *A. scopulorum. A. bernieri* has been recorded in two northern localities (Poum and Thiebagui). However, the herbarium samples coming from those two localities have a morphology similar to *A. scopulorum*, apart from one specimen dated 1966 (H.S. MacKee, 14349). *A. bernieri* is also confused with *A. subulata* as the adult trees have a very similar shape (tall and columnar). This can be a problem when the populations are inaccessible and species identification is based on observation from a distance. This also happen for the *A. biramulata / A. luxurians*, whose tree and leaf shapes share similarities (adult trees tall and columnar, leaves around 8mm long x 4mm wide, acute). To illustrate how some of these problems can be encountered and solved, three ambiguous species identifications will be dealt with in this chapter.

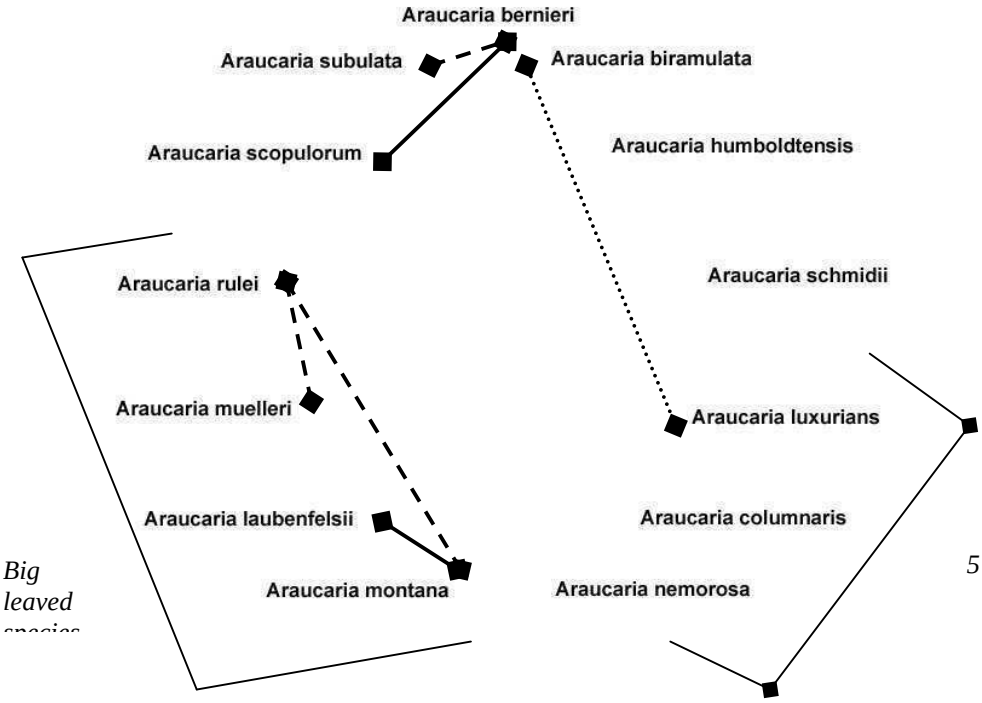


Fig. 4.1: Diagram showing the possible confusions between each pair of species (no line: no confusion; …: some confusion possible; ---: one or two known case of misidentification; ___: species delimitations issues often raised

4.1.3 Species delimitations/ Identification case studies.

1) *A. bernieri*'s known distribution would be reduced if the species is not present in two northern population mentioned in the Flora (Delaubenfels, 1972; Chapter 5). The clear delimitation of its population is therefore a priority. How accurate is the current understanding of the species distribution?
2) The 'coastal group' clade is defined by containing species with a littoral distribution (*A. columnaris, A. luxurians, A. nemorosa*). However, they have the potential to survive on in-land sites and a population of *A. luxurians* has been recorded in the middle of the island. Is this record correct? Is it possible to find *A. luxurians* inland in forest habitats?
3) Finally, *A. muelleri* and *A. rulei* are two species with a totally separated distribution. *A. muelleri* is located in the south of the island and *A. rulei* in the centre and north. However, a number of southern populations of *A. rulei* have been recorded by Nasi (1982) and recent fieldwork has found an unusually large leaved type of *A. rulei* in the region of Kouaoua. Is the north/south distribution, therefore, a reality, or is there cryptic overlap in the range of these species?

The aims of this chapter are therefore to raise the problems of species delimitation/ identification, and see to what extent the use of morphological and molecular data can contribute to clarifying species determination. The approach and material and methods used will be reviewed in each separated section.

4.2 How accurate is the current understanding of *A. bernieri*'s distribution?

The identification of all populations of *Araucaria* is important for future conservation measures. Information has been given (Jaffré, pers. comm. 2002) that

an *A. bernieri* population was present in the Reserve of the Montagne des Sources. In order to check this information (among others), preliminary fieldwork was undertaken in 2002. However, two populations were observed in the region stated. The identification of each population was difficult due to the inaccessibility of the trees. Species identification was made from a distance. In the valley *A. bernieri* (22d09'19. 37''S /166d35'28. 61''E; 494 m) was identified whereas at the entrance of the reserve (22d09'27. 91''S /166d35'29. 65''E; 443m) *A. subulata* was tentatively identified. In order to obtain a definitive identification of the two populations, a second field trip was organised in 2003. Specimens of the two populations were collected. Upon collection of the specimens, doubts were raised over the species identification. In order to reconfirm the identification, material was brought back for comparison with material from other populations. To support the morphological observations, molecular data was also collected from the samples.

4.2.1 Approach

The first comparisons were conducted on populations of *A. bernieri* and *A. subulata* available from the database of the Royal Botanic Garden Edinburgh.

The second set of examinations compared samples from the two population collected from Montagne des Sources, labelled as Population B (supposedly *A. bernieri*) and Population S (supposedly *A. subulata*).

The two results were compared in order to confirm or refute the putative species determinations.

4.2.2 Material and methods

4.2.2.1 Morphological observations

Three populations of *A. bernieri* were available for comparison (Riviere des Lacs, Pic des Pins and Lac de Yaté) together with one population of *A. subulata* (Dzumac).

The list of herbarium samples available for the measurements is given in Table 4.1. The morphological information was obtained by measuring the leaf width and length of both leaves on twigs and leaves on branches bearing the twigs (Fig. 4.3). Two sets of observations were then made to detect the presence of papillae on the margin of the leaf and stomata on the adaxial surface of the leaf (Fig. 4.2 and 4.3). Due to variation in the leaf morphology of juvenile, shaded and adult foliage, the observations focused solely on adult specimens.

In order to check whether the species measured had significantly different character means, an analysis of variance (ANOVA) was be run on the datasets using Minitab v.14 (Minitab Inc.). The null hypothesis for each test was that the sets of data had the same mean. This was rejected where $P < 0.05$ (probability of the null hypothesis being true less than 5%).

4.2.2.2 Molecular data

The list of samples used for the molecular analysis is given in Table 4.2. The molecular data are based on a set of chloroplast microsatellite markers obtained from the *trn*S-*trn*Fm and *psb*A-*trn*H regions (Chapter 2). For each population between 6 and 10 samples were scanned for the three microsatellites. The chloroplast molecule being non–recombinant, the results of the three markers were combined in one final haplotype. Chapter 2 also revealed the existence of 2 nucleotide variants in the chloroplast region *psb*A-*trn*H. 2 samples from each population of Montagne des Sources were sequenced for comparison.

4.2.2.2.1 DNA extraction

DNA was extracted from 0.5g of silica dried leaf material using Plant DNeasy kit (Qiagen, UK). Leaf material was placed in a 1.5 ml eppendorf tube and frozen by immersion in liquid nitrogen. DNA was extracted following the manufacturer's instructions using all the steps

Species name	Collector	Collector's number	Country	Origin	Barcode
A. bernieri	New Caledonia Araucaria Expedition	361	Lac de Yate, NC	Royal Botanic Garden of Edinburgh	E00141606
A. bernieri	New Caledonia Araucaria Expedition	362	Lac de Yate, NC	Royal Botanic Garden of Edinburgh	E00141605
A. bernieri	New Caledonia Araucaria Expedition	669	Pic des Pins, NC	Royal Botanic Garden of Edinburgh	E00137601
A. bernieri	Third New Caledonia Araucaria Expedition	4001	Riviere des Lacs, NC	Royal Botanic Garden of Edinburgh	E00166489
A. bernieri	H. S. McKee	15393	Riviere des Lacs, NC	New York Botanical Garden	-
A. subulata	New Caledonia Araucaria Expedition	679	Mont Dzumac, NC	Royal Botanic Garden of Edinburgh	E00131569
A. subulata	New Caledonia Araucaria Expedition	680	Mont Dzumac, NC	Royal Botanic Garden of Edinburgh	E00137880
A. subulata	Gordon McPherson	5039	Mont Dzumac, NC	New Zealand, DSIR, Botany division	-
A. subulata	Gordon McPherson	5038	Mont Dzumac, NC	New Zealand, DSIR, Botany division	-
Population B	Third New Caledonia Araucaria Expedition	4272	Montagne des Sources, NC	Royal Botanic Garden of Edinburgh	E00166507
Population S	Third New Caledonia Araucaria Expedition	4258	Montagne des Sources, NC	Royal Botanic Garden of Edinburgh	E00166512
Population S	Third New Caledonia Araucaria Expedition	4261	Montagne des Sources, NC	Royal Botanic Garden of Edinburgh	E00131781

Table 4.1: List of herbarium material examined for *A. bernieri*, *A. subulata* and the individuals of Population B and S

Species	Locality	Collector	Collector number	Number of samples
A. bernieri	Lac de Yaté	New Caledonia Araucaria Expedition	369-375	7
A. bernieri	Pic des Pins	New Caledonia Araucaria Expedition	669-678	10
A. bernieri	Rivière des Lacs	Third New Caledonia Araucaria Expedition	4000-4009	10
A. subulata	Dumac	New Caledonia Araucaria Expedition	679-687+689	10
Population B	Montagne des Sources	Third New Caledonia Araucaria Expedition	4266-4275	10
Population S	Montagne des Sources	Third New Caledonia Araucaria Expedition	4251-4260	10

Table 4.2: List of material sample use for the molecular analysis for *A. bernieri*, *A. subulata* and the individuals of Population B and S

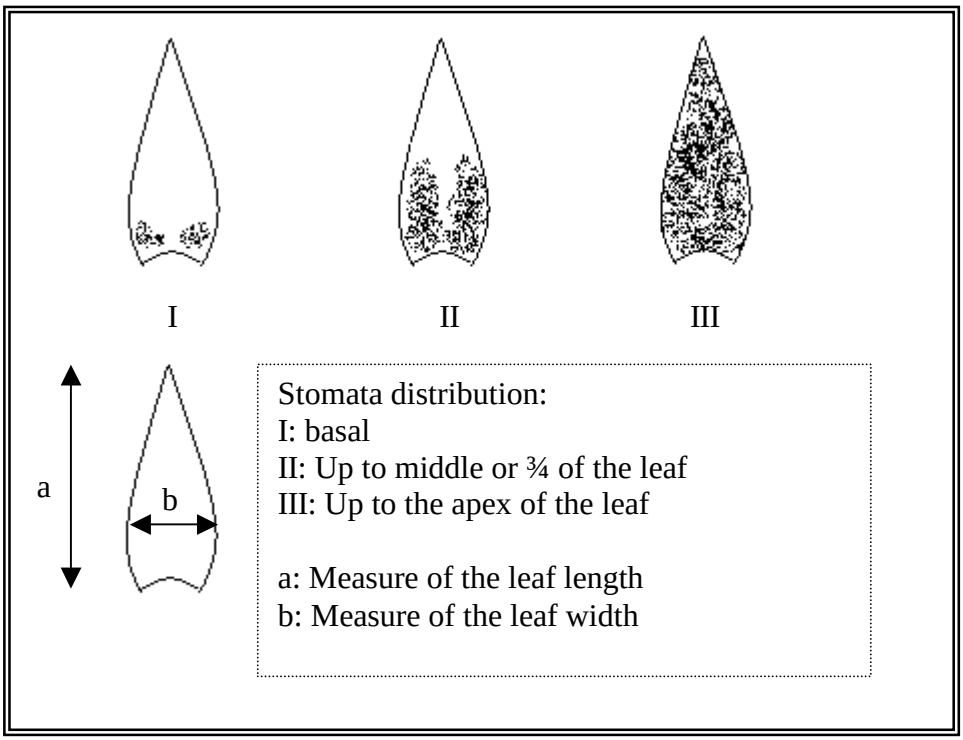


Fig. 4.2: Diagram showing the three types of stomata distribution of stomata on adaxial face of leaves as well as the type of measurement made on leaves

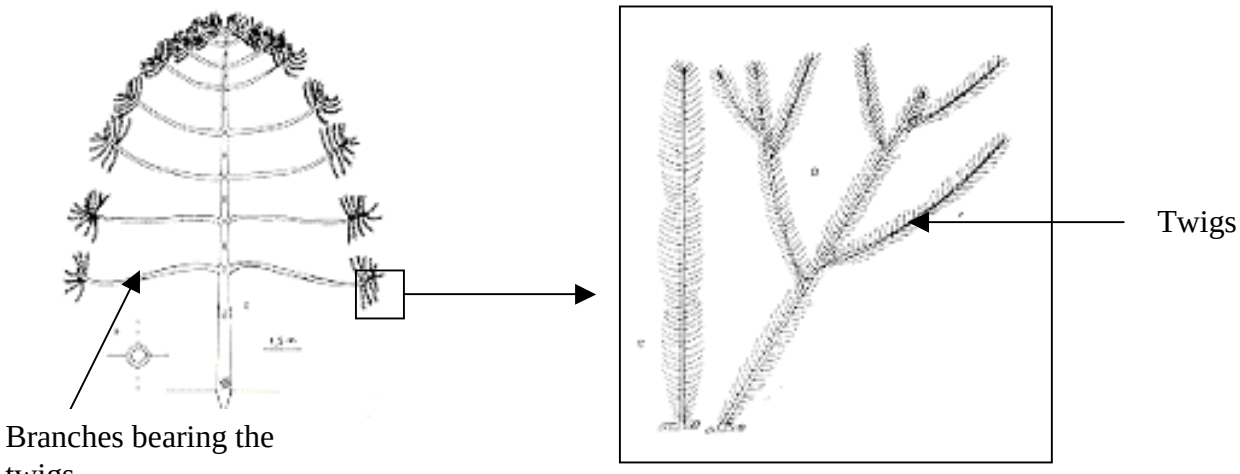


Fig. 4.3: Diagram showing the two part of the tree whose leaves were measured: the branches bearing the twigs and the twigs

4.2.2.2.2 Microsatellite analysis (AP1, AP2, AP3, PSBA)

For the PCR, 1 µl of DNA was combined in a 10 µl PCR with 1µl of 10X NH_4 buffer (Bioline), 1 µl of dNTPs (2 µM), 0.4 µl of 50mM $MgCl_2$, 1 µl of each primer, 0.25units of Biotaq DNA polymerase (Bioline) and 5.05 µl of distilled water.

The amplifications were performed in a MJ Research PTC-200 Thermal Cycler with a first denaturising step of 12 min at 94 ºC, followed by 30 cycles [15s of denaturising at 94 ºC, 15s of annealing at 60 ºC and 25s of extension at 72 ºC, with a final extension step of 72 ºC for 30 min (Grivet *et al.*, 2001).

Microsatellites were run with the size standard 400 on the CEQ8000 Beckman sequencer. Electropherograms were analysed using the Default parameters in the Fragment Analysis module of the CEQ software package version 8.0.

4.2.2.2.3 DNA sequencing (*trn*S-Fm, *psb*A-H)

DNA analyses were run on a sample of 5 to 10 individuals per population.

- Polymerase chain reaction

For the polymerase chain reaction (PCR), 2 µl of DNA were added in a 50 µl PCR containing 5µl of 10X NH_4 buffer, 5µl of 2mM dNTPs, 2.5µl of 50mM $MgCl_2$, 1.5µl of each 10µM primer, 1.25units of Biotaq polymerase (Bioline, UK) and 32.5µl of distilled water.

The amplifications were performed in a MJ Research PTC-200 Thermal Cycler with a first denaturising step of 4 min at 94 ºC, followed by 30 cycles [45s of denaturing at 94ºC, 45s of annealing (Ta shown in Table 2.4) and 1-4 min of extension at 72 ºC, with a final extension step of 72 ºC for 10 min (Grivet *et al.*, 2001). PCR products were purified using the Qiaquick PCR purification kit (Qiagen, UK) according to the manufacturers' instructions.

- Sequencing reaction

Sequencing was performed in both directions using the same forward and reverse primers as the PCR. For the sequencing reaction, 1 µl of DNA was combined in a 10 µl PCR containing 4µl of Quickstart DTCS mix (Beckman Coulter, UK), 1 µl of 10 µM primer and 4 µl of distilled water. The PCR conditions were as follows: 35 cycles of [20 sec at 96°C, 20 sec at 50°C, 4min at 60°C].

- PCR sequencing purification

For each reaction, 10 µl of distilled water was added to the 10 µl PCR product, which was then transferred to a fresh 0.5 ml tube containing 4 µl of "stop solution" (1.5M NaOAc + 50 mM EDTA) and 1 µl of 20mg/mL glycogen. 60 µl of 100% cold (-20ºC) ethanol was then added to each reaction, mixed throughly centrifuged in a microcentrifuge (~13 000 rpm) at 4 ºC for 15 mins to precipitate the DNA. The supernatant was removed, and 200 µl of cold ethanol (70%) were added to wash the pellet then the tubes were centrifuged in a microcentrifuge (~13000 rpm) for 5 mins. The ethanol wash was repeated a second time. The pellet was dried in a vacuum centrifuge for 2-5 mins and resuspended in 40 µl of Sample Loading Solution (Beckman Coulter, UK).

- Sequencing electrophoresis, trace analyses, and matrix assembly

Sequences were run on a Beckman CEQ8000 sequencer and analysed using the Default Analysis parameters from the Analysis module of the CEQ8000 software version 8.0. Pre-peak reduction was applied when enzyme slippage occurred. Analysed sequences were exported into Sequencher software version 4.5 for automated alignment. The alignment was then checked manually. The completed matrix was saved as a Nexus file.

4.2.3 Results

4.2.3.1 First set of observations: quantity of morphological and molecular variation due to species identity

- The morphological data

The lengths of the leaves are bigger in *A. subulata* samples with an average size of 4.02 mm on the branches bearing the twigs and 3.82mm on the twigs against respectively 1.06 and 2.9 mm in *A. bernieri* (Fig. 4.5, Table 4.3). However the analysis of variance showed that the leaf length on the branches bearing twigs was significantly different ($F_{1,6}$=44.54, P<0.001) in the two species whereas the leaf length of the twigs was not ($F_{1,6}$=2.43, P<0.17) (Table 4.5 a and b). The leaf width of twigs and branches bearing twigs are almost the same in the two species with a size range between 1 and 2 mm.

The stomata distribution varies slightly between the two species. The rows of stomata tend to go to the apex of the leaves on the adaxial face in *A. subulata.* However it stops around mid-leaf in *A. bernieri*, sometime reaching ¾ of the leaf but almost never reaching the apex.

- The molecular data

Five haplotypes were found over all the populations (Table 4.6, 4.7 and Fig. 4.8). The population of *A. bernieri* shared haplotype 2, 3 and 4, whereas the individuals of the population of *A. subulata* shared haplotypes 1 and 5.

Species	Leave length of the branch bearing the twigs (mm)	Leaf width of the branch bearing the twigs (mm)	Leaf length of the twigs (mm)	Leaf width of the twigs (mm)	Number of population	Number of specimen
A. bernieri	1.06 ±0.16	1.05 ±0.15	2.9 ±0.86	1.36 ±0.29	3	5
A. subulata	4.02 ±0.93	1.23 ±0.24	3.82 ±1.04	1.42 ±0.39	1	4

Table 4.3: Summary of the result obtained from herbarium material observations of *A. bernieri* and *A. subulata* (each result is based on an observation of 10 random leaves on each specimen). The full details are given in ANNEXE 4.1

Species	Leave length of the branch bearing the twigs (mm)	Leaf width of the branch bearing the twigs (mm)	Leaf length of the twigs (mm)	Leaf width of the twigs (mm)	Number of population	Number of specimen
Population B	2.2 ±0.4	1 ±0.0	3.2 ±0.74	1 ±0.0	1	1
Population S	1.07 ±0.17	1 ±0.0	3.35 ±0.79	1.1 ±0.2	1	2

Table 4.4: Summary of the result obtained from herbarium material observations of Population B and S (each result is based on an observation of 10 random leaves on each specimen). The full details are given in ANNEXE 4.1

Source of variation	DF	SS	MS	F	P	Pooled St Dev
Between species	1	17.553	17.553	44.54	0.001	0.6277
Within species	6	2.364	0.394			
Total	7	19.917				

Table 4.5a: One way ANOVA run on the dataset of the leaf length of the branch bearing the twigs measures of the populations of *A. bernieri* and *A. subulata*

Source of variation	DF	SS	MS	F	P	Pooled St Dev
Between species	1	1.711	1.711	2.43	0.17	0.8394
Within species	6	4.228	0.705			
Total	7	5.939				

Table 4.5b: One way ANOVA run on the dataset of the leaf length of the twigs measures of the populations of *A. bernieri* and *A. subulata*

Fig. 4.4: Details of the leaves of an individual from the population S. The stomata rows on the abaxial surface are easily visible and don't go up to the apex of the leave (X6.5)

Fig. 4.5: Details of leaves on the first axis (small, and close on the axis) of *A. bernieri* (X4)

Fig. 4.6: Details of a leaf of an individual from population B. Stomata are clearly visible as white rows up to the middle of the leaf (X 6.5)

Fig. 4.7: Details of the margin of the leaves of an individual from population B. Barely any papillies are visible (X40)

Haplotype	AP1	AP2	M13	Populations
1	8	9	1	Dzumac, Population B
2	8	9	2	Riviere des Lacs, Lacs de Yate, Pic des Pins, Population S
3	9	9	2	Lacs de Yate
4	8	11	2	Riviere des Lacs
5	8	10	1	Dzumac

Table 4.6: composition of the haplotypes found in *A. bernieri*, *A. subulata* and the individuals of Population B and S, and nucleotide changes in the *psb*A-*trn*H region, as well as their distribution among the populations

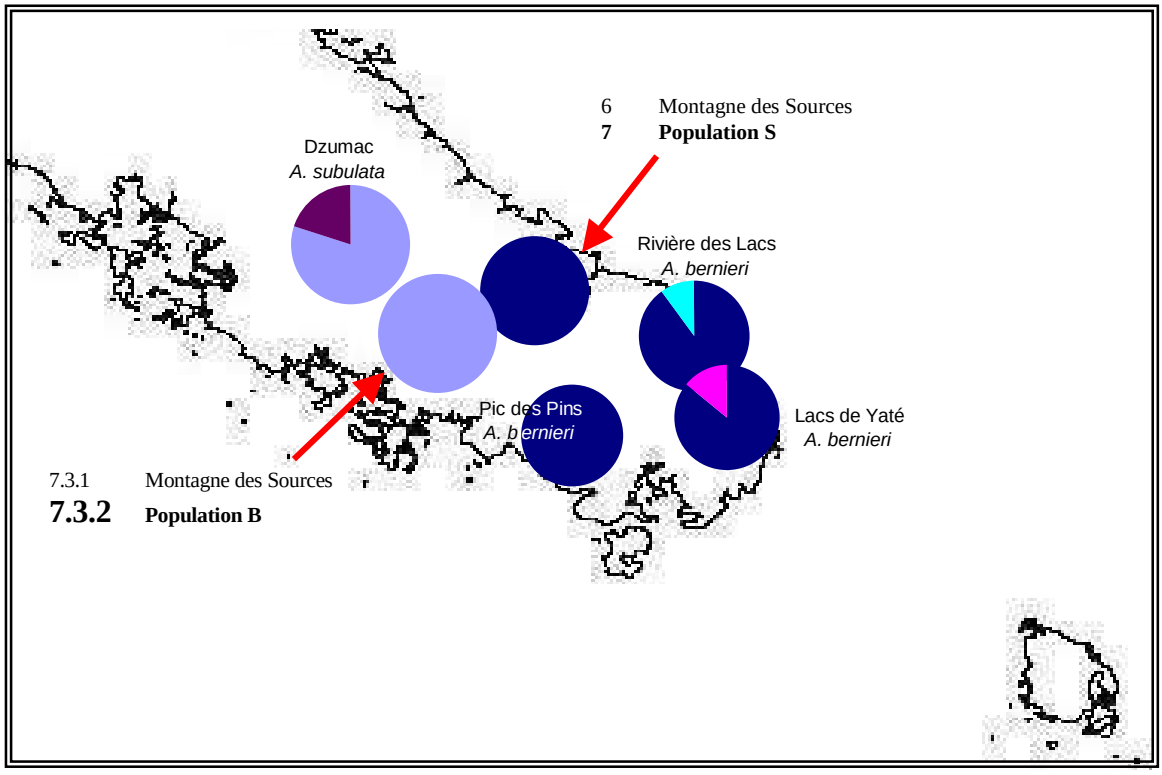


Fig. 4.8: Distribution map of the haplotypes for the population of *A. bernieri*, *A. subulata* and the Population B and S.

Taxon	Population Name	1	2	3	4	5	Total
A. bernieri	Lacs de Yaté	0	6	1	0	0	7
A. bernieri	Pic des Pins	0	10	0	0	0	10
A. bernieri	Rivière des Lacs	0	9	0	1	0	10
A. subulata	Dzumac	8	0	0	0	2	10
-	Population B	10	0	0	0	0	10
-	Population S	0	10	0	0	0	10

Table 4.7: Distribution of the haplotypes *for A. bernieri, A. subulata* and the individuals of Population B and S

Taxon	Populations	Site 156	Site 473
A. bernieri	Riviere des Lacs, Lacs de yate, Pic des Pins	C	G
A. subulata	Dzumac	T	A
-	Population S	T	A
-	Population B	C	G

Table 4.8: List of nucleotide changes in the *psb*A-*trn*H region for *A. bernieri, A. subulata* and the individuals of Population B and S.

The individuals of *A. bernieri*, *which* were sequenced, showed the nucleotide pair C/G at sites 156 and 473 of the *psb*A-*trn*H region, whereas the individuals of *A. subulata* had T/A at the corresponding sites (Table 4.8).

4.2.3.2 Second set of observations: observations concerning the two populations of Montagne des Sources

- The morphological data

For adult foliage the leaf lengths of the branches on twigs are higher in Population B (with an average of 2.20) than in Population S (mean 1.07 mm (Table 4.4). The results for *A. bernieri* and *A. subulata* have already shown that the leaf length on the branches bearing the twigs was significantly different in the two species. In order to see if the specimens from each population have leaves lengths similar to one of the two species, we can calculate the 95% confidence interval for the leaf length in each

species (Dytham, 1999). The upper 95% confidence interval for *A. berneri* is 1.06 + 1.96x 0.6277= 2.29. The lower 95% confidence interval for *A. subulata* is 4.02 – 1.96 x 0.6277= 2.79. The mean value for the single sample from population B = 2.2 mm falls within the 95% confidence interval for *A berneri* but not *A. subulata*. The mean values for the two samples from population S are 1 and 1.15 mm and fall within the 95% CI of *A. bernieri* but not *A. subulata.*.

The presence of papillae is variable in both populations (Fig. 4.7), but the stomatal distribution on the adaxial face of the leaves goes up to the apex in Population B (Fig. 4.6) whereas it usually stops half way in Population S (Fig. 4.4).

- The molecular data

Only two haplotype were found in the study (Table 4.6, 4.7 and Fig. 4.8). Individuals of Population B only had haplotype 1, whereas individuals of Population S had only haplotype 2. The two individuals of Population B sequenced had T/A at the site 156 and 473 of the chloroplast region *psb*A-*trn*H, whereas the individuals of Population S showed the nucleotides C/G (Table 4.8).

4.2.4 Discussion

The leaf lengths of the branches bearing twigs in the two doubtful populations fell into the 95% CI of *A. berneri*. However, it is noticeable that the CI was quite high due to the limited number of samples available (Dytham, 1999). Thus, though the specimen of Population B fell within the 95% CI of *A. bernieri*, this information is to be interpreted with caution as no measures from *A. berneri* ever reached 2 mm, whereas they do in the specimen of Population B. The stomatal distribution was similar between individuals of *A. subulata* and Population B, and between the individuals of *A. bernieri* and Population S. Therefore, on the basis of morphological observations, Population B seems to belong to *A. subulata* and Population S to *A. bernieri.*

The molecular data agreed with this statement as Population B shared the haplotype 1 with the population of Dzumac of *A. subulata*, when Population S shared the haplotype 2 with all 3 populations of *A. bernieri*.

In this case, the molecular data is giving support to the morphological observation and reinforcing the determination of the two populations. There is no overlap in the haplotype distribution, which suggests that there has been no hybridisation between the two populations. Therefore, the population at the entrance of Montagne des Sources seems to be *A. berneri* and the population down in the valley seems to be *A. subulata*.

The main problems of species determination with this two species often result from their tall size and similar tree shape which can make them difficult to distinguish, as well as their impenetrable habitat, which can prevent sampling. In order to avoid any confusion in the future between the two species and to confirm the identity of either a *A. subulata* or *A. bernieri*, observations of the leaf lengths from the branches bearing the twigs should be done in the field or on herbarium material. In addition the presence of the two nucleotides change A/T in the *psb*A-*trn*H region can be used as an autapomorphy of *A. subulata.* It should however be noted that the C/G sequence is not restricted to *A. bernieri*.

4.3 Is the record of the inland population of *A. luxurians* correct?

The second doubtful population was sampled in Col d'Amieu (Fig. 4.12). Local authorities (Papineau, pers. comm., 2001) indicated the presence of an inland *A. luxurians* population in the forest of Col d'Amieu. This species has so far only been found in coastal localities (Fig. 4.9) and the existence of an inland population was considered as unlikely. However in 2002, a planted population of another coastal species, *A. columnaris*, was observed alongside the road of Col d'Amieu (the tall trees are used as safeguard to prevent cars from falling down cliffs) (the localities for *A columnaris* are listed in Fig. 4.10). Furthermore in 2003, Manauté *et al.* stated that the population in Col d'Amieu population was actually *A. biramulata* (Fig. 4.11). In order to resolve the identity of this population, specimens of the population collected during fieldwork in 2002 were analyzed with molecular tools in order to compare the result with other populations of the three species. The morphological characters of the population were not be used in this study because only low branches were collected for this locality due to bad sampling conditions. The foliage of these shaded branches shows juvenile characters which are similar in all three species, and therefore cannot be used to discriminate the species.

4.3.1 Approach

The first set of analyses was done on populations of *A. biramulata*, *A. columnaris* and *A. luxurians* from the database of the Royal Botanic Garden Edinburgh. The second set of examinations was conducted on samples from the population collected in Col d'Amieu. The two results were compared in order to draw conclusions on the species identities.

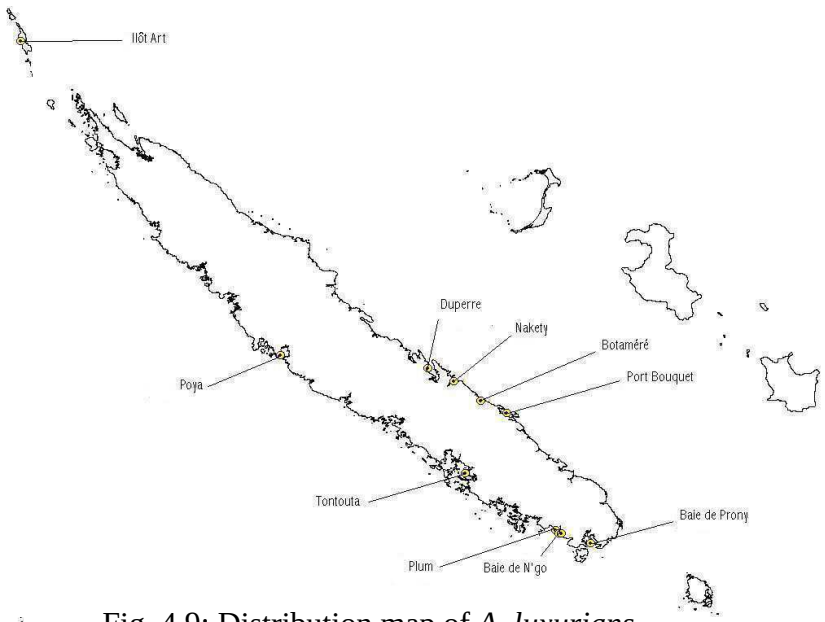


Fig. 4.9: Distribution map of *A. luxurians*

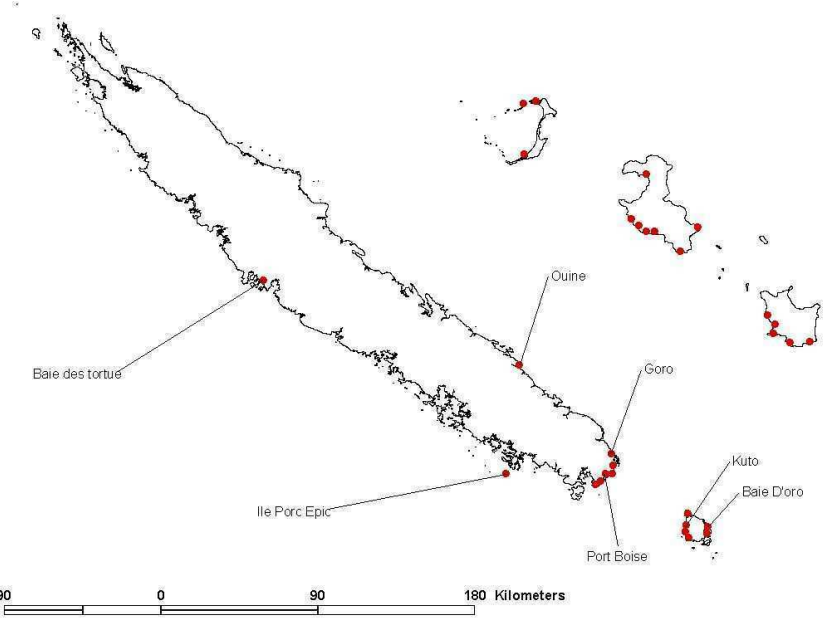


Fig. 4.10: Distribution map of *A. columnaris*

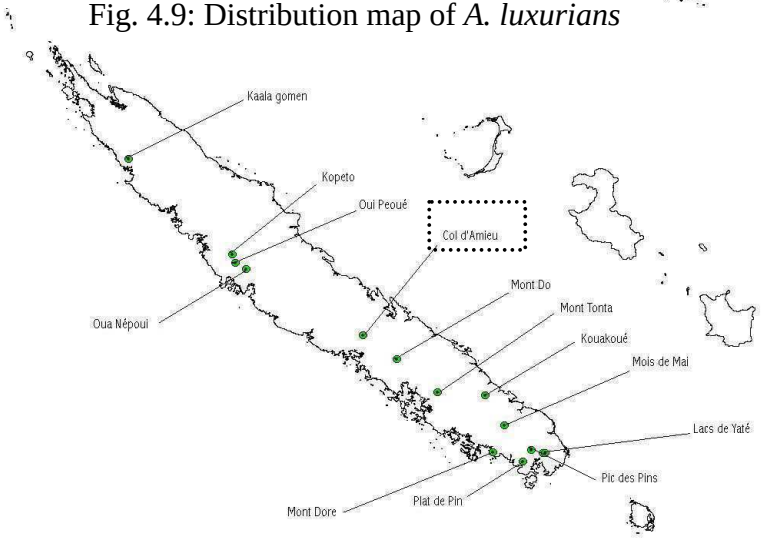


Fig. 4.11: Distribution map of *A. biramulata* (The doubtful population has been highlighted)

Fig. 4.12: Photo of the *Araucaria* population of Col d'Amieu (Photo credit: the author)

species	locality	collecto	collector number	number of samples
Population A	La Foa	Kettle C. J.; Kranitz M. L.	55-61+65	9
A. luxurians	Botamere	Third New Caledonia Araucaria Expedition	4014-4023	10
A. luxurians	Plum	New Caledonia Araucaria Expedition	930+935+940+943+946+947+954	7
A. biramulata	Foret Nord	Kettle C. J.; Kranitz M. L.	99-106	8
A. biramulata	Mont Do	Third New Caledonia Araucaria Expedition	4081-4089	9
A. columnaris	Baie des Tortues	Third New Caledonia Araucaria Expedition	5-14	10
A. columnaris	Baie d'Oro	Kettle C. J.	121-130	10
A. columnaris	Port Boisé	Kettle C. J.; Kranitz M. L.	730-739	10

Table 4.9: List of samples use for the molecular study of *A. biramulata*, *A. columnaris* and *A. luxurians*

4.3.2 Material and methods

The populations used for comparison (Table 4.9) were located at:

- Forêt Nord (FND) and Mont Do (MDO) for *A. biramulata*
- Baie d'Oro (ORO), Baie des Tortues (BDT) and Port Boisé (PBS) for *A. columnaris*
- Plum (PLU) and Botaméré (BOT) for *A. luxurians*

The molecular data were derived from a set of three chloroplast microsatellite and one minisatellite marker obtained from the *trn*S-*trn*Fm and *psb*A-*trn*H regions (Chapter 2, Chapter 4, case study 1). For each population, ten individuals were scanned for the four markers (AP1, AP2, AP3, M13). The results were then combined into one final haplotype. Chapter 2 had also revealed the existence of a 2-nucleotide variant in the chloroplast region *trn*S-*trn*Fm. 2 samples from the population of Col d'Amieu were therefore sequenced for comparison.

4.3.2.1 Microsatellite/Minisatellite analysis (AP1, AP2, AP3, M14)

Details of the analysis are given in page 101, paragraph 4.2.2.2.2, Chapter 4, case study 1

4.3.2.2 DNA sequencing (*trn*S-Fm, *psb*A-H)

Details of the analysis are given in page 101, paragraph 4.2.2.2.3, Chapter 4, case study 1

4.3.3 Results

4.3.3.1 First set of observations: quantity of morphological and molecular variation due to species identity

The AP1 microsatellite had 2 alleles, the AP2 microsatellite had 2 alleles, the AP3 microsatellite was invariant, and the M13 minisatellite had 2 alleles (Table 4.10 and 4.11). Among the studied populations, six different haplotypes were found. Their distribution among the different populations is showed on Fig. 4.13. The populations of *A. columnaris* and *A. luxurians* shared the haplotypes 3, 4 and 5 and the populations of *A. biramulata* shared the haplotypes 1 and 2. The two individuals sequenced of *A. luxurians* and *A. columnaris* showed the nucleotide A/T at the site 3 and 185 of the *trn*S-*trn*Fm region, whereas the individuals of *A. biramulata* showed C/C.

4.3.3.2 Second set of observations: observations concerning the population of Col d'Amieu (Population A)

Two haplotypes were found in the population of Col d'Amieu, haplotype 3 and 4. The sequences for the chloroplast region *trn*S-*trn*Fm were A/T at sites 3 and 185.

4.3.4 Discussion

The population of Col d'Amieu shares two haplotypes with the populations of Baie d'Oro, Botaméré, Plum and Port Boise. It shares no haplotype with the populations of Mont Do and Forêt Nord. It is mostly probable that the samples collected in Col d'Amieu belonged to the coastal species group. The overall morphology of the tree

Haplotype	AP1	AP2	AP3	M13	Sq1	Sq2	Population showing the haplotype
1	8	9	6	2	C	C	Forêt Nord, Mont Do
2	8	9	6	1	C	C	Mont Do
3	8	9	6	1	A	T	Baie des Tortues, Botaméré, Baie d'Oro, Port Boisé, Plum, Population A
4	8	10	6	1	A	T	Botaméré, Baie d'Oro, Port Boisé, Plum, Population A
5	9	10	6	1	A	T	Botaméré
6	9	9	6	1	A	T	Botaméré

7.3.3 Table 4.10: Haplotype composition of the populations of *A. columnaris*, *A. luxurians* and *A. biramulata*

Taxon	Population name	1	2	3	4	5	6	Total
A. columnaris	Baie des Tortues	0	0	10	0	0	0	10
A. columnaris	Baie d'Oro	0	0	7	3	0	0	10
A. luxurians	Botaméré	0	0	2	6	1	1	10
A. biramulata	Forêt nord	3	5	0	0	0	0	8
A. biramulata	Mont Do	0	9	0	0	0	0	9
A. columnaris	Port Boisé	0	0	7	3	0	0	10
A. luxurians	Plum	0	0	2	5	0	0	7
-	Population A	0	0	8	1	0	0	9

5.3.1 Table 4.11: Distribution of haplotypes among populations of *A. columnaris*, *A. luxurians* and *A. biramulata*

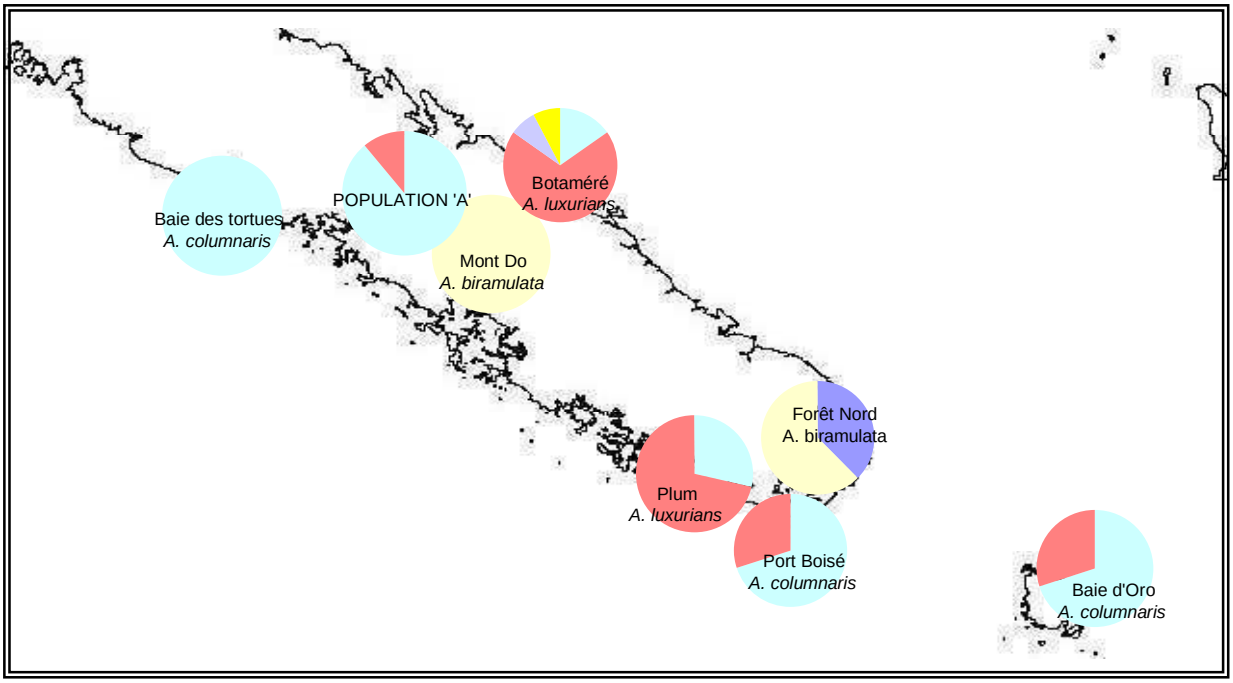


Fig. 4.13: Distribution of haplotypes among populations of *A. columnaris*, *A. luxurians* and *A. biramulata*

suggest the species might be either *A. columnaris,* as other tree have been observed in a cultivated state alongside the road or in nearby villages, or *A. luxurians* which has a similar tree shape. In order to be certain of their identity, a collection of adult material would be useful. It is not possible either to tell them apart on the basis of the foliage of lower branches, or on the current molecular data, as the chloroplast types are identical in both coastal species and are present in the population of Col d'Amieu. An extensive collection of the adult leaves from higher part of the trees (with proper material adapted to high sampling conditions) would be appropriate. However, the presence of an inland population for a species with a known coastal distribution is interesting from an evolutionary perspective. Jaffré (pers. comm., 2002) suggested that the original habitat of *Araucaria* was inland forests and the presence of these species in coastal localities might have been a secondary adaptation. This statement is supported by another similar case known from an inland population of *A. nemorosa* in Foret Nord. It would be worth investigating whether these two inland populations show greater genetic diversity. As chloroplast data failed to reveal much variability, nuclear DNA should be tried next. Microsatellite primers have been developed for Araucaria species (Robertson *et al.,* 2004) and have already been used on *A. nemorosa* and *A. columnaris* (Kettle, unpublished data). However, it cannot be ruled out that the Col d'Amieu population is simply derived from seed dispersed from the near-by planted population of *A. columnaris*.

4.4 Is the north/south distribution of *A. rulei* and *A. muelleri* a reality or is there cryptic overlap in the range of these species?

Populations of *A. rulei* are usually small and dispersed on ultramafic rocks and soils of the Main Island (Fig. 4.15). These populations have undergone an estimated decline of more than 40% due to the expansion of mining activities and their regeneration is quite poor and very slow. Adding to this is the pressure due to repetitive wild fire. As a result, *A. rulei* has been classified among the endangered species under IUCN criterion C1 (Watt, 1999). However this species is not protected in any current reserve and its conservation has become a priority. Both the Société Minière le Nickel and the Société Minière Goro Nickel have started the development of a nursery program on several mining sites, in order to reintroduce the species in non-exploited soil. Lately, there has also been recognition of uncertainty concerning the status of *A. muelleri* (Manauté *et al*., 2003). *A. muelleri* has been considered as restricted to the south of the Island (Fig. 4.14). Unlike *A. rulei*, this species is present in a few natural reserves (e.g. Montagne des Sources, Rivière Bleue), and has therefore been labeled as a Lower Risk species by Watt (1999). Nevertheless Manauté *et al* (2003) stressed that this species is being endangered in the Southern massif and should have a revised status. In such a context, the existence of populations of uncertain identity is an important matter and should be dealt with.

During two periods of fieldwork in 2001 and 2003, four populations were sampled. These were in the locality of Bwa Meyu, on the road between Kouaoua and Houailou, on Mt Dzumac on the Ouinee side of the site, in Le Trou, and finally in Mamié (Fig. 4.16). These populations were suspected to be either *A. rulei* or *A. muelleri*. It was therefore important to confirm or not the presence of *A. muelleri* at such a high latitude as Bwa Meyu and check the nature of the population at Ouinée, Le Trou and Mamié to clarify the distribution of these species. One of the aims of this study was to identifying taxon specific markers in order to strengthen future conservation measures.

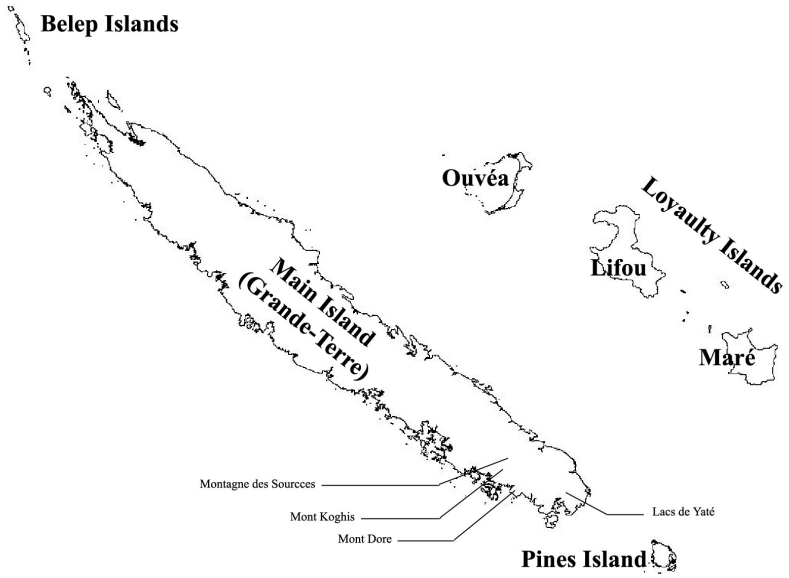


Fig. 4.14 : Distribution map of *A. muelleri* populations

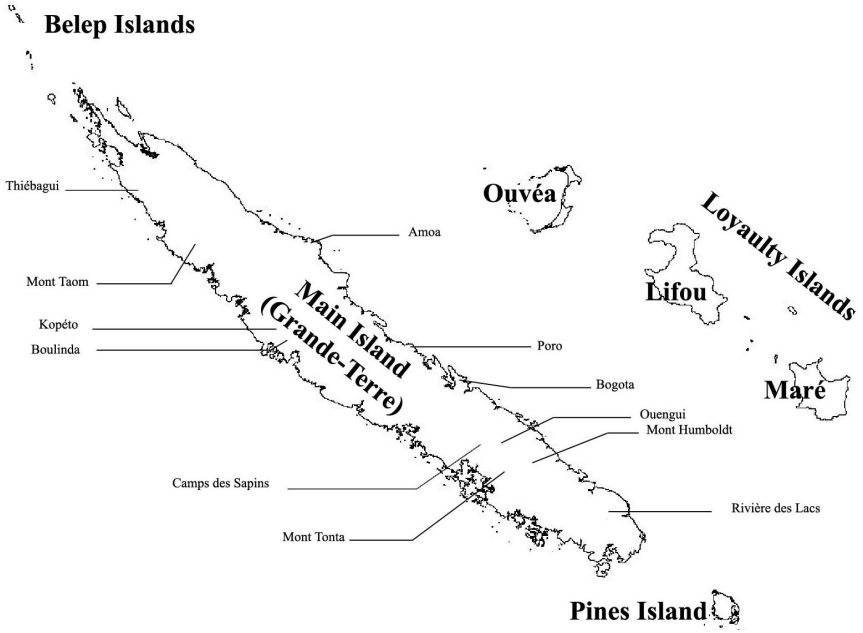
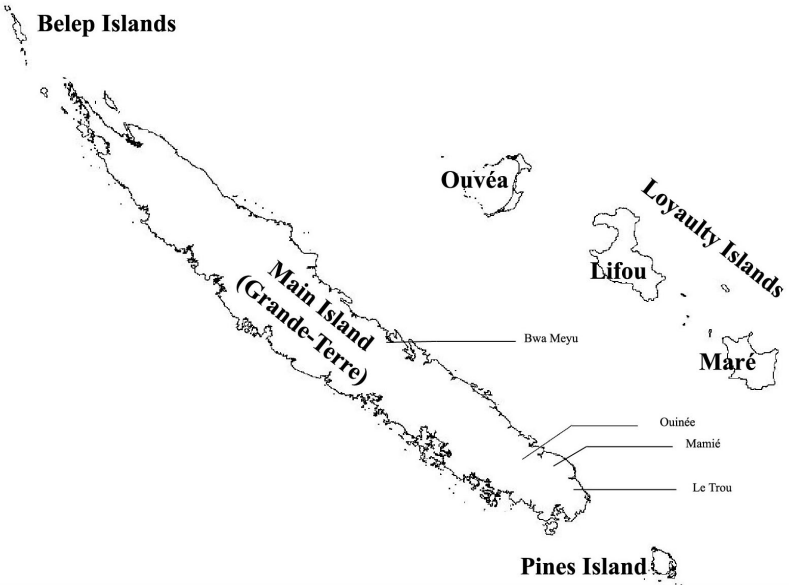


Fig. 4.15: Distribution map of *A. rulei* populations



← Fig. 4.16: Distribution map of populations of uncertain identity

4.4.1 Approach

In order to study these problems, a first set of analyses were conducted on available populations of *A.rulei* and *A. muelleri* from the database of the Royal Botanic Garden Edinburgh. The second set of studies was carried out on samples from the four populations at Bwa Meyu, Ouinée, Le Trou and Mamié. The two sets of results were compared in order to draw conclusions as to the species identities.

4.4.2 Material and methods

To assess general differences in morphological and molecular characters, individuals were selected from a range of populations to encompass the geographical range of the two species.

For the molecular analyses, 3 populations of *A. muelleri* (Koghis, Montagne des Sources, Pic des Pins), and 6 pops of *A. rulei* (Bogota, Boulinda, Camps des Sapins, Kopéto, Poro, Thiebagui) were sampled as well as the four populations of Le Trou, Mamié, Ouinée and Bwa Meyu. The list of all specimens is given in the appropriate sections.

4.4.2.1 Morphological observations

The list of herbarium samples available for morphological measurement is given in Table 4.12. Herbarium specimens from the 4 populations of *A. muelleri* (Koghis, Montagne des Sources, Mont Mou, Pic des Pins), and 5 populations of *A. rulei* (Bogota, Camps des Sapins, Rivière des Lacs, Poro, Thiebagui) were obtained from the Royal Botanic Garden of Edinburgh. Materials from these populations were

Species name	Collector's number	Collector	Country	Origin	Barcode
A. muelleri	7	Cretinon L. & Gardner M. F.	Koghis		E00107129
A. muelleri	919	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	Koghis	Royal Botanic Garden of Edinburgh	E00137435
A. muelleri	1024	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	Koghis	Royal Botanic Garden of Edinburgh	E00137433
A. muelleri	3554	H. S. McKee	Mont Mou	Royal Botanic Garden of Edinburgh	E00070963
A. muelleri	653	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	Pic des Pins	Royal Botanic Garden of Edinburgh	E00137598
A. muelleri	[TNCA] 3006	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Montagne des Sources	Royal Botanic Garden of Edinburgh	E00119771
A. muelleri	[TNCA] 3007	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Montagne des Sources	Royal Botanic Garden of Edinburgh	E00166516
A. muelleri	[TNCA] 3019	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Montagne des Sources	Royal Botanic Garden of Edinburgh	E00166515
A. rulei	241	Gardner M. F., Herbert J., Hollingsworth P. M., Ponge A.	Bogota	Royal Botanic Garden of Edinburgh	E00141268
A. rulei	242	Gardner M. F., Herbert J., Hollingsworth P. M., Ponge A.	Bogota	Royal Botanic Garden of Edinburgh	E00141270
A. rulei	1040	Gardner M. F., Herbert J., Hollingsworth P. M., Ponge A.	Bogota	Royal Botanic Garden of Edinburgh	E00141269
A. rulei	311	Gardner M. F., Herbert J., Hollingsworth P. M., Ponge A.	Camps des sapins	Royal Botanic Garden of Edinburgh	E00137883
A. rulei	312	Gardner M. F., Herbert J., Hollingsworth P. M., Ponge A.	Camps des Sapins	Royal Botanic Garden of Edinburgh	E00141033
A. rulei	86	Cretinon L. & Gardner M. F.	Poro	Royal Botanic Garden of Edinburgh	E00107123
A. rulei	194	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	Poro	Royal Botanic Garden of Edinburgh	E00137878
A. rulei	[TNCA] 5006	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Rivière des Lacs	Royal Botanic Garden of Edinburgh	E00166484
A. rulei	38	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	Thiebagui	Royal Botanic Garden of Edinburgh	E00141839
Population 1	[TNCA] 870	Gardner M. F., Herbert J., Hollingsworth P. M., Ponge A.	Le Trou	Royal Botanic Garden of Edinburgh	E00137600
Population 2	331	Gardner M. F., Herbert J., Hollingsworth P. M., Ponge A.	Mamié	Royal Botanic Garden of Edinburgh	E00141384
Population 2	333	Gardner M. F., Herbert J., Hollingsworth P. M., Ponge A.	Mamié	Royal Botanic Garden of Edinburgh	E00141379
Population 3	[TNCA] 2233	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Ouinée	Royal Botanic Garden of Edinburgh	E00131815
Population 3	[TNCA] 2237	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Ouinée	Royal Botanic Garden of Edinburgh	E00131810
Population 3	[TNCA] 2251	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Ouinée	Royal Botanic Garden of Edinburgh	E00131817
Population 4	[TNCA] 4099	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Bwa Meyu	Royal Botanic Garden of Edinburgh	E00166511
Population 4	[TNCA] 4106	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Bwa Meyu	Royal Botanic Garden of Edinburgh	E00166508
Population 4	[TNCA] 4111	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	Bwa Meyu	Royal Botanic Garden of Edinburgh	E00166487

Table 4.12: List of the herbarium material of *A. rulei* and *A. muelleri*

compared with material from populations of uncertain identity: Le Trou, Mamié, Ouinée and Bwa Meyu.

The morphological information involved measurement of the leaf width and length of the leaf of twigs (Fig. 4.2 and 4.3, case study 1). Two sets of observations were then made concerning the presence of papillae on the margin of the leaf and stomata on the adaxial surface of the leaf (Fig. 4.2, case study 1). The study only included adult specimens.

In order to check whether the measures observed have significantly different means an analysis of variance (ANOVA) was run on the datasets using Minitab v.14 (Minitab Inc.). The null hypothesis for each test was that the sets of data had the same mean.

4.4.2.2 Molecular data

Populations were sampled within a broad geographic range for both species (Table 4.16). The molecular data were derived from a set of chloroplast microsatellite/minisatellite markers obtained from the *trn*S-*trn*Fm and *psb*A-*trn*H regions (Chapter 2; Chapter 4, case 2). For each population 10 samples were scanned for the four markers.

Two sites with a single nucleotide variant were also detected in the *psb*A-*trn*H region (Chapter 1). In order to scan these sites for variation, the sequences were input in Webcutter 2.0 (http://tools.neb.com/nebcutter2) to identify which enzyme would cut at the specified nucleotide base. A PCR-RFLP analysis was then run using the two restriction enzymes *Adh*1 and *Msi*1.

The chloroplast molecule being non–recombinant, the results of the four markers and the PCR-RFLP analysis were combined into one final haplotype.

4.4.2.2.1 Microsatellites (AP1, AP2, AP3, PSBA)

Details of the analysis are given in page 101, paragraph 4.2.2.2.2, Chapter 4, case study 1

4.4.2.2.2 RFLP (*Adh*1, *Msi*1)

- Step 1

For the psbA-trnH PCR, 1 µl of DNA was combined in a 12.5 µl volume with 1.25 µl of Taq Polymerase Buffer (Bioline), 1.25 µl of 2 µM dNTPs, 0.625 µl of 50mM $MgCl_2$, 0.375 µl of each primer, 0.3215 units of Biotaq DNA polymerase (Bioline) and 8.125 ml of distilled water.

The amplifications were performed in a gradient cycler with a first denaturising step of 4 min at 94 ºC, followed by 30 cycles [45s of denaturising at 94 ºC, 45s of annealing at 60 ºC and 3 min of extension at 72 ºC], with a final extension step of 72ºC for 10 min (Grivet *et al.*, 2001).

- Step 2

Both AdhI and MsiI enzyme restrictions were performed by adding 2.5 µl of psbA-trnH PCR product with 1 µl of NEBuffer (50mM NaCl, 10mM Tris-HCl, 10 mM $MgCl_2$, 1mMl dithiothreitol, pH 7.9) supplied with the restriction enzyme by BioLabs Inc., 0.25 µl of the restriction enzyme and 2.5 µl of distilled water. Digestions were carried out for 2 h at 37 ° C.

- Step 3

Digested fragments were separated and visualised on a 2% agarose gel containing 3µl of ethydium bromide, and was run for 2 hours

In order to see how the molecular characters defined group of species, an analysis of molecular variance (AMOVA) was run using Arlequin 2000 (Schneider *et al.*, 2000).

4.4.3 Results

4.4.3.1 First set of observations: quantity of morphological and molecular variation due to species identity

- The morphological data

The leaf lengths of the twigs are generally longer in specimens of *A. muelleri* (average length above 22 mm) than in *A. rulei*, whose average leaf lengths rarely exceed 20 mm except in the sample of Riviere des Lacs, where the average is 21.5 mm (Table 4.13). The width of the leaf in *A. muelleri* is generally twice the size of those of *A. rulei* individuals, except again for the population of Riviere des Lacs. The analysis of variance showed that the leaf length and width were significantly different (respectively $F_{1,15}$=31.79, P< 0.0001 and $F_{1,15}$=21.32, P< 0.0001) in the two species (Table 4.14 a and b)..

Stomata were observed only at the very base of the abaxial face of every specimen of *A. rulei* (Fig. 4.18). On the other hand, all *A. muelleri* individuals showed abundant stomata covering going up to the apex of the leaves (Fig. 4.17 and 4.17). Dispersed or absent papillae were observed randomly on the margin of every population (Fig. 4.20).

- The molecular data

The three microsatellites of the *trn*S-*trn*Fm chloroplast regions were variable in *A. rulei* and *A. muelleri*. In *A. rulei*, 5 different alleles were found for AP1, 4 for AP2

Taxon	Population	Mean of leave length on the twigs (mm)	Mean of leaf width on the twigs (mm)	Number of specimen
A. muelleri	Koguis	27.06 ±5.21	11.56 ±3.35	3
	Mt des Sources	22.95 ±4.42	9.33 ±5.21	3
	Mt Mou	29 ±1.79	14.7 ±0.64	1
	Pic des Pins	22.9 ±2.38	13.2 ±0.87	1
A. rulei	Bogota	15.57 ±2.34	5.53 ±1.28	3
	Camps des Sapins	18.3 ±1.38	5.65 ±0.46	2
	Riviere des Lacs	21.5 ±2.34	10.9 ±0.28	1
	Poro	15.7 ±1.68	5.75 ±1.22	2
	Thiebagui	14.3 ±1.42	5.8 ±0.87	1
Population1	Le Trou	26.6 ±3.35	12.8 ±1.33	1
Population2	Mamie	20.85 ±3.60	8.3 ±3.35	2
Population3	Ouinee	16.9 ±3.06	7.67 ±2.04	3
Population4	Bwa Meyu	22.6 ±5.60	7.47 ±2.95	3

Table 4.13: Summary of the result obtained by observing ten random leaves of herbarium specimen of population of *A. rulei* and *A. muelleri*. The table also includes the results for the four questioned populations (List of full detailed measures of specimen in ANNEXE 4.1)

Source of variation	DF	SS	MS	F	P	Pooled St Dev
Between species	1	339.5	339.5	31.79	0.0001	3.268
Within species	15	160.2	10.7			
Total	16	499.6				

Table 4.14a: One way ANOVA run on the dataset of the leaf length measures of the populations of *A. rulei* and the populations of *A. muelleri*

Source of variation	DF	SS	MS	F	P	Pooled St Dev
Between species	1	150.78	150.78	21.32	0.0001	2.659
Within species	15	106.06		7.07		
Total	16	256.84				

Table 4.14b: One way ANOVA run on the dataset of the leaf width measures of the populations of *A. rulei* and the populations of *A. muelleri*

Source of variation	DF	SS	MS	F	P	Pooled St Dev
Between species	4	191.5	47.9	2.44	0.103	4.426
Within species	12	235.1	19.6			
Total	16	426.5				

Table 4.15a: One way ANOVA run on the dataset of the leaf length measures of the populations 1 to 4 and the populations of *A. muelleri*

Source of variation	DF	SS	MS	F	P	Pooled St Dev
Between species	4	87.59	21.9	2.21	0.129	3.145
Within species	12	118.69	9.89			
Total	16	206.28				

Table 4.15b: One way ANOVA run on the dataset of the leaf width measures of the populations 1 to 4 and the populations of *A. muelleri*

Source of variation	DF	SS	MS	F	P	Pooled St Dev
Between species	4	162.2	40.5	3.1	0.054	3.617
Within species	13	170	13.1			
Total	17	332.2				

Table 4.15c: One way ANOVA run on the dataset of the leaf length measures of the populations 1 to 4 and the populations of *A. rulei*

Source of variation	DF	SS	MS	F	P	Pooled St Dev
Between species	4	42.96	10.74	2.03	0.149	2.298
Within species	13	68.63	5.28			
Total	17	111.6				

Table 4.15d: One way ANOVA run on the dataset of the leaf width measures of the populations 1 to 4 and the populations of *A. rulei*

Species	Locality	Collector	Collector number	Number of samples
A. muelleri	Koghi	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	895+913-917+888-889+893+896	10
A. muelleri	Montagne des Sources	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	[TNCA] 3000-[TNCA] 3006+[TNCA] 4263-[TNCA] 4265	10
A. muelleri	Pic des Pins	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	633-639+662-663+666	10
A. rulei	Bogota	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	241+244+246+249+250+254+255+259-261	10
A. rulei	Boulinda	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	[TNCA] 2571-[TNCA] 2574+[TNCA] 2593-[TNCA] 2594+[TNCA] 2596-[TNCA] 2599	10
A. rulei	Camp des sapins	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	301-309+312	10
A. rulei	Kopeto	Kettle C. J., Kranitz M. L.	17-25+52	10
A. rulei	Poro	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	183-184+188-191+193-195+207	10
A. rulei	Thiebagui	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	38+40-41+44-50	10
Population 1	Le trou	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	858-877	10
Population 2	Mamié	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	331-336+341+345+348+362	10
Population 3	Ouinée	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	[TNCA] 2230-[TNCA] 2239	10
Population 4	Mine Bokaine	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	[TNCA] 4110-[TNCA] 4118+[TNCA] 4126	10

Table 4.16: List of material sample use for the molecular study of *A. rulei* and *A. muelleri*

and 2 for AP3. In *A. muelleri*, AP1 showed 2 different alleles, 3 for AP2 and 3 for AP3. The minisatellite M13 was variable in both *A. muelleri* (2 alleles) and *A. rulei* (2 alleles) and variation in the nucleotide sequence was found in both *A. muelleri* and *A. rulei*.

Overall, 13 haplotypes were retrieved (Fig. 4.21, Table 4.17 and 4.18). The individuals of *A. rulei* showed the most polymorphism with nine different haplotypes versus six for *A. muelleri.* The specimens from Kopeto and Poro were the ones showing the highest number of haplotypes with five different haplotypes. The individuals from Thiebagui and Pic des Pins were the least variable with only one haplotype. Chloroplast type 2 and 6 were the most widespread in *A. rulei* whereas chloroplast type 16 was dominant in *A. muelleri*. The individuals of Koghis and Pic des Pins showed no chloroplast type in common with the specimens from *A. rulei* populations. The populations of Montagne des Sources had haplotype 2 and 9 in common with some *A. rulei* individuals.

4.4.3.2 Second set of observations: observations concerning the four populations of uncertain identity

- The morphological data

The average leaf length for the four populations ranged from 16.9 mm in Ouinee samples to 26.6 mm in Le Trou's individuals (Table 4.13). The average leaf width ranged from 7.47 mm in Bwa Meyu's specimens to 12.8 mm in Le Trou's individuals.

An analysis of variance was made for each set of measurements in order to test whether the four populations had significantly different leaf length or width from *A. muelleri* or *A. rulei*. The results are presented in Tables 4.15 a to d. In each case, P was greater than 0.05 and the null hypothesis, i.e. the means of each populations tested are not different, was not rejected.

Fig. 4.17: Details of the leaves of an individual of *A. muelleri* from the population of Montagne des Sources (NCO3 3006). The stomata on the abaxial surface are easily visible in white row on the picture. The scale is in millimeters (X 6.50)

Fig. 4.18: Details of stomata rows on the abaxial face of a leaf of *A. muelleri* (NCO3 3006). (X40)

Fig. 4.19: Details of a leaf of an individual *A. rulei* (TNCA] 5006). Stomata are clearly visible as white rows only at the base of the leaf. (X 6.50)

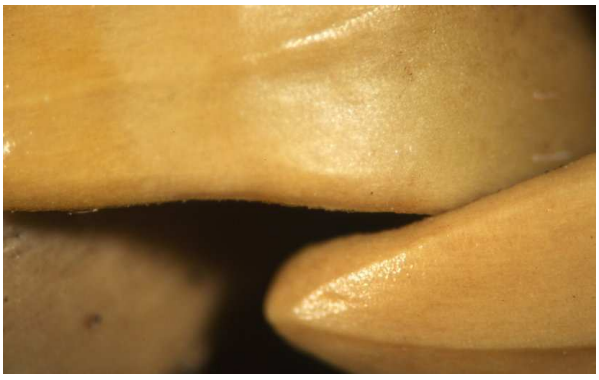


Fig. 4.20: Details of the margin of the leaves of an individual from population 3 (NCO3 2233). Barely any papillies are visible, as well as no stomata on the top of the abaxial surface (X40)

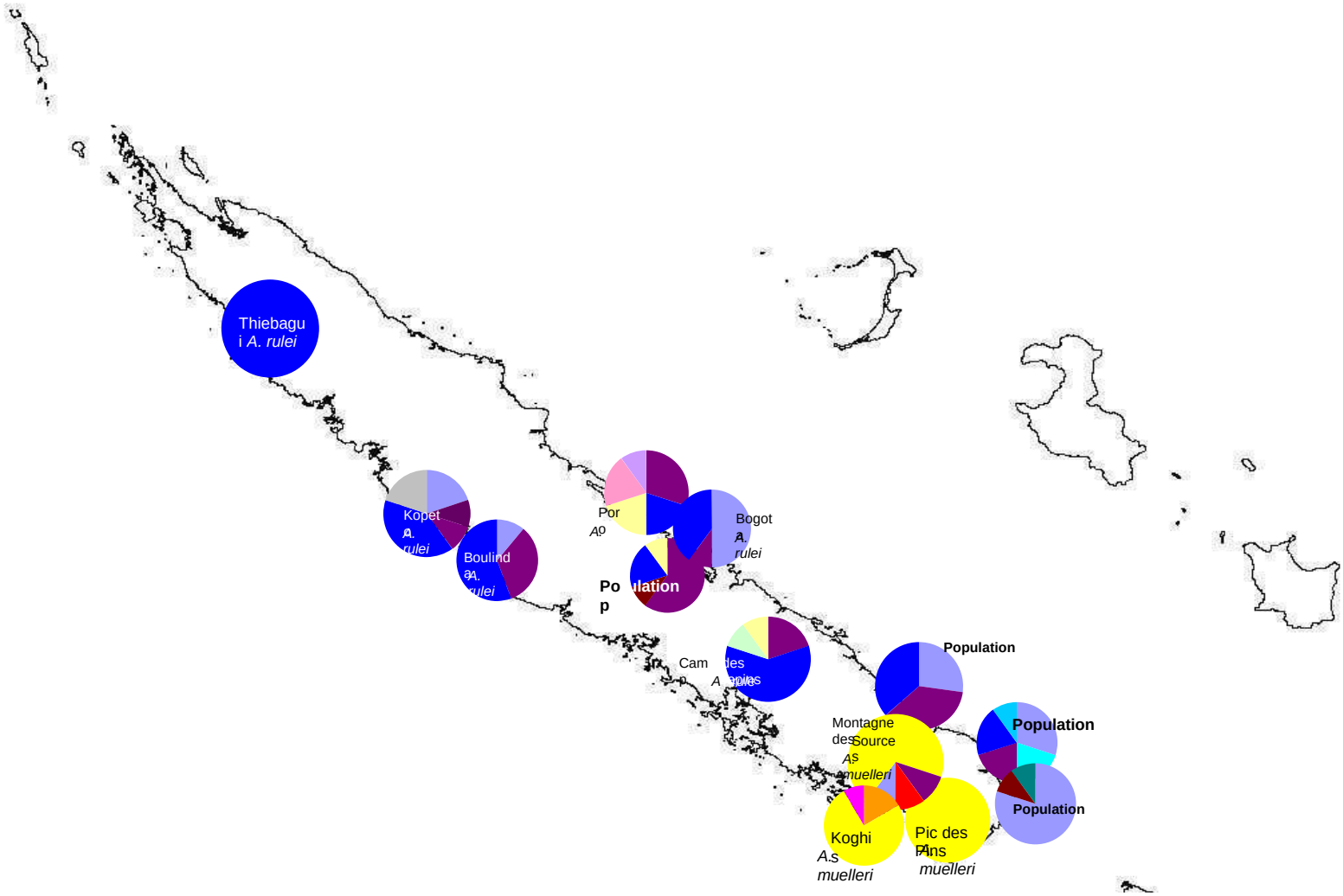


Fig. 4.20: Map of the haplotypes resulting from the combination of six molecular markers in *A. rulei* and *A. muelleri*

Haplotype	AP1	AP2	AP3	M13	M1	M2	Population showing the haplotype
1	10	9	5	3	A	A	Camps des Spains
2	8	9	6	2	A	G	Population1, Population 2, Population 3, Bogota, Boulinda, Kopeto, Montagne des Sources
3	10	11	6	3	A	A	Kopeto
4	8	9	6	3	A	A	Population 2
5	8	9	6	2	G	G	Population 2
6	10	9	6	3	A	A	Population 2, Population 3, Population 4, Bogota, Boulinda, Camps des Sapins, Kopeto, Poro, Thiebagui
7	9	8	6	3	A	G	Population 1
8	8	10	6	2	A	G	Kopeto
9	9	9	6	3	A	A	Population 2, Population 3, Population 4, Bogota, Camps des Sapins, Kopeto, Poro
10	9	9	5	3	A	A	Poro
11	9	10	6	3	A	A	Poro
12	8	9	6	1	G	G	Mont Koguis
13	8	9	7	1	A	G	Montagne des sources
14	10	10	6	3	A	A	Population 1, Population 4
15	9	9	6	2	A	G	Koghis
16	8	9	6	1	A	G	Koghis, Montagne des Sources, Pic des Pins
17	11	9	6	3	A	A	Population 4, Camps des Sapine, Poro

Table 4.17: Composition of each haplotype of *A. rulei* and *A. muelleri*

Taxon	Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total
A. muelleri	Koghi	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2	7	0	10
A. muelleri	Montagne des Sources	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	7	0	10
A. muelleri	Pic des Pins	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	10
A. rulei	Bogota	0	5	0	0	0	4	0	0	1	0	0	0	0	0	0	0	0	10
A. rulei	Boulinda	0	1	0	0	0	6	0	0	3	0	0	0	0	0	0	0	0	10
A. rulei	Camp des sapins	1	0	0	0	0	6	0	0	2	0	0	0	0	0	0	0	1	10
A. rulei	Kopeto	0	2	2	0	0	4	0	1	1	0	0	0	0	0	0	0	0	10
A. rulei	Poro	0	0	0	0	0	2	0	0	3	1	2	0	0	0	0	0	2	10
A. rulei	Thiebagui	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	10
-	P opulation 1	0	8	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	10
-	Population 2	0	3	0	1	2	2	0	0	2	0	0	0	0	0	0	0	0	10
-	Population 3	0	3	0	0	0	3	0	0	4	0	0	0	0	0	0	0	0	10
-	Population 4	0	0	0	0	0	2	0	0	6	0	0	0	0	1	0	0	1	10

Table 4.18: Distribution of the haplotypes among populations of *A. rulei* and *A. muelleri*

	PERCENTAGE OF VARIATION						
	Groupe 1 = All A. rulei population +	Groupe 2 = All A. muelleri population +	Among group of species	Among populations within group of species	Within populations	FST	Number of populations
A	Population 1 to 4	-	36,11	10,87	53,02	0.46980	9
B	Population 2 to 4	Population 1	25,78	15,85	58,37	0.41627	9
C	Population 1,3,4	Population 2	22,89	17,72	59,38	0.40617	9
D	Population 1 to 3	Population 4	26,20	15,57	58,23	0.41775	9
E	Population 1,2,4	Population 3	19,93	19,57	60,50	0.39504	9
F	Population 1	Population 2 to 4	11,04	24,97	64,00	0.36003	9
G	Population 2	Population 1,3,4	12,46	23,98	63,56	0.36441	9
H	Population 4	Population 1 to 3	12,71	23,81	63,48	0.36519	9
I	Population 3	Population 1,2,4	16,29	21,34	62,38	0.37624	9
J	-	Population 1 to 4	11,27	24,89	63,83	0.36168	9

Table 4.19: Successive results of the AMOVA realised with two groups of species (Group 1 and 2). Group 1 is a combination of *A. rulei* population and none to four of the questioned populations. Group 2 is a combination of *A. muelleri* population and none to four of the questioned populations

The papillae distribution was random but the stomata were only observed at the base of all the specimens.

- The molecular data

Overall from the four populations, 8 different haplotypes were retrieved (Table 4.17). The specimens from Population 2 (Mamie) showed the most variability with 5 different haplotypes. Populations 1 and 3 were the least variable with 3 different haplotypes. Each population shared one to two common haplotypes (haplotype 2 or 9) with the *A. muelleri* population of Montagne des Sources, but none with the populations from Koghis or Pic des Pins. Population 2 to 4 shared at least one common haplotype with all the *A. rulei* populations, and population 1 shared one common haplotype with 3 of the *A. rulei* populations (Kopeto, Boulinda and Bogota).

In the analysis of molecular variance (Table 4.19), the percentage of variation among the group of species was the highest (36.11 %) when all four unknown populations were grouped with *A. rulei* (A). It was the lowest (11.27 %) when all populations were grouped with *A. muelleri* (J). In addition the percentage of variation within the group of species is the lowest (10.87 %) when the populations are grouped with the *A. rulei* populations (A). The level of variation within populations is stable around 60 %.

4.4.4 Discussion

According to the morphological data, all four populations showed stomata only at the base of the adaxial face of the leaves, which is a character found in the populations of *A. rulei*. Although the size of the leaves is not significantly different between the four

populations and either *A. muelleri* or *A. rulei*, on the basis of the stomata distribution, the four populations would be put in the *A. rulei* group.

According to the molecular data, the four populations show common haplotypes with both species, however there are more affinities between the four populations' haplotypes and the populations of *A. rulei*. The AMOVA confirmed that variability between the species was maximized when the four populations where attached to *A. rulei.* It seems appropriate to suggest that the four populations belong to *A. rulei*.

The presence of a particularly big leave size in the population of Le Trou suggests possible hybridization between the two species. The population of Le Trou share one haplotype with the population of Montagne des Sources. However this haplotype is also present in three of the *A. rulei* populations. In order to obtain more information on possible hybridization, one of the next steps would be the use of nuclear microsatellite markers. The primers developed by Robertson *et al.* (2004) have already successfully been used in *A. rulei* and would therefore constitute a first useful tool to deal with this question.

Nevertheless, the results demonstrated that the actual distribution of *A. muelleri* might be more restricted than previously thought and further emphasis should be made on the conservation of this species.

4.5 General discussion

New Caledonia has one of the richest conifer biodiversities in the world. However, the knowledge of this biodiversity is still incomplete and contains uncertainty, which has retarded the conservation process. These three species delimitation problems are only one example among the wide number of problem encountered, not only in *Araucaria*, but also in several other genera. The collections in herbaria are invaluable resources for clarifying some species determinations. However they have their well-known limits (e.g. limited number of specimens available, lost of the original colour, desiccation and loss of shape, damaged material etc.) and the addition of other data

from field observation and of course, molecular analyses can greatly enhance the studies. Not only are molecular data useful to corroborate morphological observations, they can also compensate when no valuable morphological information is retrieved from the observation of the sample. In *Araucaria*, despite the low level of genetic variability retrieved in the previous chapter, relevant combinations of molecular markers were still defined for specific pairs of species. In the first case of study, the molecular data were complementary to the morphological data as no morphological observation was sufficiently different to enable identification of the specimens. In the second case study, the morphological information was totally lacking and the molecular data were the only source of information. This resulted in an ambiguity in the final determination of the species. In the third case of study, the molecular data supported the morphological observation as the stomata distribution was a robust morphological character to separate the two species. However more work on the isolation of relevant molecular markers remains to be done on specific pairs of species like *A. montana/A. laubenfelsii.* The use of nuclear microsatellite developed specifically for New Caledonian *Araucaria* by Robertson *et al.* (2004) seems the most appropriate next step to study the genetic distinction of the species.

This study has highlighted the weaknesses of our knowledge of the genus and the urgent need to update the flora. Such work also helps in conservation to assess the genetic diversity of each population and work out more efficient seed sampling for nursery programmes. Watt (1999) made a review of the status of New Caledonian Conifers, including *Araucaria*. Threats to several species were already pinpointed and the creation of 12 new nature reserves was recommended in a conservation perspective. In the Araucariaceae review of 2003, Manauté *et al.* (2003) also suggested the revision of the status of some species, among which were *A. muelleri* and *A. luxurians* whose populations are being threatened by wild fire and the beginning of mining exploitation of the southern soils for nickel. This study recommends an extensive review of the populations of *Araucaria* in order to reassess a proper conservation status for each species.

CHAPTER 5 - An overview of the biology and taxonomy of New Caledonian *Araucaria*

5.1 Introduction

Previous chapters have highlighted the problems of species delimitation, identification and low genetic divergence existing between the New Caledonian *Araucaria*. One of the main problems in working with these species is the dated nature of their account in the Flora of New Caledonia (DeLaubenfels, 1972). Since the completion of the flora (published in French), new localities have been discovered and further herbarium material has been collected and studied, and additional papers have been published. Particularly important works are those of Veillon (1978) who used produced a key for the species, and Nasi (1982) who observed the wood structures of the trees, as well as the coning season, and reviewed the existing populations for each species. Finally, habitat preferences and IUCN criteria (IUCN, 1994) were reviewed by Manauté *et al.* (2003).

The aim of this chapter is to bring together what is known about New Caledonian *Araucaria* from the literature, and to add to this personal observations made during the 3 years of this PhD based on field, herbarium and laboratory studies.

5.2 Material and methods

Previous morphological knowledge is taken from: the Flora of New Caledonia (DeLaubenfels, 1972), and papers by Veillon (1978), Nasi (1982), Jaffré (1995), and Manauté *et al.* (2003). Other authors will be cited for specific points within this chapter. Translations from the Flora of New Caledonia into English were made by Robert Mill (RBGE), and sections of this are repeated in the text under the sections entitled ‘Summary of Previous Work’.

During three successive field seasons (December 2001-2002-2003), information has been gathered on *Araucaria* species. 54 populations were visited

across New Caledonia, including all the 13 species and various types of habitats. Field measurements were undertaken for some specimens and pictures of the different parts of the trees have been taken. Herbarium specimens have been collected for most of the populations. Additional information was gathered from herbarium loans from 13 herbaria worldwide. (The full list of specimens examined is given in the CD appendix). The description in the next paragraphs will use the following terminology (see also Fig. 5.1):

Seedling: young plant of less than a year old

Juvenile: young tree of a few years old

Branches: main branches derived from the trunk

Twigs: branchlets, smaller branches of secondary or tertiary order

Reiterations: generation of new branches from epicormic shoot of the bark

Rauh model: orthotropic branches and no reiteration

Massart model: plagiotropic branches and presence of partial reiteration

For each species, details of previous knowledge will be summarized, followed by personal observations.

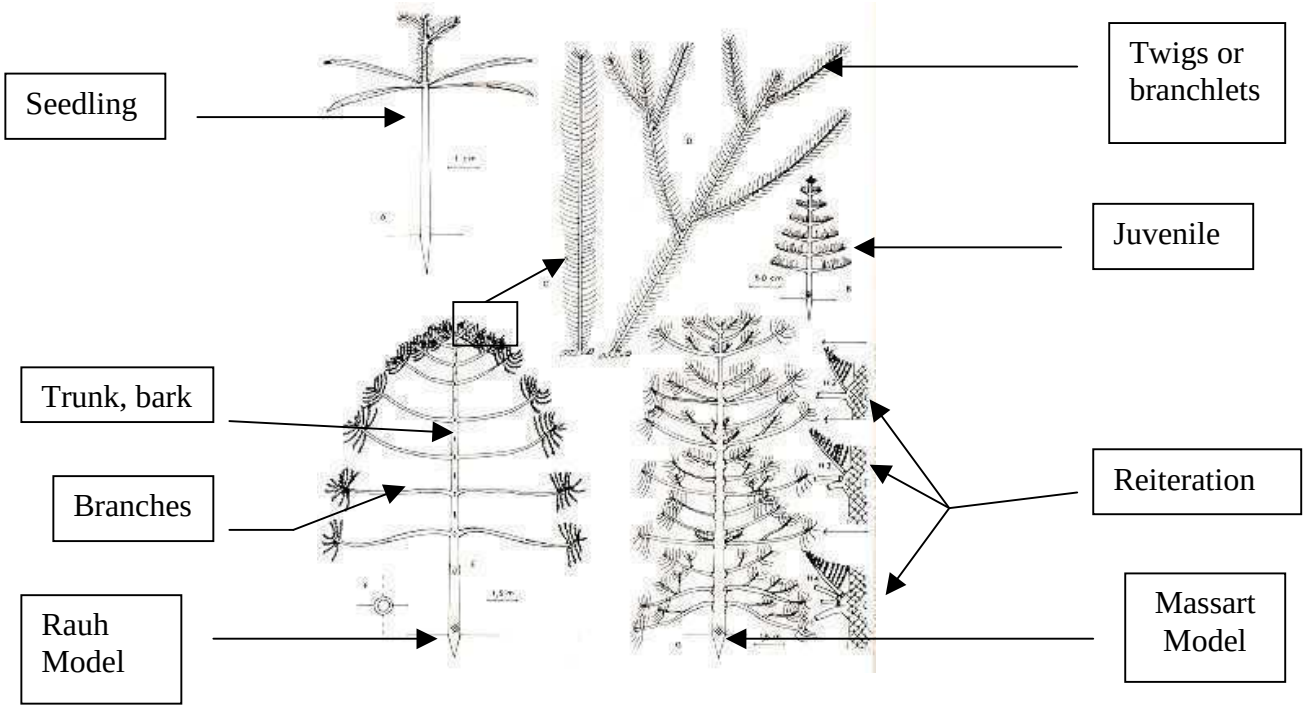


Fig. 5.1: Terminology used to describe New Caledonian *Araucaria*. The illustration is taken from Veillon (1978)

5.3 Species description and updated taxonomy

5.3.1 *Araucaria bernieri*

5.3.1.1 Summary of previous work

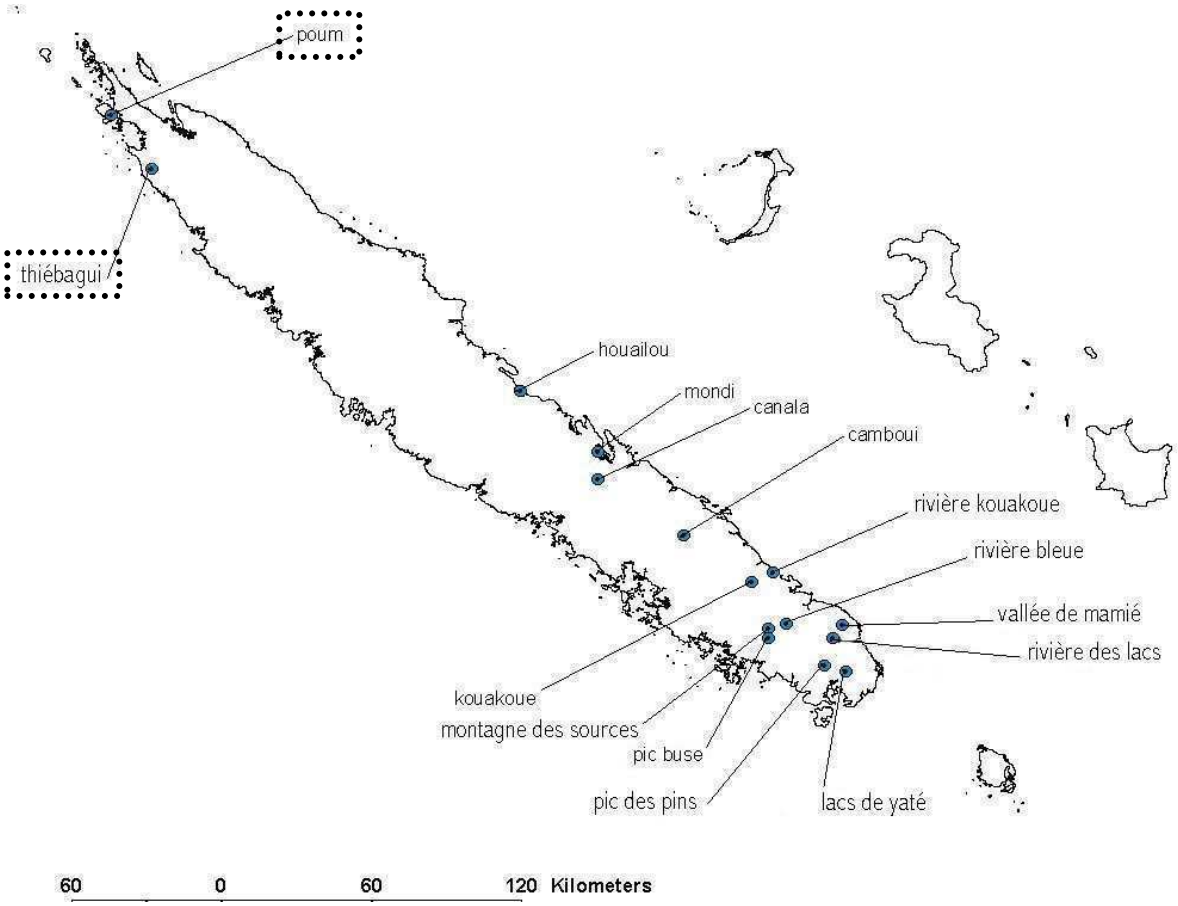


Fig. 5.2: Distribution map of *A. berneri* based on DeLaubenfels (1972), updated with field observations and herbarium specimen data (The map is annotated to indicate questionable populations)

Araucaria bernieri usually grows in evergreen humid rainforest of low and middle altitude on ultramafic rocks. It is found between 100m and 700m, usually on steep slopes of mountains or in deep valley. The species is abundant in the south of the island. Two populations have also been recorded in the north of the island in Poum and Thiebagui (DeLaubenfels, 1972).

Adult individuals are tall and can reach 50m high. They have a columnar shape, and are flat topped. Veillon (1978) describe the growing structure as the Massart model, with branches growing horizontally with reiterations at the base of

the branches. The branchlets grow almost horizontally on each side of the axis, which result in a “V” structure. The bark of the tree is grey, exfoliating horizontally. The leaves of juvenile *A. bernieri* are divergent, thin, flattened bilaterally with a keel on both faces and around 7 mm long. The colour is glaucous and remains so in the adult trees. The leaves of lower branches are divergent and curved forwards; the tip is parallel to the branch, strongly keeled, more or less acute, slightly narrowed and thickened at the base. The leaf length is 1.5 to 3 mm and the leaf width 1.5 to 2 mm. The branches with adult foliage are whip-like, 4 to 6 mm in diameter including the leaves. The leaves on the branches are very small, 1-1.5 x 1 mm. The leaves of adult foliage of twigs are divergent, but inclined forwards and parallel to the branch, becoming almost imbricate, strongly keeled on the back and on the lower part of the axial side. They are triangular to acute, 2-3.5 x 1.5-2.5 mm, slightly narrowed and thickened at the base.

The male cones are white to glaucous, cylindrical, 4-9 cm long and 8-16 mm in diameter, accompanied below by lanceolate sterile bracts attaining 3 x 1 mm. The blades of the microsporophyll above the pollen sacs are imbricate, triangular, acute, 2.5 x 2.5 mm, and each scale has 4-6 pollen sacs. The female cones are 8-10 cm long and 7.5-8 cm in diameter, and glaucous. The seed scales are around 30 mm long, with an elongated tip of 5 mm, erect on the edge of the seed scale, which is strongly bossed in front.

5.3.1.2 Personal notes

The two populations from the north of New Caledonia (Thiébagui and Poum) are of questionable identity. Many of the herbarium specimens from these localities (McKee 23153, Nothis 480, Veillon 7928, Schmid 2675) that are labelled as *A. bernieri* show greater morphological affinities to *A. scopulorum*. The stomata go up to the apex of the leaves in *A. scopulorum*, when they stop halfway on most *A. bernieri* leaves. The foliage colour of *A. scopulorum* is generally green, but glaucous in *A. bernieri*. *A. bernieri* also has thinner branchlets than *A. scopulorum* and the leaves on the branches are almost flat and pressed to the axis, giving a smooth feeling, whereas in *A. scopulorum*, they are curved and acute, giving more a rough

sensation. Finally, the leaves of *A. scopulorum* (which appear like scales) are broader than those of *A. bernieri* (which have a feathered appearance).

The only specimen from the north of the island that looks like *A. bernieri* is a specimen in the New Caledonian herbarium (H.S. McKee, 14349) dating back to 1966, and collected from Poum. It has leaves on branches less than 1.5mm long and 1mm wide, and its stomata distribution restricted to the middle of the blade. However, on visiting these sites, no matching specimens were observed during fieldwork. The evidence for *A. bernieri* having northern disjunct populations thus remains equivocal. It is possible, that the species did exist here, but has been wiped out by mining extraction (both localities are the sites of current nickel mines). A second possibility is that northern populations of *A. bernieri* exist outwith areas recently searched (this is possible as the sites are large and complex). Finally, accepting that most of the northern records for *A. bernieri* appear to be simple misidentification errors, it could be that the H.S. McKee, 14349 sheet has been mislabelled and the material stems from a different locality, or has been taken from a juvenile individual of *A. scopulorum.* (Although this last explanation seems doubtful, as even juvenile material is relatively straightforward to distinguish in this species pair). For any future identification challenges between these two taxa, the use of a nuclear microsatellite (AS167, Robertson *et al*., 2004) is useful, as A. *bernieri* and A. *scopulorum* have a different allele range (164 to 166 for *A. bernieri*, 168 to 178 for *A. scopulorum* (unpublished data).

Another possible identification confusion for *A. bernieri* is with A. *subulata* (Chapter 4). In this case, a comparison of the leaf length on the branches is useful. They are needle-like (3 to 12 mm long for 1 mm wide) and parallel to the axis in *A. subulata,* whereas they are very short (< 2 mm) and pressed to the axis in *A. bernieri*. Sampling to date also suggests there is a molecular discrimination between these two species. In the chloroplast region *psb*A-*trn*H there are two nucleotide differences at bp 156 and bp 473 (T and A in *A. subulata* and C and G in *A. bernieri* respectively), as well as an extra copy of the 13 base pair minisatellite in A. *bernieri* (two copies in *A. subulata* and three in *A. bernieri*).

5.3.2 *Araucaria biramulata*

5.3.2.1 Summary of previous work

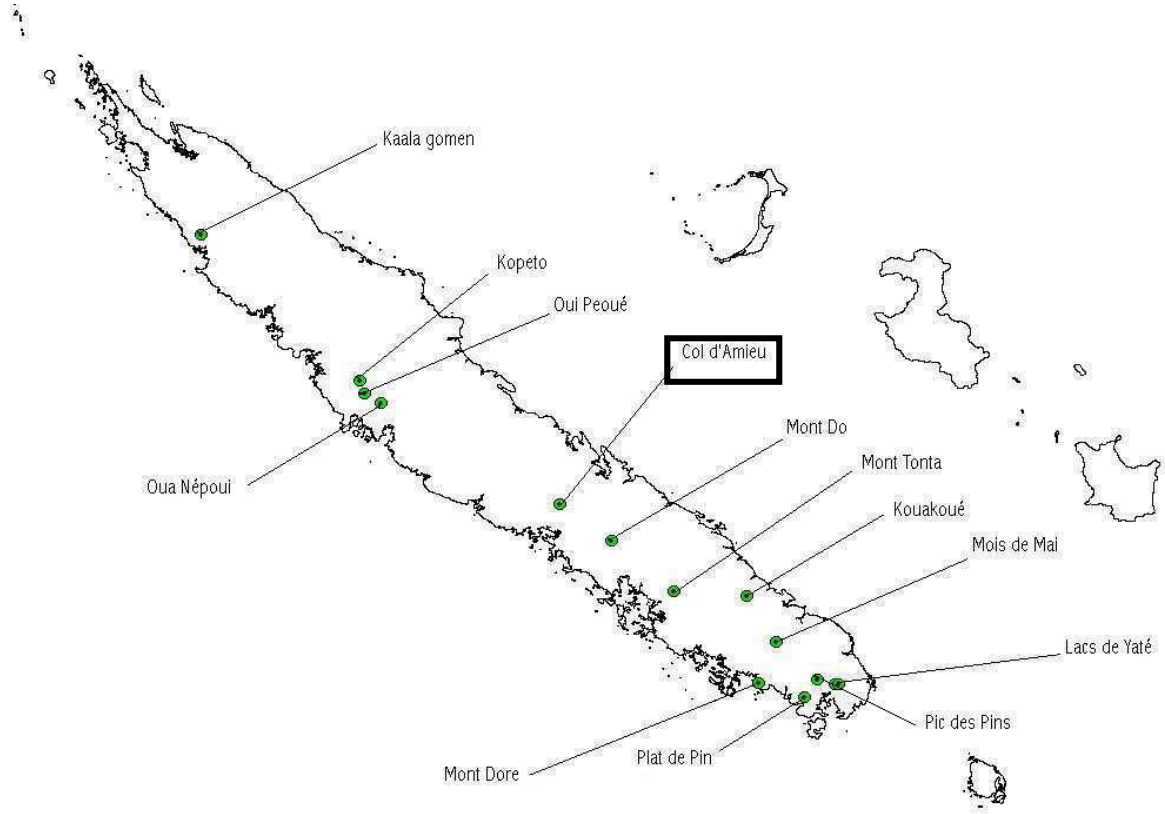


Fig. 5.3: Distribution map of *A. biramulata* based on DeLaubenfels (1972), updated with field observations and herbarium specimen data (The map is annotated to indicate questionable populations)

Araucaria biramulata usually grows in humid rainforest of low and middle altitude on ultramafic rocks. It is found between 150m and 1100m, usually on steep slopes of mountains or in deep valleys. It is frequent in the south of the island and also in the western massif of the Main Island, in places that are often hard to reach.

Adult individuals grow up to 30 m high, with a more or less columnar shape. It grows on the Rauh model, also called 'candelabra type'. In this model, branches have a vertical growth tendency. However, as the trees grow, they diverge from the model by having reiterations at the base of the branches. In juveniles, the branchlets almost grow horizontally on each side of the axis, which result in a pseudo "V"

structure. In adult trees, the branchlets grow on a spiral scheme, which result in a helicoidal structure at the end of branches. The terminal branchlets have a tendency to divide in two, which gave the species its name. The bark of the tree is grey to brownish, exfoliating horizontally. The leaves of juveniles are thin, flattened bilaterally and around 1 cm long.

The leaves of low branches are divergent, subulate, 5-6 x 4-5 mm and strongly keeled on both sides. The branches of adult foliage are 8-13 mm in diameter, including the leaves. The leaves of adult foliage are divergent, stiff, subulate, narrowed and thickened at the base, concave above or almost flat, with a strong keel on the back and 7-9 x 5-6 mm.

The male cones are cylindrical, 6-7 cm long and 15-20 mm in diameter, accompanied below by sterile bracts, which reach a size of 10 mm and narrow to the base, which is 4 mm wide. The blades of the microsporophylls above the pollen sacs are divergent, triangular, acute, 5-6 x 4 mm, each with 7-8 pollen sacs. The female cones are 9-10 cm long and 8-9 cm in diameter. The seed scales are 30 mm long, with an erect tip of 8 mm.

5.3.2.2 Personal notes

This species was defined on the basis of the split of its branchlets. However, on its own this is not a sufficient character for identification. A similar phenomenon occurs in species like *A. montana*, *A. humboldtensis* and *A. scopulorum*, the latter in which it is quite frequent. Thus identification of material as being *A. biramulata* purely on the presence of bifurcating branchlets is questionable.

Any confusion between *A. biramulata* and *A. scopulorum* should not be a problem, as the two species have very different leaf types: *A. biramulata* adult leaves have a flat blade and a length of up to 1 cm whereas the leaves of *A. scopulorum* have a strong keel on the back, do not exceed 7 mm, and are more claw-like. Confusion can occur between *A. luxurians* and *A. biramulata*. The general shape of the trees is similar and the leaf shapes can be confused. The stomata rows on the back of both species leaves go up to the apex. However, the leaves of *A. biramulata* are a little smaller than those of adult *A. luxurians* (up to 8mm length in *A.*

biramulata and up to 10 mm in *A. luxurians*), and they are a little divergent from the axis, which gives the branchlets of *A. biramulata* a slender appearance. Though the flora account (DeLaubenfels, 1972) mentions some resemblance between *A. biramulata* and *A. humboldtensis* or *A. columnaris*, these two latter species are quite different. The leaves of *A. humboldtensis* are strongly keeled on the abaxial face and dense on the branchlets. Its trees do not exceed 15m high and the lowest branches usually fall down and are not replaced, which gives the trees a very typical candelabra shape. *A. columnaris* has a similar tree shape to *A. biramulata,* but its adult leaves are usually as long as wide, whereas *A. biramulata* has leaves longer than wide. Furthermore, the stomata on the abaxial face of the leaves of *A. columnaris* are only present at the base and the apex. Molecular data can also discriminate *A. columnaris* from *A. biramulata*. In the chloroplast region *trn*H-*trn*Fm, there are two-nucleotide substitutions between the two species at base-pair positions 3 and 185 (respectively A and T in *A. columnaris* and C and C in *A. biramulata* (Chapter 4)).

5.3.3 *Araucaria columnaris*

5.3.3.1 Summary of previous work

Araucaria columnaris grows on coastal limestone on calcareous soil. These forests can occur up to 200m inland, and occupy calcareous platforms made of uplifted coral reefs. This species is the only conifer found in this type of forest, which also lacks members of the typical ancient Gondwanan flora. The largest populations of *A. columnaris* are on the Loyalty Islands and the Isle of Pines, where it forms very dense populations dominating low forests exposed to winds. Smaller populations are also found on sedimentary rocks in Bay of the Turtles near Bourail (molecular and demographic data suggests this population may be planted; Kettle, 2005), and on some islands between Noumea and Isle of Pines. The species has been widely exploited for wood (Nasi, 1982) and is planted on the border of roads as a safety barrier and indicator for cars. It is important from a cultural perspective as a representation of male fertility, and this has increased the planted range of the

species (newly married couples traditionally planted a tree of this species it in front of their home, alongside a coconut tree as symbol of female fertility).

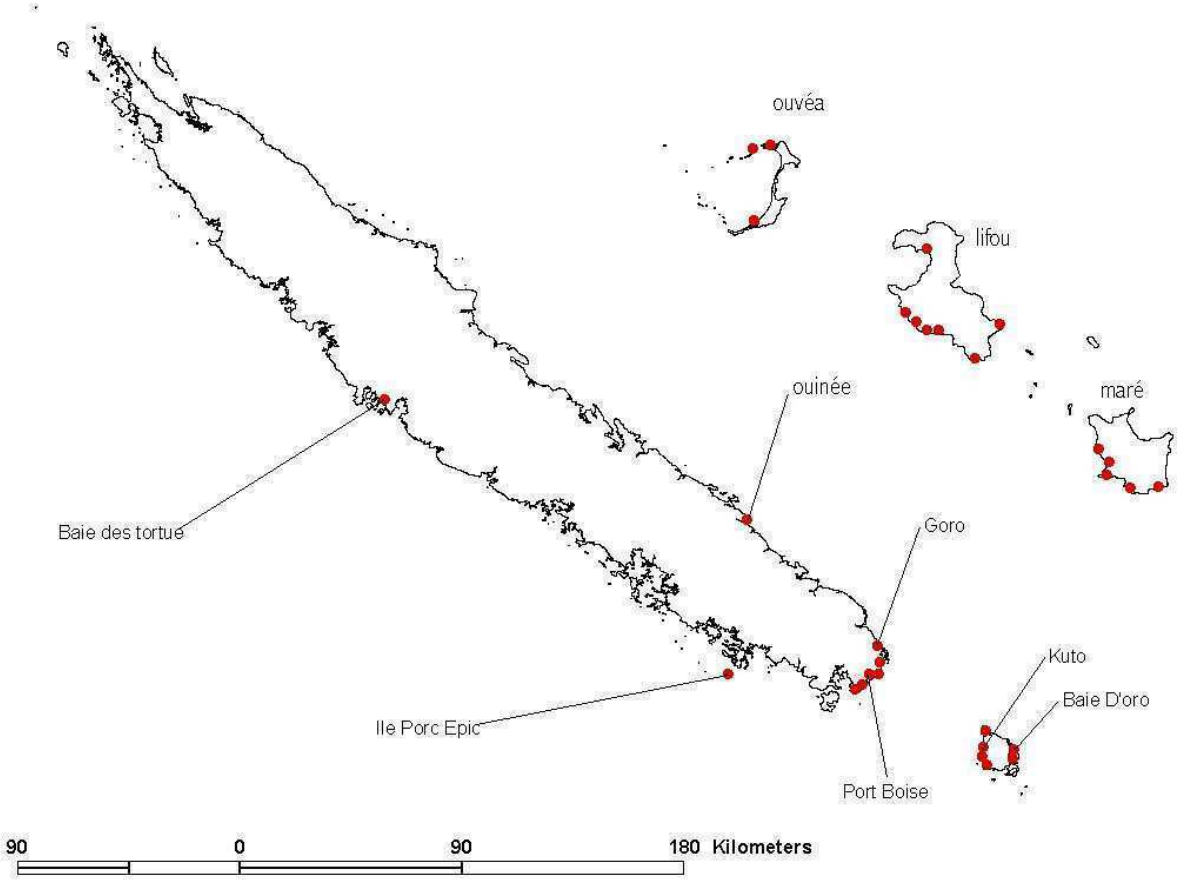


Fig. 5.4: Distribution map of *A. columnaris* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data

Adult trees can reach height of 60m. They have a columnar shape and are flat topped. They grow on the Massart model, with abundant reiterations at the base of the second axis. Main branches can grow up to 2 m long. The first branches are caduceus and replaced by adventitious branches, so that the tree has the appearance of a column with a round or pointed capital, and if the first branches persist for longer, the tree can take the form of two superposed cones, the base of the upper cone resting on the top of the lower one. In juveniles, the branchlets almost grow horizontally which result in a pseudo “V” structure. In adult trees, the branchlets grow on a spiral scheme, which result in a helicoidal structure at the end of branches. The bark of the tree is grey, exfoliating horizontally. Leaves of juveniles are thin, flattened bilaterally with a keel on both sides, and are around 1 cm long. The branches with adult foliage

resemble straps or belts, 9-10 mm in diameter including the leaves. The leaves of adult foliage are strongly imbricate, stiff, almost triangular, rounded at the apex, narrowed and thickened at the base and concave above. They have an extra-central crest on the back and are 5-7 x 4-5 mm.

The male cones are acute, of variable size, 5-10 cm long and 15-22 mm in diameter. They are accompanied below by sterile bracts, which are 7 mm, acute, tapering from a 5 mm wide base. The blades of the microsporophylls above the pollen sacs are divergent, more or less cuspidate, rounded to broadly acute, slightly enlarged at the base, 7-10 x 4 mm, thin, flexible and curved, each with about 10 pollen sacs. The female cones are 10-15 cm long and 7-11 cm in diameter. The seed scales are 30-35 mm long, with an erect tip of 7 mm long and clearly curved forwards, sometimes curving downwards outside.

5.3.3.2 Personal notes

A. columnaris is phylogenetically closely related to *A. luxurians* and *A. nemorosa* (Chapter 2), all species with a general coastal distribution. Its leaves are highly variable, even on the same branchlets, though the adult foliage always maintains the same ratio of leaves nearly as long as wide. Similar leaf size variation is found in all three coastal species. The variation of the leaf size might be due to regrowth of broken branchlets because of exposure to strong winds. Another possibility is sensitivity to seasonal variations and water availability. At any rate, the determination of the species is often straightforward, due to its unusual habitat, and when found elsewhere, the almost square shape of its leaves. It also has very characteristic male cones, orange when ripe, and with a fluffy aspect due to the numerous papillie on the margin of the blade of the microsporophylls.

The species can sometimes be confused with *A. luxurians*, especially when only the lower branches with juvenile characters are available. The observation of the stomata rows on the outside of the leaves can be used, as they are only located at the base and at the apex of the leaves in *A. columnaris*, when in *A. luxurians* they go all the way up to the apex (however, in leaves showing juvenile characters, the stomata

distribution of *A. luxurians* might be slightly incomplete and absent on some part of the leaves).

5.3.4 *Araucaria humboldtensis*

5.3.4.1 Summary of previous work

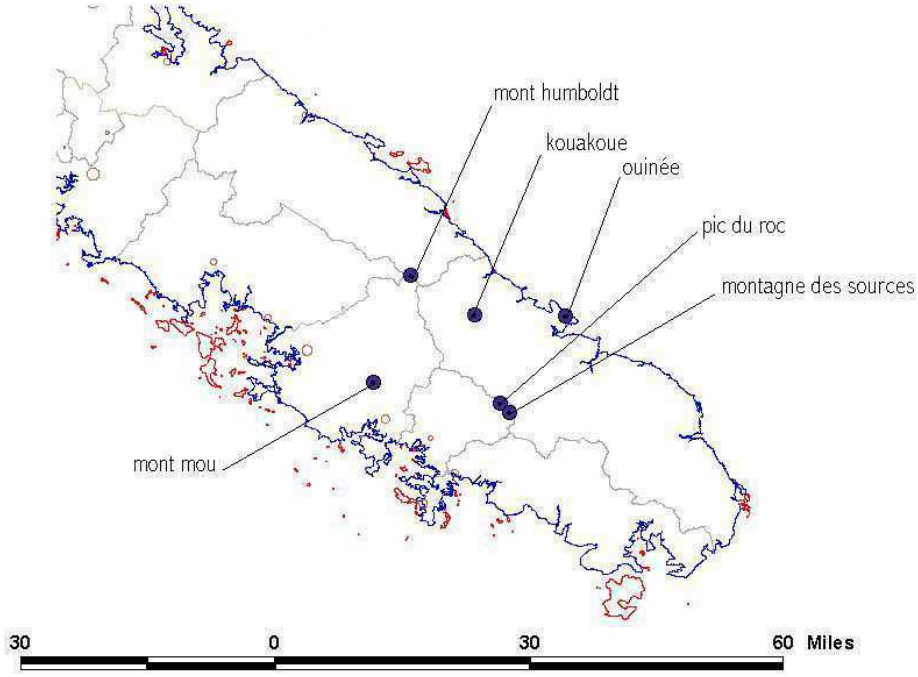


Fig. 5.5: Distribution map of *A. humboldtensis* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data

Araucaria humboldtensis grows in evergreen humid rainforest of high altitude on ultramafic rocks. It is found in low forest (6 to 14m) on maquis of high altitude between 800m and 1600m. These kinds of forests are abundant on southern massif and are rich in other conifer species.

The trees are small, not exceeding 15m. They grow on the Massart model; the lowest branches usually fall down and are not replaced, which results in a flattened crown at the top of the tree. The branchlets grow horizontally on each side of the axis, which results in a “V” structure. The bark is grey, exfoliating in small quadrangular scales or in horizontal strips. The leaves of juveniles are triangular in transversal section, divergent and curved, so that the acute apex is directed to the inside. The leaves are imbricate, strongly keeled on both sides and 2.5-4 x 2-3 mm at the base. The branches with adult foliage are more robust, often rather short (less than 20 cm) and 8-10 mm in diameter including the leaves. The leaves of adult foliage are divergent and curved as in the leaves of young trees, more or less flat with an extra-central keel on the back, 5-6 x 4-5 mm, slightly narrowed and strongly thickened at the base. They are imbricate, robust and have a glaucous colour.

The male cones are cylindrical, 6 cm long and 15 mm in diameter, accompanied below by sterile bracts reaching 8 mm and tapering from a base around 4 mm wide, rounded or acute. The blades of the microsporophylls above the pollen sacs are imbricate, triangular, acute, 3 x 3 mm and each scale bearing around 6 pollen sacs. The female cones reach 9 cm long by 8 cm in diameter. The seed scale is around 30 mm, with an elongated tip around 6 mm long, strongly leaning forwards, sometimes finally curved on outside and more or less erect.

5.3.4.2 Personal notes

The flattened crown, very candelabra like shape, of *A. humboldtensis* is quite characteristic of the species. Another important feature is the presence of white exudates found here and there on the leaves of older trees. The branchlets are very short (rarely exceeding 20 cm) and the leaf distributions very regular, which give a robust aspect to the foliage. The species is sometime found sympatrically with other *Araucaria* like in Montagne des Sources, where it grows next to *A. muelleri*. However, the species maintain their difference in sympatry.

The flora account (DeLaubenfels, 1972) mentions a resemblance with *A. scopulorum.* However, this species has much smaller branchlets and different colour leaves. *A. humboldtensis* leaves are glaucous and very dark, whereas *A. scopulorum*

leaves are light green with the extremity of the branchlets bearing nearly yellowish leaves. In addition, the leaves on the branches are very dense and almost 1cm long in *A. humboldtensis*, when they are more sparse and shorter (2-3 mm) in *A. scopulorum*.

5.3.5 *Araucaria laubenfelsii*

5.3.5.1 Summary of previous work

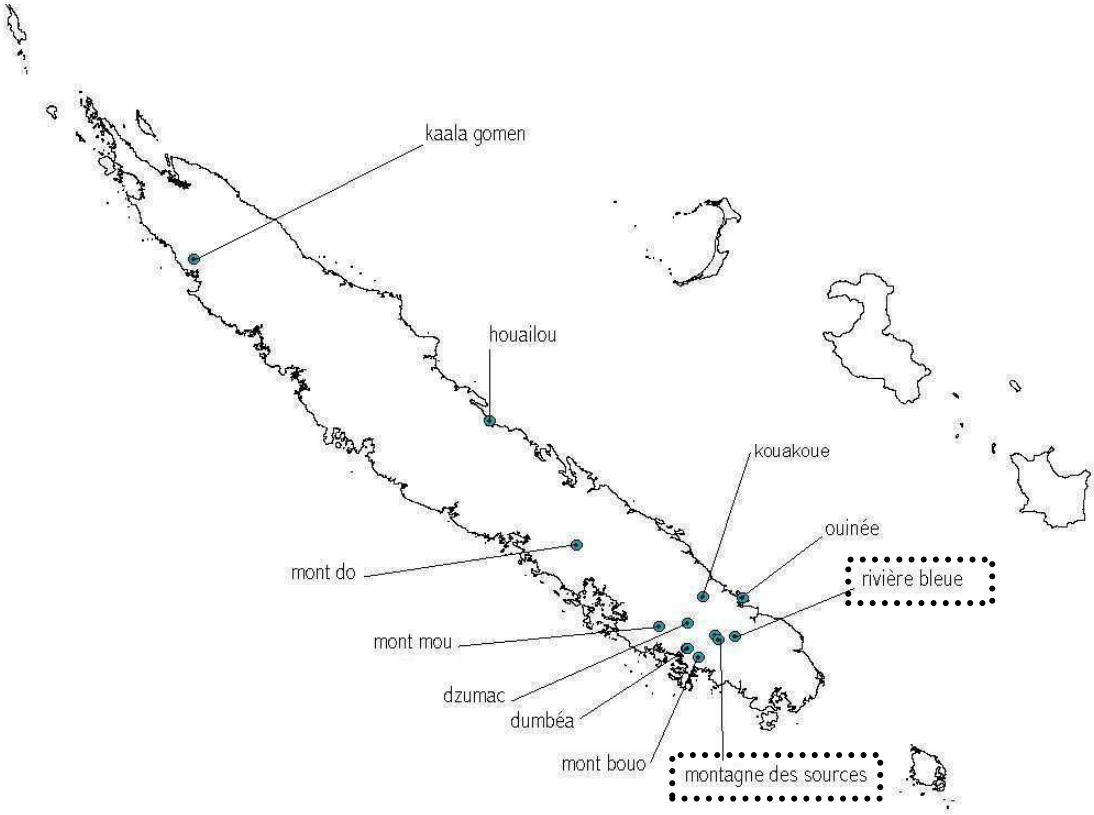


Fig. 5.6: Distribution map of *A. laubenfelsii* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data (The map is annotated to indicate questionable populations)

Araucaria laubenfelsii grows on ultramafic soils in humid rainforest and in maquis. It is found between 400m and 1300m and occupies rocky lateritic or ferritic platforms. Some individuals can reach 20m. The species is common in the southern mountain (between La couvelée and Ouinée), in Mont Do and in Kaala Gomen. Several studies (Riggs et al, 1998; Enright et al, 2000) have highlighted the role of

fire in the recruitment of seedlings of this species and its distribution in the landscape.

The adult individuals have a columnar shape. The species morphology matches the Rauh model (Veillon, 1978), but as it matures it diverges from the model by having reiterations at the base of the branches. The top of the tree is flat. The main branches can grow up to 2.5m. In juveniles, the branchlets almost grow horizontally on each side of the axis, which results in a pseudo “V” structure. In adult trees, the branchlets grow on a spiral scheme, which result in a helicoidal structure at the end of branches. The bark is grey, exfoliating horizontally. The juvenile leaves are divergent, needle-like at first, then flattened and concave above, 10-15 mm long, progressively wider and longer. The leaves of low branches are divergent, narrow, lanceolate, curved at the tip, variable in length on the same branch and 12-19 x 7-10 mm. The branches with adult foliage are 18-28 mm in diameter, including the leaves. Adult leaves are more or less imbricate, coriaceous, subulate, tapered to a small curved or incurved tip, thickened near the base, concave towards the top with an extra-central crest on the back. They tend to vary in length on the same branch and are 12-20 x 8-10 mm.

The male cones are cylindrical, 12-15 cm long, 22-28 mm in diameter, accompanied below by sterile bracts reaching 16 mm in length. The sterile bracts are cuspidate, around 5 mm wide at the base. The blades of the microsporophylls above the pollen sacs are divergent, triangular, rounded to acute, 5-6 x 4.5-5 mm, each with c. 12 pollen sacs. The female cones are 10-12 cm long, 8-9 cm in diameter. The seed scales are around 30 mm long, with a beak 8-10 mm long, slightly curved forwards.

5.3.5.2 Personal notes

Araucaria laubenfelsii was described in 1972 by Corbasson (DeLaubenfels, 1972). Its populations used to be referred to *A. montana* and several herbarium specimens predating 1972 are still labelled as *A. montan*a. However even since the description of the species, problems of identification have remained as the two species have a very similar morphology. The flora account (DeLaubenfels, 1972) stresses that the

apex of the leaves of *A. montana* are more curved than *A. laubenfelsii*, but this distinction can be treacherous when the specimens are dried as the leaves tend to curve in the process. On several occasions during the current study, specimens collected with pointed leaves and therefore labelled as *A. laubenfelsii*, ended up with curved leaves (and a rounded apex) upon drying. There is some correlation between stomatal distribution and leaf morphologies, in that specimens collected as *A. laubenfelsii* have stomata that go up to the leaf apex, whereas they do not in specimens collected as *A. montana*. However, though the flora account mentions that "even when growing in similar places, each species retains its own characteristics", specimens were observed having all range of intermediates characters (leaf length, width, curve, stomata distribution) in localities like Mine Bokaine, on the east coast of the island. The other difference between the two species is suggested to be the altitude at which they grow, *A. laubenfelsii* being found at higher altitude (up to 1300m) than *A. montana* (800m). However, given the difficulties listed above this should be treated with caution so that it does not become self-perpetuating (e.g. a tree is *A. laubenfelsii* if it looks like *A. montana* but was collected at 1100m).

Using chloroplast microsatellites on the samples from the locality of Mine Bokaine revealed that all specimens shared the same chloroplast type, for the 4 markers studied (AP1, AP2, AP3, M13). When the study was extended to other *A. laubenfelsii* and *A. montana* populations (Mont do, Mont Mou, Ouinée for *A. laubenfelsii* and Boulinda, Kaala Gomen, Kopeto, Mont Panié for *A. montana*), only the populations of *A. montana* from Mont Panie and Kaala Gomen showed a different kind of chloroplast haplotype. There is thus no chloroplast DNA evidence for the presence of two distinct taxa. Certainly given the threats faced by these species (mining exploitation and wild fire) and the uncertainty over their status and distribution, it would be worth further study to see if the two should be considered synonymous.

One other minor source of confusion involving *A. laubenfelsii* involves herbarium samples of *A. rulei*. To distinguish these taxa, the total absence of stomata on the back of the leaves of *A. rulei* (except some at the apex and at the base, usually hidden by overlapping leaves) is a robust character to tell the two species apart when

adult leaves are not available. In adult specimens, the leaf size of *A. rulei* (1.5-3 cm) is far large than that of *A. laubenfelsii* (0.9-1.2 cm).

Finally, it is noticeable that during the visit of the locality of Montagne des Sources and Riviere Bleue, no specimen from the species was observed.

5.3.6 *Araucaria luxurians*

5.3.6.1 Summary of previous work

Araucaria luxurians grows on maquis and dense forest of low altitude in localities with a rainfall below 2000mm. It is found between 0 and 200m, on windy ridges, most often on hyper-magnesium brown soils issued from serpentines. There are dense populations in the south of the Main Island. Two herbarium specimens have also been sampled from the Belep Islands off the north coast of New Caledonia.

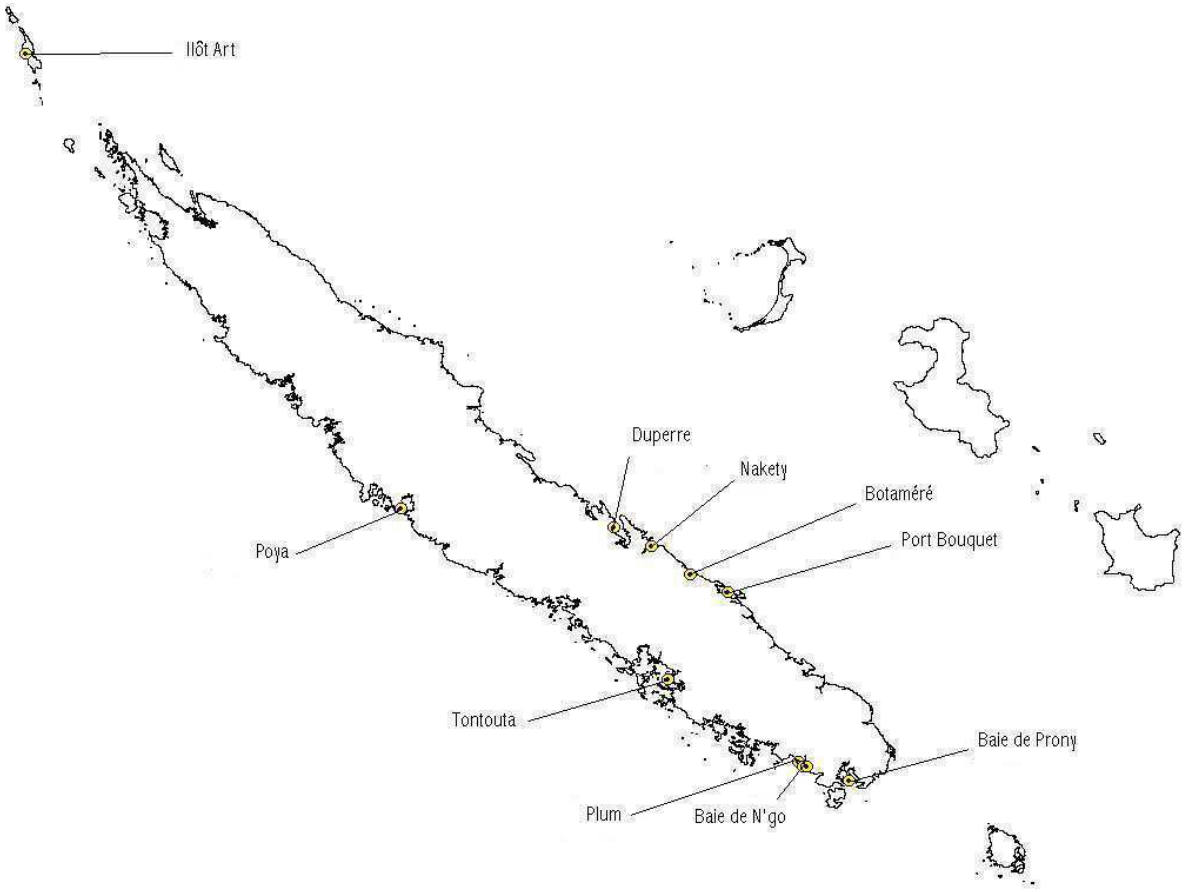


Fig 5.7: Distribution map of *A. luxurians* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data

Adult trees grow up to 30m and have a columnar shape. They grow on the Massart model with abundant reiterations at the base of the second axis. In juveniles, the branchlets grow almost horizontally which results in a pseudo “V” structure. In adult trees, the branchlets grow on a spiral scheme, which result in a helicoidal structure at the end of branches. The main branches can grow up to 3 m long. The bark of the tree is grey, exfoliating horizontally. The juvenile leaves are divergent, 6 to 12 mm long, larger on old branches. The leaves of low branches are spread, more or less narrow, subulate, and very variable along the same branch. They are 7-13 x 4-8 mm. The branches of adult foliage are 10 to 18 mm in diameter, including the leaves, and the foliage. Leaves of adult foliage are more or less imbricate, coriaceous, subulate, tapered to a small curved tip, narrowed and thickened at the base, concave above, often with an extra-central crest on the back and 5-7 x 4-5 mm.

The male cones are cylindrical, 12-17 cm long and 25-28 mm in diameter, accompanied beneath by sterile bracts reaching 15 mm in length; the sterile bracts are cuspidate, around 4 mm wide at the base. The blades of the microsporophyll above the pollen sacs are divergent, oval, acute, 8-9 x 4 mm, each with 12-15 pollen sacs. The female cones are 10-12 cm long by 8-10 cm in diameter. The seed scales are 30-35 mm long with an elongated tip around 10 mm, which is slightly sloping forwards and finally curved backwards.

5.3.6.2 Personal notes

The leaf size varies along the branchlets. Like in *A. columnaris*, it is possible to observe successions of bigger leaf segments to smaller leaf segments, ranging from a length of 5 mm x 3 mm to a length of 10 mm x 5 mm.

The species could potentially be confused with *A. biramulata* in some places (c.f. *A. biramulata*’s notes), or with A. laubenfelsii, though *A. laubenfelsii* and *A. luxurians* have very different habitats and are never found growing together, except in a nursery. The variation in leaves along the branchlets is not present in *A. laubenfelsii*. In addition, by sequencing the chloroplast region trnS-trnFm, two nucleotide substitutions separate *A. luxurians* and *A. biramulata* or *A. laubenfelsii* in

base pair positions 3 and 185 (respectively A and T in *A. luxurians* and C amd C in *A. biramulata* (Chapter 4) or *A. laubenfelsii* (Annexe 5.1)).

5.3.7 *Araucaria montana*

5.3.7.1 Summary of previous work

Araucaria montana is the species with the most widespread distribution. It grows on plateaus or mountain tops in the main massif of the island. It is more frequently found on ultramafic soils, but also occurs on acidic soils in Mont Panié. The species forms dense populations dominating maquis with bushes and also low forests. It can grow above 800m and in areas of heavy rainfall like on Mt Panié it can be found as low as 300m.

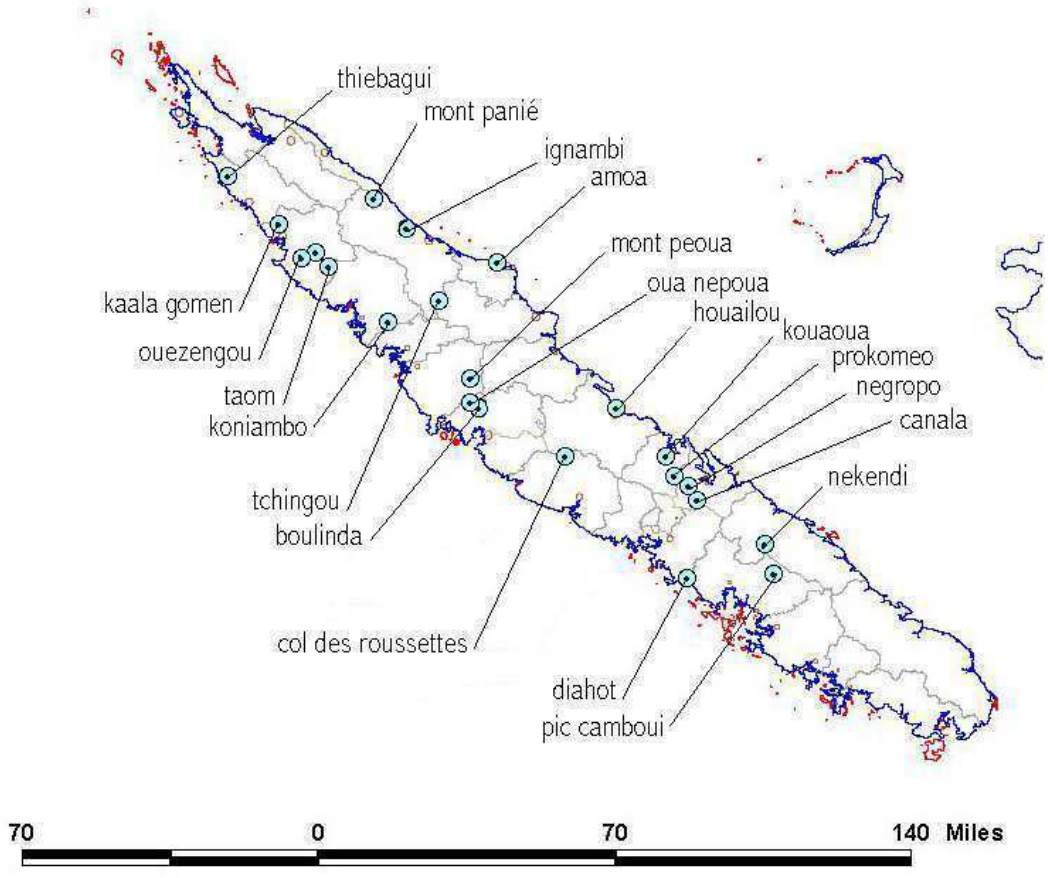


Fig. 5.8: Distribution map of *A. montana* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data

The adult individuals have a columnar shape and can reach 30m high and generally conform to the Rauh model. However, as they grow they diverge from the model by having reiterations at the base of the branches. The top of the tree is flat and the main branches can grow up to 2.5m. The bark is light brown to grey and exfoliating horizontally.

The leaves of juveniles and on low branches are lanceolate, imbricate, 10 x 4-5 mm and becoming progressively larger. The branches of adult foliage are 15-22 mm in diameter, including the leaves, generally rather uniform along the same branch. The adult leaves are stiff, strongly divergent, but clearly curved, resulting in a tip turned inside and imbricate leaves. They are oval, strongly concave above, with an extra-central crest on the back, 11-14 x 7-8 mm, narrowed and thickened at the base.

The male cones are cylindrical, 8-13.5 cm long and 20-28 mm in diameter, but often nearer to 20 than 28, accompanied below by sterile bracts reaching 10 mm and tapered from a base around 4 mm wide. The blades of the microsporophylls above the pollen sacs are divergent, triangular, acute, 4 x 4 mm, each with about 12 pollen sacs. The female cones grow to at least 8-9 cm long and 6-8 cm in diameter (perhaps more when completely mature). The seed scales reach 32 mm long, with an elongated tip 5-10 mm a little curved forward.

5.3.7.2 Personal notes

See note on *A. laubenfelsii*. There are extreme difficulties in distinguishing these taxa and it is possible that they are synonymous.

5.3.8 *Araucaria muelleri*

5.3.8.1 Summary of previous work

Araucaria muelleri grows in humid rainforest and maquis on ultramafic rocks, between 150 and 1000m. It forms small relictual populations on the maquis on

eroded soils. It is also found in the mountains as bigger populations like at Montagne des Sources and Mont Koghis.

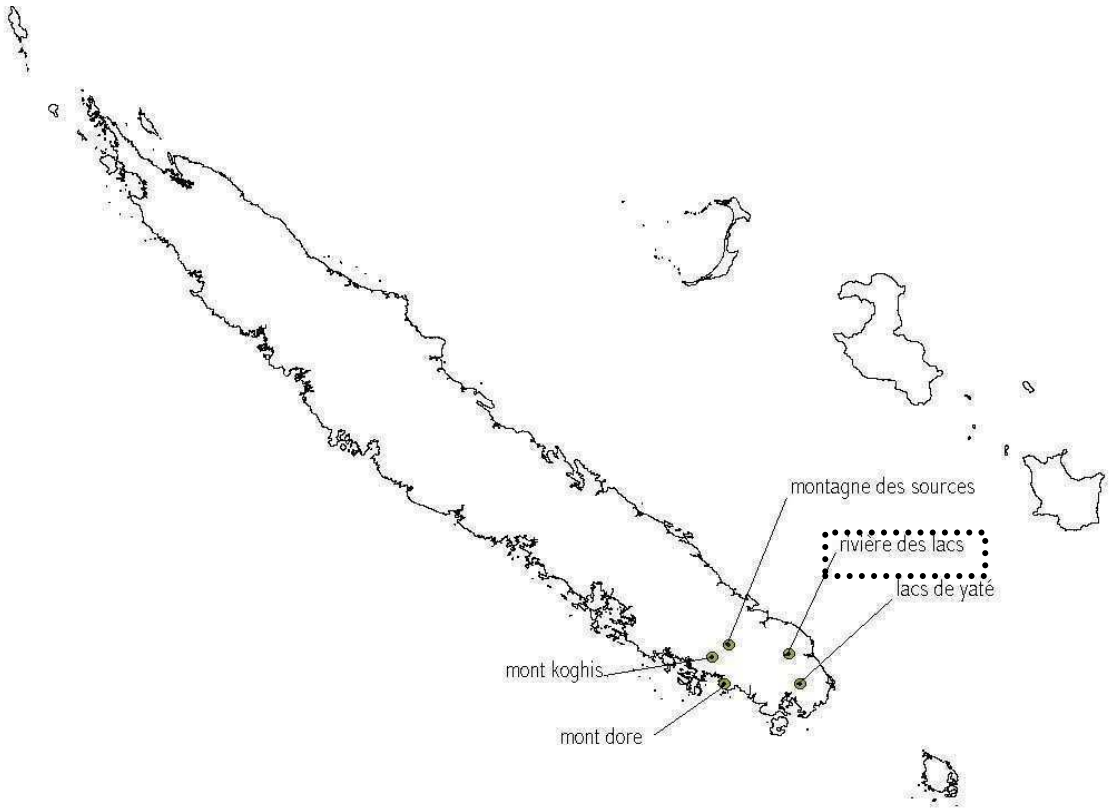


Fig. 5.9: Distribution map of *A. muelleri* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data (The map is annotated to indicate questionable populations)

The adult trees can grow up to 25 m. They grow on the Rauh model. The crown forms a perfect candelabrum with a flattened top composed of branches curved towards the top. The bark is white to light grey and exfoliates in scales or horizontal strips. The juvenile leaves are 20 to 25 mm long, strongly divergent, becoming as long as the adult leaves and enlarging as the plant passes into the adult state. The leaves of low branches are divergent, narrow, lanceolate and 30-35 x 13-15 mm. The branches of adult foliage are 30-50 mm in diameter, including the leaves. The leaves of adult-type foliage are more or less imbricate, more or less narrow and concave above, marked by a weak extra-central ridge on the adaxial face (turned towards the axis). They are 30-35 mm long and 15-20 mm wide, and thickened at the base in a quadrangular attachment.

The male cone are cylindrical, 13-25 cm long and 28-37 mm in diameter, accompanied below by sterile bracts attaining a length of 16 mm; sterile bracts are cuspidate, around 4 mm wide at the base. The blades of the microsporophyll above the pollen sacs are divergent, cuspidate and blunt, around 5 x 5 mm with about 20 pollen sacs per scale. The female cones are 11-15 cm long by 8-10 cm in diameter. The seed scales are 30-32 mm long with a beak of 10-20 mm long strongly curved forwards.

5.3.8.2 Personal notes

A. muelleri is a very distinctive species that is unlikely to be confused with other species, other than *A. rulei*, with which it shares the typical white bark and candelabra shape observable even from a distance. It has the biggest leaves (up to 35 mm in length and 16 mm in width) of the New Caledonian species. As seen in Chapter 4, four populations of *A. rulei* showed very similar morphological characters (leaves size range, leaves orientation on the axis), but the absence of stomata on the back of the leaves of *A. rulei* samples is a very robust character, supported by the presence of a frequency difference in chloroplast type in the two species, revealed by the presence of one or two extra copies of a 13 bases minisatellite in the *psb*A-*trn*H region (mainly one copy in *A. muelleri*, two or three in *A. rulei* (Chapter 4).

5.3.9 *Araucaria nemorosa*

5.3.9.1 Summary of previous work:

Araucaria nemorosa grows on oxydic soils in places receiving less than 2000 mm rainfall per annum. It is only known from seven subpopulations in the south of the island in the Botanical Reserves of Foret Nord, Port Boisé and the littoral forest of the south west of the Main Island. In these two latter localities, it grows mixed with *A. columnaris*.

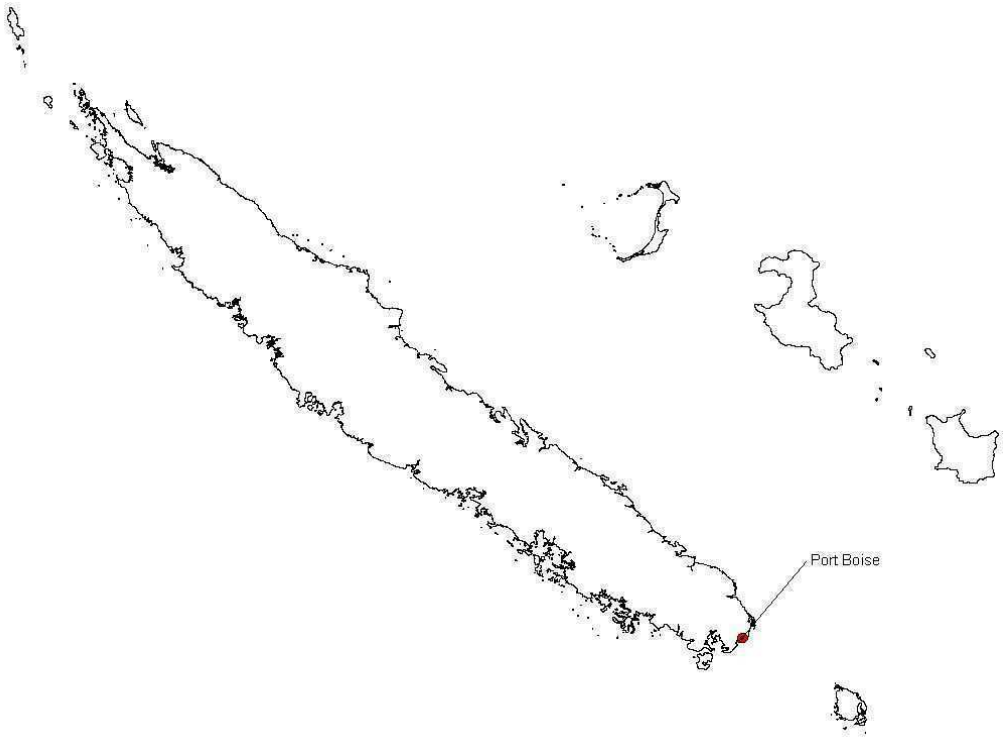


Fig. 5.10: Distribution map of *A. nemorosa* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data

The trees grow up to 15m tall and have a broadly oval shape. They follow the Massart model, with the principal branches being divergent and slightly ascending.

The branches with juvenile foliage are closely spaced, not distichous. The leaves of low branches are divergent, slightly curved forwards, quadrangular in transverse section, obtuse, sometimes clearly variable along the branch, 4-8 x 0.8-1.2 mm. The branches with adult foliage are larger, 8-12 mm in diameter, including the leaves. The latter are variable along the same branch. The leaves of adult foliage are variable, the shortest ones are divergent and the longest ones more or less imbricate, the tip slightly curved on the axial side, with a dorsal keel and triangular in cross section. They are lanceolate, blunt, more or less terminated by a curved tip and 6-10 x 1.5-3 mm.

The male cones are cylindrical, around 8 cm long and 14 mm in diameter, accompanied below by sterile bracts of 10-12 mm x 3 mm wide at their base, but clearly narrowing up to 1 mm, linear with the tip blunt. The blades of the microsporophyll above the pollen sacs are strongly divergent, triangular, blunt, 3 x 2

mm, each with around 6 pollen sacs. The female cones are 11 cm long by 8.5-9 cm in diameter. The seed scales are around 30 mm long, with an elongated tip 12-20 mm long, narrow and divergent.

5.3.9.2 Personal notes

This species represent one of the rarest conifers in the world with only one locality known and seven sub-populations. It belongs to the monophyletic coastal group, although one of its sub-population occurs slightly inland at Foret Nord, near Port Boise. This population maybe a relict locality and it may well have previously been more widespread in this type of habitat (T. Jaffré, pers.comm. 2002). A. nemorosa is rather different from the other species of the genus. Its leaves tend to keep their juvenile characters, with a slight widening at near the base of the leaves. The sterile bracts below the male cones are narrow, linear and longer than the microsporophylls, which is unique in the genus. The tree shape is rounded and even in the distance it can be differentiated from *A. columnaris*, whose populations are proximal to those of *A. nemorosa* around the coastline at Port Boise.

5.3.10 *Araucaria rulei*

5.3.10.1 Summary of previous work

Araucaria rulei grows on ultramafic rocks, on rocky slopes, or lateritic plateaux. It forms scattered population often very degraded, from Thio to the massif of Thiebagui in the north. One of the richest populations is found on Mt Boulinda on ferralitic soils, at the base of summit. It is a reliable indicator of nickel rich soils.

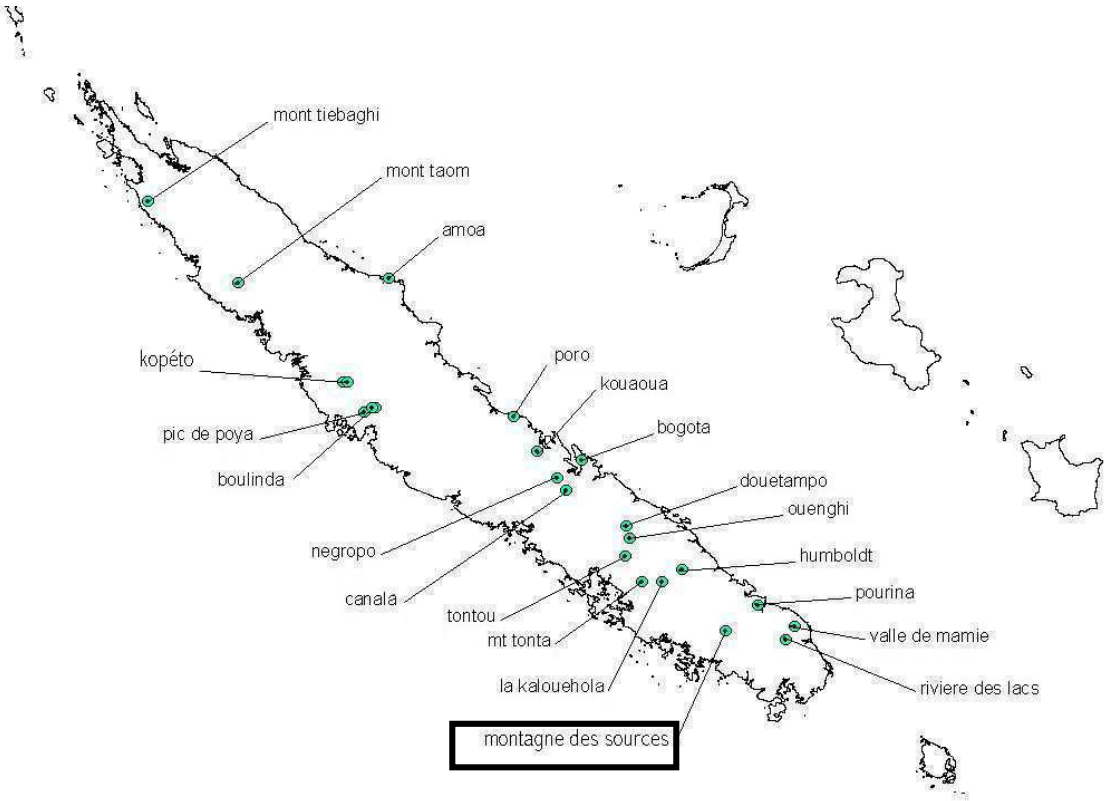


Fig. 5.11: Distribution map of *A. rulei* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data (The map is annotated to indicate questionable populations)

The adult trees can reach 30m high though this is rarely observed. They are usually smaller, with a rounded crown. They grow on the Rauh model. The primary branches are rather sparse, persisting for several years, turned upwards and each one bearing a dense bunch of stout secondary branches. The bark is white, exfoliating horizontally or in irregular scales in older trees. The leaves of juveniles are lanceolate, imbricate, slightly curved and the apex is acute. They rang in size from 12 to 15 mm in length and 2 to 5 mm in width. The adult foliage is very uniform, imbricate, dense hard and shiny. The leaves are lanceolate, acute, and divergent at first, but curved so that the tip points towards the inside. They are slightly veined on the back, 20 to 25 mm in length for a width of 11 to 14 mm.

The male cones are cylindrical, around 13 cm long and 30 mm in diameter, accompanied beneath by subulate, acute sterile bracts reaching a length of around 15 mm, tapering from a base reaching 5 mm in width. The blades of the

microsporophyll above the pollen sacs are divergent, elongate-triangular, acute, 7 x 4 mm and each scale with about 15 pollen sacs. The female cones are 12 cm long by 8 cm in diameter. The seed scales are around 30 mm long with an elongated tip around 15 mm long, divergent and slightly tilted forwards.

5.3.10.2 Personal notes

A. rulei populations are good indicators for nickel concentration. This results in most of its populations being wiped-out by mining extraction. It grows on very open ground in localities like Bwa Meyu or Ouinee, but the biggest populations grow in primary forest habitat in Mont Boulinda (this locality is also threaten by mining extraction).

It can be confused with two species depending on the state of its leaves. The young individuals can be similar to adult *A. laubenfelsii*. When the trees get older, they loose the leaves on the main branches and the blade of the leaves of the twigs widen (reaching 8 to 14 mm in populations like Bwa Meyu), making it more look like *A. muelleri*. However, in both cases, the absence of stomata in the middle of the abaxial face of the leaves can be used tell the species apart, as both *A. laubenfelsii* and *A. muelleri* have stomata up to the apex of the leaves. One dubious case was observed with a young specimen collected under the name of *A. rulei*. When put to dry, the leaves curled and the resulting morphology was one of *A. montana*, which also does not have any stomata on the back of its leaves. A molecular analysis has also revealed a relationship between the two species. *A. rulei* has a very distinctive chloroplast haplotype, with three repeats (two in very isolated cases) of the 13 base pair minisatellite in the *psb*A-*trn*H region. This chloroplast type has been observed in a few individuals of the population of *A. montana* of Mont Panie (Chapter 6). However, in the latter case, the morphology of the species was clearly not *A. rulei*, the adult leaves size not exceeding 1.2 cm, the bark being dark grey and not whitish, and finally the apex of the leaves were slightly rounded and not acute.

5.3.11 *Araucaria schmidii*

5.3.11.1 Summary of previous work

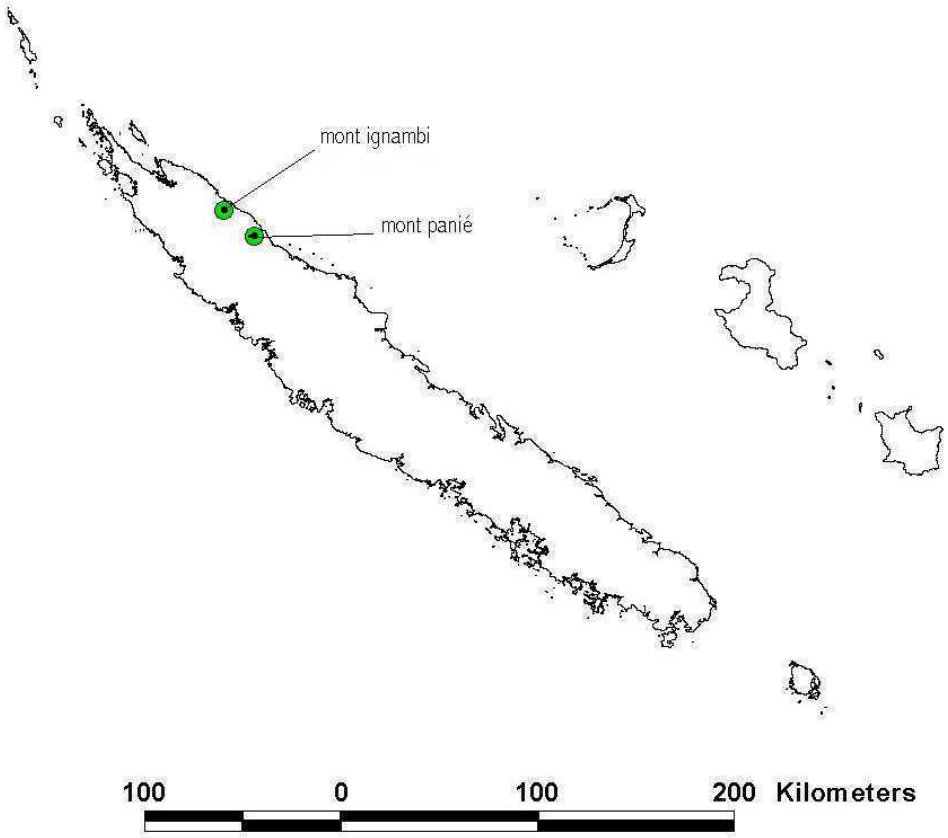


Fig. 5.11: Distribution map of *A. schmidii* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data

Araucaria schmidii grows in evergreen rainforest at high altitude on acidic soils. It is found on steep slopes, near the summit plateau of Mt Panié, above 1400m. It is only known from the massif of Mt Panié (which includes Mt Ignambi).

The trees are around 30m tall, with a flattened top. They grow on the Massart model. The bark is grey, exfoliating in horizontal strips. The juvenile leaves are divergent and curved forward, lanceolate and strongly keeled, reaching 18 x 2 x 1.5 mm. the branches with adult foliage are 6 to 9 mm in diameter, including the leaves.

The leaves of adult foliage are imbricate, lanceolate and acute, curved inwards with a keel on the back. Their size range is 7-10 mm in length for 1.5-2 mm in width.

The male cones are cylindrical, 3-5 cm long and 7-11 mm in diameter, accompanied beneath by subulate sterile bracts reaching 6 mm long. The female cones are almost round, glaucous, 8 cm long x 8 cm in diameter. The seed scales are around 20 mm long with an elongated tip around 12 mm long, divergent and slightly tilted forwards.

5.3.11.2 Personal notes

A. schmidii is a distinctive species. One of its characteristics is the trend to generate multiple stems. This phenomenon is also observed in other species but far less frequently. Its limited range of populations (Massif of Mont Panié) make it easy to identify in the field, as it can not be confused with the only other *Araucaria* growing there (*A. montana*), which is a large leaved species. However, based only on herbarium sheets, the regular aspect of its branchlets (due to the regular distribution of its leaves) and the subulate shape of its leaves are similar to the morphology of A. *subulata*. However, the density of the leaves is higher in *A. schmidii*. The cones are very different as well, being glaucous in *A. schmidii* and light green in *A. subulata*.

5.3.12 *Araucaria scopulorum*

5.3.12.1 Summary of previous work

Araucaria scopulorum grows on maquis and dense forest of low-medium altitude from 0 to 700m, in localities with a rainfall below 2000mm, most often on hypermagnesium soils issued from serpentine. It occupies rocky slopes and windy ridges next to the sea up to 300m in Thio and Houailou. Several populations have been found on the massif of Poum at altitude of 700m. It is rarely found in forests but this may be the result of extreme reduction of its distribution.

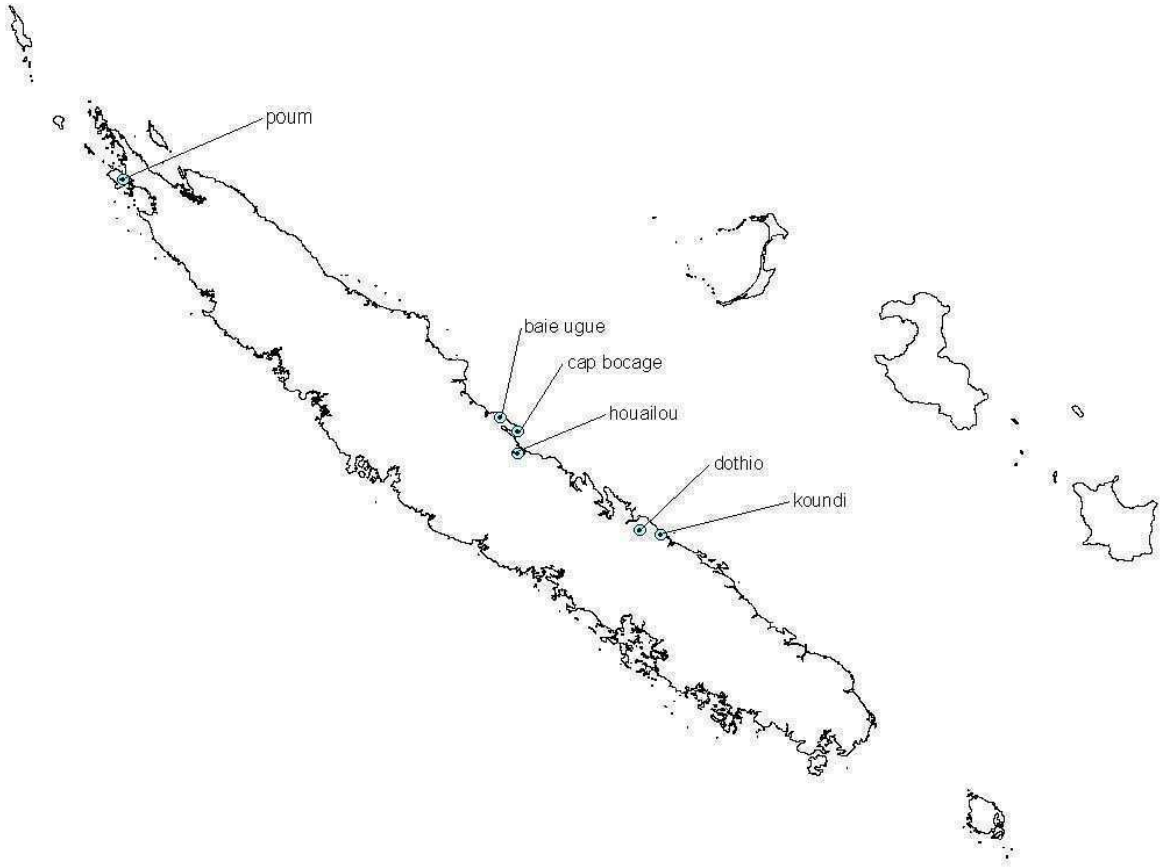


Fig. 5.13: Distribution map of *A. scopulorum* based on DeLaubenfels (1972) updated with field observations and herbarium specimen

The adult trees grow up to 20m tall, with a columnar shape. The top of the trees is flattened. They grow on the Massart model. The juvenile leaves are divergent, around 7mm long and flattened bilaterally. The bark exfoliates in horizontal strips, light grey and almost white. The juvenile leaves are divergent, 7 mm long and flattened bilaterally at first. The leaves of second-order branches are reduced, 1-2 x 1-1.5 mm. The branches with adult foliage are long, 6-8 mm in diameter, including the leaves. The leaves of adult foliage are divergent, but incurved at the tip, imbricate, furnished with a strong dorsal keel and on the lower part of the axial side, subulate, 3-4 x 2.5-3 mm, slightly narrowed and strongly thickened at the base.

The male cones are cylindrical, 3-5 cm long and 7-11 mm in diameter, accompanied beneath by subulate sterile bracts reaching 6 mm long, tapering from a

base 2 mm wide. The blades of the microsporophylls above the pollen sacs are imbricate, triangular, acute, 2.5 x 2.5 mm, each scale with about 6 pollen sacs. The seed scales are around 30 mm long, with an elongated tip around 5 mm strongly inclined forwards and finally sometimes curved outside and more or less flexed.

5.3.12.2 Personal notes

4 new sub-populations were discovered in October 2004 on the top of the mining site of Poum (Jaffré, pers. comm. 2004). The fact that new populations can still be discovered is very important, especially on a mining site. I visited this site in 2002, and the land managers were convinced that there was no *Araucaria* growing on the mining site. However, the *A. scopulorum* population was found to be very widespread but bushy-like. It is possible that other populations have still not been discovered due to the small size of individual trees.

There is an important confusion involving the northern populations of this species and *A. bernieri* (see note for *A. bernieri*), and the species may also be confused with *A. biramulata* (see note on *A. biramulata*) and *A. humboldtensis* (see note on *A. humboldtensis*).

5.3.13 *Araucaria subulata*

5.3.13.1 Summary of previous work:

Araucaria subulata grows in everegreen rainforest of low and middle altitude on ultramafic soils. It is an exclusively forest species that is found between 300 and 1000m. It is mainly found in the south of the island from 500m to 900m.

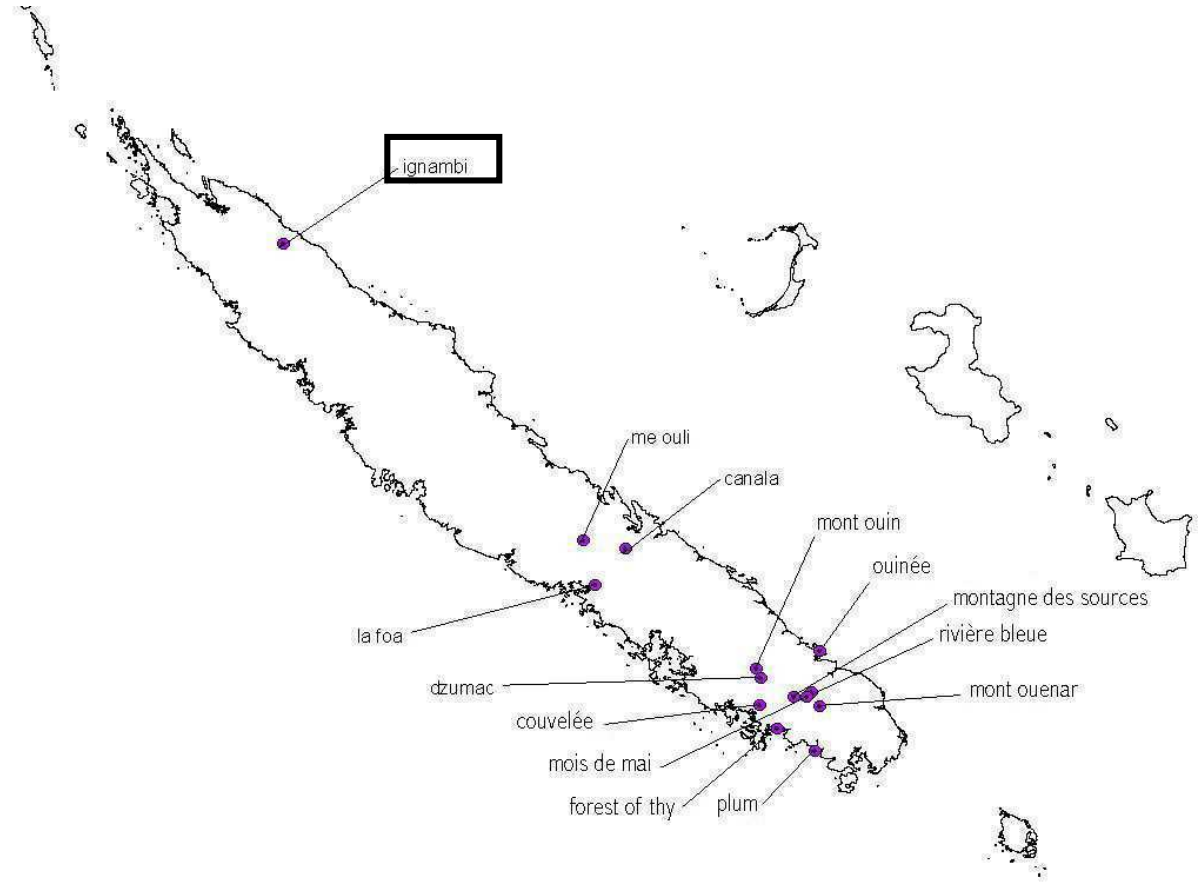


Fig. 5.14: Distribution map of *A. subulata* based on DeLaubenfels (1972) updated with field observations and herbarium specimen data (The map is annotated to indicate questionable populations)

The adult trees are narrowly columnar, attaining a height of 50 m. The first branches are rapidly falling and replaced by adventitious branches. The bark is grey, exfoliating in horizontal strips. The leaves of low branches are lanceolate, divergent then curved parallel to the branch, imbricate, strongly keeled and acute. The branches with adult foliage are more robust, resembling whips, more or less arranged in 2 ranks, 5-9 mm in diameter, including the leaves. The leaves of adult foliage are divergent, but curved inwards at the tip, imbricate, strongly keeled on the back and also almost as far as the tip on the axial side, subulate, acute, 4-6 x 2-2.5 mm, slightly narrowed and thickened at the base.

The male cones are cylindrical, 5-10 cm long and 12-13 mm diameter, accompanied beneath by lanceolate sterile bracts reaching a length of 4 mm and 2 mm wide at the base. The blades of the microsporophylls above the pollen sacs are

imbricate, triangular, acute, 3 x 2.5 mm, each microsporophyll with about 10 sacs. The female cones are at least 11-12 cm by 7-9 cm in diameter. The seed scale is around 30 mm long, with an elongated tip around 6 mm strongly curved forwards, then sometimes curved towards the top and erect.

5.3.13.2 Personal notes

A. subulata is usually identified by its subulate and needle-like leaves, generally 4 mm long. Their shape resembles *A. schmidii* though the latter has much longer leaves ranging from 5 mm to 8 mm. In very old *A. subulata*, the blade of the leaves widen (going from 1mm to 2.5 mm), giving the leaves a scaly aspect. This has not been observed in *A. schmidii*. The tall trees have a similar columnar shape to *A. bernieri*, reaching up to 50 m. The flora account (DeLaubenfels, 1972) mentions that this close architectural affinity prevented the discovery of *A. bernieri* for a long time, the two species being considered as one. This confusion still exists in localities like Montagne des Sources, where the trees are difficult to access, and the sheer height of the tree prevents leaf sampling, and the overall shape of the trees is the only element available to determine the species name. Some information is sometimes available thanks to fallen twigs. This approach is not perfect, but some confidence can be achieved when all fallen twigs from a given tree show similar morphological characteristics. Moreover, *A. bernieri* foliage is glaucous when *A. subulata* is generally green, but this characteristic can disappear in very old *A. subulata* trees where the foliage becomes darker. Morphological leaf differences have been reviewed in Chapter 4. The main differences are based on the leaves on the main branches, small (less than 1.5mm) and pressed on the bark in *A. bernieri*, needle-like and curved in *A. subulata*. The chloroplast type is also different in the two species, *A. bernieri* having three copies of the 13 bp minisatellite present in the *psb*A-*trn*H region (Chapter 3), and *A. subulata* only two. The distribution of *A. subulata* is predominantly restricted to the south of New Caledonia. The isolated population is the north on Mont Ignambi warrants rechecking. The record for this locality is based on Schmid, 2463. However the soil type of Mount Panie is of acidic type and not ultramafic. Then the specimen shows rather shaded foliage and the leaf type (small leaves, less than 6mm, needle-like, not subulate) could easily be *A. schmidii*. The

population would be worth checking using *psb*A-*trn*H chloroplastic region. The two species have a three-nucleotide difference in this region. At the sites 156, 473 and 568, A. schmidii has repectively C/G/C when A. subulata has T/A/T.

5.4 Discussion

Species	Type of substrate	Shape	Three top	Growth model	External bark color	Altitude	Adult leaf length of twigs	Adult leaf width of twigs
A. bernieri	Ultramafic	Columnar	Flat	Massart	Grey	100-700	2-3.5	1.5-2.5
A. biramulata	Ultramafic	Columnar/ Candelabra	Flat	Rauh (alterated)	Grey	150-1100	7-9	5-6
A. columnaris	Calcareous	Columnar	Flat	Massart	Grey	0-50	5-7	4-5
A. humboltensis	Ultramafic	Columnar/ Candelabra	Flat	Massart (alterated)	Grey	800-1600	5-6	4-5
A. laubenfelsii	Ultramafic	Columnar/ Candelabra	Flat	Rauh (alterated)	Grey	400-1300	12-20	8-10
A. luxurians	Ultramafic	Columnar	Flat	Massart	Grey	0-200	5-7	4-5
A. montana	Ultramafic/ Acidic	Columnar/ Candelabra	Flat	Rauh (alterated)	Grey	300-800	11-14	7-8
A. muelleri	Ultramafic	Candelabra	Rounded	Rauh	White	150-1000	20-30	12-18
A. nemorosa	Ultramafic	Columnar	Rounded	Massart (alterated)	Grey	0-50	6-10	1.5-3
A. rulei	Ultramafic	Candelabra	Rounded	Rauh	White	150-1200	20-25	11-14
A. schmidii	Acidic	Columnar	Flat	Massart	Grey	1400-1628	7-10	1.5-2
A. scopulorum	Ultramafic	Columnar	Flat	Massart	Grey	0-700	3-4	2.5-3
A. subulata	Ultramafic	Columnar	Flat	Massart	Grey	300-1000	4-6	2-2.5

Table 5.1: Summary of main characteristics of New Caledonian *Araucaria* species

The flora (DeLaubenfels, 1972) gives a good account of the species and their distributions; however, subsequent data enables the refinement of some of the morphological characters as well as the relationships between the species.

5.4.1 Distribution of the species

The distribution of some species has been updated because of recent field observation and new methods of investigations available. In the case of *A. bernieri*, the possibility of the species having disappeared in the locality of Poum and Thiebagui needs to be taken in account. In the same context, several populations of *A. rulei* and *A. laubenfelsii* are being threatened. In sites like Kopéto, ongoing deforestations are slowly wiping out the two populations. Individuals observed were covered in red dust and very unhealthy, with only a few twigs remaining at the top of the trees. The only healthy individuals were located down in the valley in a remnant primary forest surrounded by the mining site. These exploitations will slowly change the current distribution of the species if nothing is done to preserve them.

Another source of change in the distribution of species is due to the re-identification, like the population at Le Trou (cf. Chapter 4) or Montagne des Sources (cf. Chapter 4). The discovery of new localities is also adding to the information from the flora. The four new subpopulations of *A. scopulorum* found in Poum in 2004 suggest that this species is fairly cryptic, unlike other *Araucaria* species that tend to be more visible in the landscape. The adult trees can be as small as 4 m and disappear in the surrounding vegetation. This character is also present in another species, *A. humboldtensis*, and it seems reasonable to assume that other populations of this, and other *Araucaria* are still to be discovered.

5.4.2 Morphology of the species

Though all New Caledonian species belonged to the section *Eutacta* and share common features like the epigeal germination and the four cotyledons, high levels of morphological variation makes some species difficult to define. The identification

problems linked to bifid branches are a good example of this and it seems questionable to base identifications on such a labile character.

Tree habit can also vary, and damaged trees can show altered growth forms compared to undamaged trees (Veillon, 1978). Thus while the major differences between species like *A. muelleri* and *A. rulei* compared to e.g. *A. columnaris* are clear cut, habit is only of limited value as an identification method, and identifications made from helicopter reconnaissance trips should be considered provisional in the extreme.

Leaf measurements are also potentially variable characters. The development of each species starts with a similar phase of young specimens showing needle-like leaves that eventually widen during the maturation of the tree. Hence, the first phases of the development can be misleading and it is preferable to look for adult individuals in order to proceed to the determination. Even then, lower branches of the trees are often quite troublesome. The foliage of these branches can oscillate between the juvenile state to the adult state. To get stable leaf morphologies, higher branches are recommended, and when inaccessible, a collection of fallen twigs can help resolving conflicts in the determination of the species. The latter method requires caution as fallen twigs can be mixed from multiple trees. A thorough observation of the surrounding vegetation is necessary when using this method, in order to be sure no bias enters the identification process. More confidence is obtained when morphological identification is coupled with molecular identification, each method complementing one another. For example, this has been very useful to clarify the identity of southern populations of *A. rulei* (cf. Chapter 4).

5.4.3 Species of concern

The correct identification of species assumes that all of them are meaningful units. In the case of *A. montana* and *A. laubenfelsii*, some doubt remains as to whether this is the case. The extremes of these two taxa are different (i.e. leaves of twigs curved, rounded at the apex and stomata only at the base and apex on the abaxial face for *A. montana* and leaves of twigs straighter, pointed at the apex and stomata rows regular and dense up to the apex of the abaxial face for *A. laubenfelsii*). However on several

occasions, intermediate states were observed, such as leaves straight but rounded at the apex, or stomata going to the apex in discontinuous and sparse rows. Moreover, chloroplast haplotypes distributions do not match the limited morphological differences observed and this suggests that further work is needed to unravel the exact nature and distribution of these two species.

5.4.4 A key to adult foliage of New Caledonian Araucaria

While cone material and genetic techniques are useful tools for unravelling taxonomic problems in the New Caledonian *Araucaria*, simple methods need developing to facilitate field identifications. As part of this process, the information contained in this study has been summarized in the following key based on adult foliage. An illustrated version of the key is also available on: http://site.voila.fr/*Araucaria*.

Key for adult foliage of twigs for New Caledonian Araucaria species.

1)

+Stomata up to the apex of the leaves of the twigs on a continuous or a discontinuous row…**2**

+Stomata mostly basal, sometime at the apex but the middle part of the leaves are empty…. **7**

2)

+Length of the leaves >=15mm…***A. muelleri*** (Var. "with Stomata")

+Length of the leaves<15mm…. **3**

3)

+Leaves size variable by repetitive scheme along the twigs…**4**

+Leaves size regular along the twigs ….**5**

4)

+Leaves wider than 3mm and presence of papillies…. ***A. luxurians***

+Leaves not wider than 3mm and absence of papillies…. ***A. nemorosa***

5)

+Leaves wider than 3mm….**6**

+Leaves not wider than 3mm….***A. scopulorum***

6)

+Leaf length <10mm, stomata rows in a non-continous lane…. ***A. biramulata***

+Leaf length >=10mm, stomata rows in a continuous lane….***A. laubenfelsii***

7)

+Leaf length >=12mm…. ***A. rulei***

+Leaf length <10mm….**8**

8)

+Leaves size variable by repetitive scheme along the twigs…*A****. columnaris***

+Leaves size regular along the twigs …**9**

9)

+Leaves on the branches have the same width as the leaves on the twigs ….**10**

+Leaves on the branches have a different width than the leaves on the twigs ….**12**

10)

+Leaves on the branches longer than the leaves on the twigs …**11**

+Leaves on the branches of the same length of the leaves on the twigs …***A. humboldtensis***

11)

+Leaves on the twigs open on the axis, adaxial surface easily visible….***A. subulata***

+Leaves closed on the axis, adaxial surface barely visible….***A. schmidii***

12)

+Leaves much wider than 2 mm….***A. montana***

+Leaves not wider than 2mm….***A. bernieri***

CHAPTER 6 - Partitioning of chloroplast haplotypes diversity within and among New Caledonian *Araucaria* species.

6.1 Introduction

New Caledonia, a small South Pacific Ocean island (19,103 km^2) contains 13 of the world's 19 species of *Araucaria*. Phylogenetic evidence (Chapter 2; Setoguchi *et al.*, 1998) suggest that the New Caledonian *Araucaria* are monophyletic, and have speciated *in situ*, and they now occupy a diverse array of altitudinal, climatic and edaphic environments on the island ranging from colonist populations on recently emerged coral outcrops, to the summit of New Caledonia's highest mountain at >1600m. Ten of the 13 species are entirely restricted to ultramafic soils (*A. bernieri, A. humboltensis, A. luxurians, A. nemorosa, A. scopulorum, A. subulata, A. biramulata, A. laubenfelsii, A. muelleri* and *A. rulei*) and an eleventh species (*A. montana*) occurs on both ultramafic and non-ultramafic soils (Chapter 5). The remaining two species occur on schists and calcareous soils respectively (*A. schmidii* and *A. columnaris*). There are taxonomic doubts over the distinctness of one species pair (*A. montana* and *A. laubenfelsii*, Chapters 4 & 5), but even if this pair proves to be synonymous, the *Araucaria* species diversity in New Caledonia exceeds that found in the rest of the world.

Estimating the time of speciation of this group has proved difficult, and the absence of a fossil record from the island precludes direct dating. Informal estimates of the time of speciation have invoked the deposition of ultramafic soils some 30 mya as being a major driving factor behind diversification (Jaffré, 1995; Manauté *et al.*, 2003; see Fig. 6.1 for the current distribution of ultramafic soils). Recently, molecular clock approaches have been explored but these have yielded widely varying estimates of the radiation timing ranging from 10 to 43 mya depending on the method of calibration used (Chapter 3). Making the best available inference from the fossil record from elsewhere (which is very good for *Araucaria*), known dates of Gondwanan fragmentation, and estimates of nucleotide substitution rates in other plant groups, a favoured interpretation of the data is that (tentatively) the species

arose some 20~44 mya. However, it should be stressed that this estimate is provisional and there are many inconsistencies in the different data sets that do not foster confidence.

Regardless of the precise date of speciation, one puzzle remains: how did so many wind pollinated species managed to speciate in such a geographically restricted area? The island contains steep gradients of climate, precipitation, altitude, and soil chemistry and thus from an ecological perspective the range of niches is high. However, given the close spatial proximity of habitats, and the efficient dispersal of wind pollinated trees leading to high levels of gene flow, it would require very strong selective gradients for speciation to occur. Put simply, there is a basic lack of geographical space in which allopatric speciation could occur on New Caledonia.

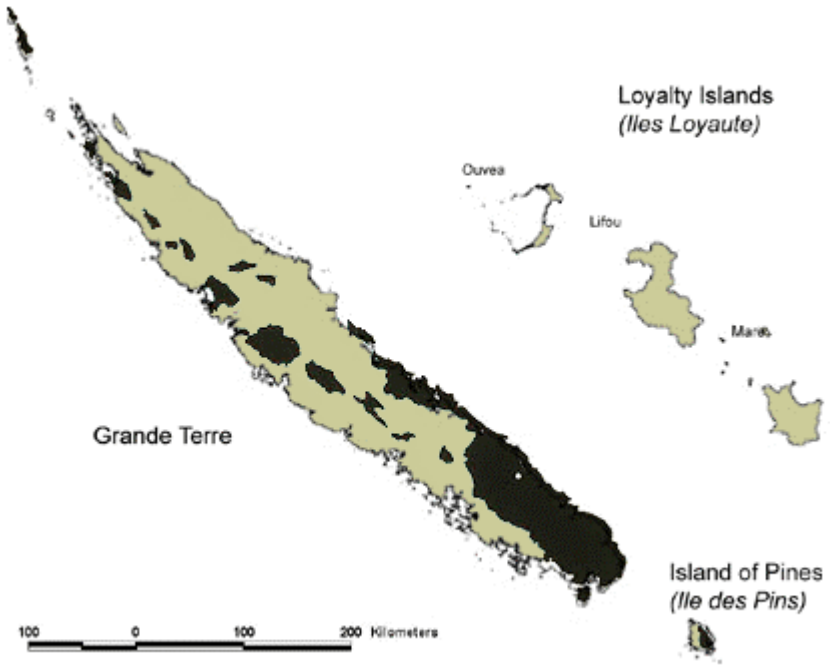


Fig. 6.1 Map of New Caledonia and with the distribution of ultramafic soils shown in black

The apparent paradox of a monophyletic radiation coupled with a small geographical space and wind pollinated trees raises the question as to the spatial scale over which differentiation can occur in these species. The often cited fact of low levels of

population differentiation among populations of conifers (e.g. Hamrick *et al.* 1992) is heavily biased by studies of temperate Pinaceae species. The confounding variables between the large northern hemisphere populations of *Pinus* species and the often fragmented distributions of tropical/sub-tropical conifers are likely to be large, and the assumption of near panmixia in all conifer species is open to question. Certainly New Caledonia contains small geographically isolated populations of *Araucaria* species and the sharp topological contrasts on the island potentially offer opportunities for genetic isolation which might contribute towards differentiation.

One aim of this chapter is to thus to undertake broad scale population surveys of chloroplast DNA markers of all species to establish the scales over which population differentiation occurs. CpDNA being paternally inherited in the Araucariaceae is likely to be a good representation of patterns of gene flow for the nuclear genome (c.f. Petit *et al.,* 2005). This contrast with the situation in angiosperms in which maternally inherited cpDNA often shows contrasting genetic structure to nuclear DNA markers which is attributable to relatively restricted of gene flow by seed compared to pollen (Ennos *et al.,* 1999; Squirrell *et al.,* 2001). By examining the distribution of cpDNA variation in multiple species is should be possible to evaluate whether there is evidence for intra-specific divergence that might give some insights into how genetic isolation might occur, and if so, in which habitats this is most evident.

A second aim of this chapter is to assess whether there is any evidence for genetic biodiversity hotspots on the island of New Caledonia. Phylogeographic studies have provided strong evidence for the importance of long term historical factors as determinants of regional genetic structure in a wide range of species (Avise, 2000). Glacial cycles can results in range expansions/contractions, and this can lead to an uneven distribution of genetic variation. Although tropical and sub-tropical regions did not experience major glaciation events like more boreal and austral regions, they did nevertheless experience climatic change which may well have altered species' distributions. Such long term historical factors, coupled with more contemporary events (e.g. levels of deforestation in different areas) could

potential lead to a situation in which some parts of a given country contain populations with higher levels of genetic diversity than others. Given that the conservation of genetic biodiversity is an integral goal of the Convention on Biological Diversity, understanding whether such hotspots are present is a necessary first stage in conserving them.

6.2 Materials and Methods

6.2.1 Plant material

Plant material was collected during 3 successive field seasons in December 2001, 2002, 2003 (Table 6.2). Populations were sampled in an effort to get maximal coverage of the distribution for each species, although this was to some extent constrained by the logistical challenges of undertaking population samples of 13 different species. In total, 49 populations were sampled, with c 10 individuals sampled per population. Species were identified using field characteristics coupled with subsequent examination of herbarium specimens. For each individual a few leaves were collected and stored in silica gel. Herbarium sheets were made for one or two individuals from most of the populations.

Species name	Locality	Population code	Sample size	5.4.5 Collector	5.5 Collector number
6 A. bernieri	Lacs de Yaté	bernLdY	7	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	369-375
A. bernieri	Montagne des Sources	bernMdS	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	4251-4260
A. bernieri	Pics des Pins	bernPdP	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	669-678
A. bernieri	Rivière des Lacs	bernRdL	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	4000-4009
A. bernieri	Thio	bernTHO	8	Kettle C. J., Kranitz M. L.	301-306+330+331
A. biramulata	Forêt Nord	birFND	8	Kettle C. J., Kranitz M. L.	99-106
A. biramulata	Mont Do	birMDO	9	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	4081-4089
A. columnaris	Baie des Tortues	colBTO	10	Kettle C. J., Kranitz M. L.	5-14
A. columnaris	Ilôt Porc Epic	colIPE	10	Kettle C. J.	334-343
A. columnaris	Lifou	colLCD	10	Kettle C. J.	461-470
A. columnaris	Maré	colMAR	10	Kettle C. J.	501-510
A. columnaris	Baie d'Oro	colORO	10	Kettle C. J.	121-130
A. columnaris	Port Boisé	colPBS	10	Kettle C. J.	730-739
A. humboldtensis	Montagne des Sources	humMdS	9	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	2001-2009
A. laubenfelsii	Bwa Meyu	lauMBK	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	4151-4158+4050-4051
A. laubenfelsii	Mont Do	lauMDO	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	603+611-616+622+631-632
A. laubenfelsii	Ouinée	lauOUE	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	2200-2204+2220-2224
A. luxurians	Botaméré	luxBOT	13	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	55-61+65+63
A. luxurians	Col d'Amieu	luxFOA	9	Kettle C. J., Kranitz M. L.	4014-4026
A. luxurians	Plum	luxPLU	7	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	930+935+940+943+946+947+954
A. montana	Boulinda	monBOU	10	Third New Caledonia Araucaria Expedition	2561-2570
A. montana	Kaala Gomen	monKAA	11	Kettle C. J., Kranitz M. L.	Ml17+26-30+889-893
A. montana	Kopéto	monKOP	6	Kettle C. J., Kranitz M. L.	Ml10+46-50
A. montana	Mont Panié	monMPA	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	4239-4248
A. muelleri	Koghis	mulKOG	12	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	895+913-919+888-889+893+896

A. muelleri	Montagne des Sources	mulMdS	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, & P. Thomas	3000-3006+4263-4265
A. muelleri	Pics des Pins	mulPdP	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	633-639+662-663+666
A. nemorosa	Cap de la Reine Charlotte	nemCRC	8	Kettle C. J., Kranitz M. L.	3050-3057
A. nemorosa	Forêt Nord	nemFND	9	Kettle C. J., Kranitz M. L.	3298-3305
A. nemorosa	Port Boisé	nemPBS	12	Kettle C. J., Kranitz M. L.	2300-2307+2444-2447
A. rulei	Bogota	rulBOG	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	241+244+246+249+250+254+255+259-261
A. rulei	Boulinda	rulBOU	9	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	[TNCA] 2571-2574 + 2593-2594 + 2596-2599
A. rulei	Camps des Sapins	rulCdS	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	301-309+312
A. rulei	Kopéto	rulKOP	10	Kettle C. J., Kranitz M. L.	17-25+52
A. rulei	Mamié	rulMAM	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	331-336+341+345+348+362
A. rulei	Bwa Meyu	rulMBK	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	[TNCA] 4110-4118+ 4126
A. rulei	Ouinée	rulOUE	11	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	[TNCA] 2230-2239
A. rulei	Poro	rulPOR	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	183-184+188-191+193-195+207
A. rulei	Thiébagui	rulTIE	5	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	38+40-41+44+50
A. rulei	Le Trou	rulTRO	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	858-877
A. schmidii	Mont Panié	schMPA	9	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	4209-4210+4217-4218+4220-4225
A. scopulorum	Cap Bocage	scoCBO	8	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	121+122+132+135+136+139+148+150
A. scopulorum	Bwa Meyu	scoMBK	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	4162-4171
A. scopulorum	Poro	scoPOR	7	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	211+212+215+216+221+222+224+226+231+233
A. scopulorum	Poum	scoPOU	10	Kettle C. J., Kranitz M. L.	2+869-876
A. scopulorum	Thio	scoTHO	11	Kettle C. J., Kranitz M. L.	307-316
A. scopulorum	Thiébagui	scoTIE	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	82-86+111-115
A. subulata	Dzumac	subDZC	10	Gardner M. F., Herbert J., Hollingsworth P. M. , Ponge A.	679-687+689
A. subulata	Montagne des Sources	subMdS	10	M. F. Gardner, M. L. Hollingsworth, P. M. Hollingsworth, C. J. Kettle, M. Kranitz, J. Manauté & P. Thomas	4266-4275

Table 6.1. Species, population localities, sample sizes and collector details of 49 sampled populations of New Caledonian *Araucaria*.

6.2.2 Molecular methods

6.2.2.1 DNA extraction

DNA was extracted from 0.5g of silica dried leaf material using Plant DNeasy kit (Qiagen, UK). Leaf material was placed in a 1.5 ml eppendorf tube and frozen by immersion in liquid nitrogen. DNA was extracted following the manufacturer's instructions using all the steps

6.2.2.2 Identification of chloroplast variants

Short repetitive sequences in chloroplast DNA (cpSSRs, chloroplast simple sequence repeats) represent a useful class of marker for distinguishing among closely related chloroplast haplotypes (Provan *et al.* 2001). Given the limited amount of cpDNA sequence variation among New Caledonian *Araucaria* species (Chapter 2), cpSSRs were employed for broader population screening. Three mononucleotide repeats (cpSSRs) were identified by sequencing the inter-genic chloroplast spacer the *trn*S-*trn*Fm in different New Caledonian *Araucaria* species (Chapter 2). One was a poly-A repeat (referred to as AP1) which ranged from A_7-A_{11}, one was a poly-C repeat (referred to as AP2) ranging from C_8-C_{11}, and one was an AT repeat, ranging from AT_6-AT_8 (referred to as AP3). A longer 13bp minisatellite (CTAAATCTAGACT) repeat unit (referred to as M13) ranging from 1-3 repeats, was identified by sequencing the inter-genic spacer the p*sb*A-*trn*H (Chapter 2). Primers pairs were designed to flank these repetitive regions using Web Primer (http://seq.yeastgenome.org/cgi-bin/web-primer) to allow for rapid assays to be undertaken discriminating chloroplast haplotypes via length variants.

6.2.2.3 Assay conditions

For the PCR, 1 µl of DNA was combined in a 10 µl PCR with 1µl of 10X NH_4 buffer (Bioline), 1 µl of dNTPs (2 µM), 0.4 µl of 50mM $MgCl_2$, 1 µl of each primer, 0.25units of Biotaq DNA polymerase (Bioline) and 5.05 µl of distilled water.

The amplifications were performed in a MJ Research PTC-200 Thermal Cycler with a first denaturising step of 12 min at 94 ºC, followed by 30 cycles [15s of denaturising at 94 ºC, 15s of annealing at 60 ºC and 25s of extension at 72 ºC, with a final extension step of 72 ºC for 30 min (Grivet *et al.*, 2001). The annealing temperature for the different markers was 60 ºC.

Microsatellites were run with the size standard 400on the CEQ8000 Beckman sequencer. Electropherograms were analysed using the Default parameters in the Fragment Analysis module of the CEQ software package version 8.0.

6.2.2.4 Data analysis

The number of repeats for each marker was coded from 1 to 6, with 1 representing the smallest number of repeats retrieved, 2 the second smallest number, and so on. The allelic state at each separate chloroplast locus was noted and then combined to produce multi-locus chloroplast haplotypes. The geographical distribution of the chloroplast haplotypes were plotted onto a map using pie charts to represent population level frequencies.

To assess the amounts of chloroplast variation, the number of populations that were polymorphic for cpDNA was recorded, and the mean gene diversity (H_E) estimated using Arlequin (Schneider *et al.*, 2000). To examine population differentiation, Analyses of Molecular Variance (AMOVA) were undertaken to partition the genetic variation within and among various groupings of the data (see below). To estimate levels of population differentiation within individual species, Arlequin was also used to estimate F_{ST}. The significance of estimates of population and taxon differentiation was undertaken using permutation tests.

To examine the distribution of genetic variation among species and regions, a hierarchical pooling approach was taken. Firstly, the amounts of genetic diversity were examined by region by splitting the main island of New Caledonia into southern, central and northern groups, and the haplotypic diversity in these regions compared (two populations of *A. columnaris* sampled from the Loyalty islands were off the east coast were treated separately in this analysis). Secondly, the patterns of haplotype diversity in various groups of species were examined. As the coastal species (*A. columnaris, A. luxurians, A. nemorosa*) represent a phylogenetically

discrete group united by their maritime distribution, they were treated as one group (the ‘coastal’ group). Secondly, the four large leaved species (*A. montana, A. laubenfelsii, A. rulei, A. muelleri*) all occur in a basal polytomy in the phylogeny. They are united by their morphology (leaf size), their occurrence on sites of high altitude (often rather open mountain tops or plateaus), and some similarity in habit (they show Rauh or modified Rauh tree shapes; Chapter 5). These species were thus considered as a group (the ‘big leaved’ group). The final group is the small leaved, non-coastal species (*A. bernieri, A. biramulata, A. humboldtensis, A. schmidii, A. scopulorum,*and *A. subulata*). These are generally phylogenetically related, albeit, rendered paraphyletic by the inclusion of the coastal group nested within this clade. They are united by similarities in habit and leaf size, and an occurrence at generally intermediate levels of elevation (but with exceptions like *A. schmidii*). *A. humboltensis* is the one species which is somewhat misplaced in this group. On phylogenetic grounds, it belongs with the large leaved species with which it co-occurs in the basal polytomy (Chapter 2). However, it is atypical for the other species in this grade. On morphological and ecological grounds it has greater affinities with the more derived ‘small leaved non-coastal species’. As such it was included in this group (which was collectively termed the ‘intermediate group’). Although this placement was rather unsatisfactory and ambiguous, as only a single population of *A. humboltensis* was available, its contribution towards signal in the data is limited anyway.

It should be stressed that these groupings are clearly diffuse. However, they are considered as a tool for exploring the data rather than fixed categories. They represent an intermediate level of hierarchy between populations within species, and between all species. By examining the amount of variation in the data set explained by different groupings, it is possible to examine whether they have any biological relevance.

6.3 Results

6.3.1 Haplotype diversity

Genetic variation was detected in all four chloroplast markers. AP1 was the most variable with five different alleles. AP3 and M13 were the least variable with three alleles each. In total 24 haplotypes were retrieved (Table 6.2). The distribution of these haplotypes among populations and species are shown in Table 6.3 and Fig. 6.2. Of the 49 populations, 40 (82%) contained more than one haplotype, the remaining 9 populations (18%) were monomorphic. 19 populations (39%) contained 2 haplotypes, 12 populations (25%) contained 3 haplotypes, 6 populations (12%) contained 4 haplotypes, and 3 populations (6%) contained 5 haplotypes (Table 6.3).

Haplotypes 1, 2, 3 and 6 are the most widespread, being present among multiple populations of multiple taxa (Tables 6.3 & 6.4). In particular, Haplotype 1 accounts for 22% of individuals (102/468), Haplotype 3 for 33% of individuals and Haplotype 6 accounts for 10% of individuals. In contrast, 10 haplotypes are found in only one population. Haplotype 4 is only present in the *A. bernieri* population of Rivière des Lacs. Haplotype 7 is only present in the population of *A. columnaris* of Maré. Haplotype 9 is only present in *A. laubenfelsi* population of Bwa Meyu. Haplotype 18 is only present in *A. muelleri* population of Montagne des Sources. Haplotype 24 is only present in *A. scopulorum* population of Poro. Finally, four populations of *A. rulei* have a unique type of chloroplast (Camps des Sapins, Kopéto, Le Trou and Poro).

6.3.2 Overall genetic structure

In the total data set there is only limited evidence of spatial geographical structure (e.g. haplotypes present at high frequency in part of the island, but not in others) (Figure 6.2, Table 6.4). Seven of the haplotypes occur in localities that span the geographical regions sampled on the main island (north, central and south). The clearest evidence for geographical structure comes from the fact that the central populations are the source of most region-specific haplotypes (Table 6.4). Of the 11

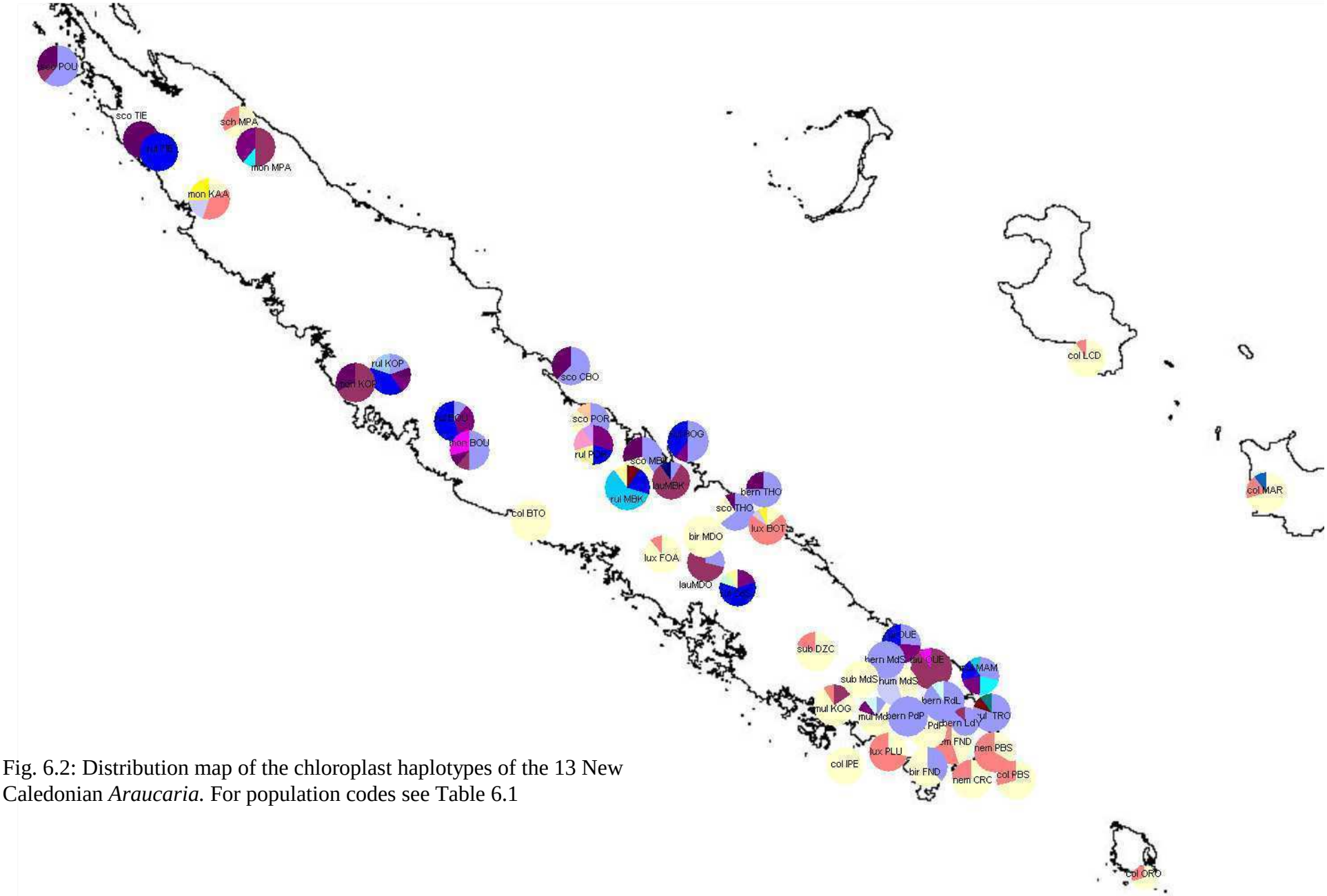


Fig. 6.2: Distribution map of the chloroplast haplotypes of the 13 New Caledonian *Araucaria.* For population codes see Table 6.1

haplotypes that are restricted to either northern, central or southern regions of the main island, 8 are restricted to the central region, and 3 are restricted to the southern region (no northern region specific haplotypes were detected). The vast majority of these central region-specific haplotypes occur in *A. rulei* (Table 6.4).

Haplotype	M13	AP1	AP2	AP3
1	2	2	2	2
2	2	3	2	2
3	1	2	2	2
4	2	2	4	2
5	2	2	3	2
6	1	2	3	2
7	1	2	1	2
8	1	3	2	2
9	2	1	2	2
10	2	3	3	2
11	1	3	3	2
12	2	4	2	2
13	3	3	2	2
14	3	4	3	2
15	3	3	1	2
16	3	4	2	2
17	3	2	2	2
18	1	2	2	3
19	3	4	2	1
20	3	5	2	2
21	3	4	4	2
22	3	3	3	2
23	3	3	2	1
24	2	2	2	3

Table 6.2. The genotypic constitution of 24 haplotypes detected among New Caledonian *Araucaria* populations based on four chloroplast markers (AP1, AP2, AP3, M13).

Population	Haplotype																							Gp
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
col BTO	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
col IPE	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
col LCD	.	.	9	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
col MAR	.	.	7	.	.	2	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
col ORO	.	.	7	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
col PBS	.	.	7	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
lux BOT	.	.	2	.	.	9	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
lux FOA	.	.	8	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
lux PLU	.	.	2	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
nem CRC	.	.	6	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
nem FND	.	.	4	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
nem PBS	.	.	4	.	.	8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	coa
bern LdY	6	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
bern MdS	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
bern PdP	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
bern RdL	9	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
bern THO	6	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
bir FND	3	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
bir MDO	.	.	9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
sch MPA	.	.	6	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
sco CBO	5	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
sco MBK	4	.	3	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
sco POR	4	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	int
sco POU	6	1	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
sco THO	7	.	3	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
sco TIE	.	.	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
sub DZC	.	.	8	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
sub MdS	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
hum MdS	.	.	4	.	.	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	int
lau MBK	1	8	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	big
lau MDO	3	7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	big
lau OUE	.	9	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	big
mon BOU	5	1	.	.	1	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	big
mon KAA	.	.	2	.	.	4	.	2	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	big
mon KOP	.	4	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	big
mon MPA	.	5	.	.	.	.	.	.	.	.	.	1	4	.	.	.	.	.	.	.	.	.	.	.	big
mul KOG	.	2	9	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	big
mul MdS	1	.	7	.	.	.	.	.	.	.	.	.	1	.	.	.	.	1	.	.	.	.	.	.	big
mul PdP	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	big
rul TRO	8	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	big
rul BOG	5	.	.	.	.	.	.	.	.	.	.	.	1	.	.	4	.	.	.	.	.	.	.	.	big
rul BOU	1	.	.	.	.	.	.	.	.	.	.	.	3	.	.	5	.	.	.	.	.	.	.	.	big
rul CdS	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	6	.	.	1	1	.	.	.	.	big
rul KOP	2	.	.	.	1	.	.	.	.	.	.	.	1	.	.	4	.	.	.	.	2	.	.	.	big
rul MAM	3	.	.	.	.	.	.	.	.	.	.	2	2	.	.	2	1	.	.	.	.	.	.	.	big
rul MBK	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	2	6	.	.	1	.	.	.	.	big
rul OUE	3	.	.	.	.	.	.	.	.	.	.	.	4	.	.	4	.	.	.	.	.	.	.	.	big
rul POR	.	.	.	.	.	.	.	.	.	.	.	.	3	.	.	2	.	.	.	2	.	2	1	.	big
rul TIE	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5	.	.	.	.	.	.	.	.	big

Table 6.3. Number of individuals in each population containing different chloroplast haplotypes based on 49 sampled populations of New Caledonian *Araucaria.* For population codes, see Table 6.1. Gp = group, coa = coastal, int = intermediate, big = big leaved species.

6.3.1.1	Region	Haplotypes																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
sch MPA	northern	.	.	6	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
sco POU	northern	6	1	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
sco TIE	northern	.	.	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
mon KAA	northern	.	.	2	.	.	4	.	2	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.
mon MPA	northern	.	5	.	.	.	.	.	.	.	.	.	1	4	.	.	.	.	.	.	.	.	.	.	.
rul TIE	northern	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5	.	.	.	.	.	.	.	.
col BTO	central	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
lux BOT	central	.	.	2	.	.	9	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.
lux FOA	central	.	.	8	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
bern THO	central	6	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
bir MDO	central	.	.	9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
sco CBO	central	5	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
sco MBK	central	4	.	3	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
sco POR	central	4	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
sco THO	central	7	.	3	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
lau MBK	central	1	8	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
lau MDO	central	3	7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
mon BOU	central	5	1	.	.	1	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.
mon KOP	central	.	4	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
rul BOG	central	5	.	.	.	.	.	.	.	.	.	.	.	1	.	.	4	.	.	.	.	.	.	.	.
rul BOU	central	1	.	.	.	.	.	.	.	.	.	.	.	3	.	.	5	.	.	.	.	.	.	.	.
rul CdS	central	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	6	.	.	1	1	.	.	.	.
rul KOP	central	2	.	.	.	1	.	.	.	.	.	.	.	1	.	.	4	.	.	.	.	2	.	.	.
rul MBK	central	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	2	6	.	.	1	.	.	.	.
rul POR	central	.	.	.	.	.	.	.	.	.	.	.	.	3	.	.	2	.	.	.	2	.	2	1	.
col IPE	southern	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
col ORO	southern	.	.	7	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
col PBS	southern	.	.	7	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
lux PLU	southern	.	.	2	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
nem CRC	southern	.	.	6	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
nem FND	southern	.	.	4	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
nem PBS	southern	.	.	4	.	.	8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
bern LdY	southern	6	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
bern MdS	southern	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
bern PdP	southern	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
bern RdL	southern	9	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
bir FND	southern	3	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
hum MdS	southern	.	.	4	.	.	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
sub DZC	southern	.	.	8	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
sub MdS	southern	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
lau OUE	southern	.	9	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
mul KOG	southern	.	2	9	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
mul MdS	southern	1	.	7	.	.	.	.	.	.	.	.	.	1	.	.	.	.	1	.	.	.	.	.	.
mul PdP	southern	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
rul TRO	southern	8	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.
rul MAM	southern	3	.	.	.	.	.	.	.	.	.	.	2	2	.	.	2	1	.	.	.	.	.	.	.
rul OUE	southern	3	.	.	.	.	.	.	.	.	.	.	.	4	.	.	4	.	.	.	.	.	.	.	.
col LCD	Loyalty Isles	.	.	9	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
col MAR	Loyalty Isles	.	.	7	.	.	2	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 6.4. Number of individuals in each population containing different chloroplast haplotypes based on 49 sampled populations of New Caledonian *Araucaria,* arranged according to different regions on New Caledonia. For population codes, see Table 6.1.

6.3.3 Diversity within species, species groups, and regions

6.3.3.1 Diversity within species

Table 6.3 provides a convenient summary of diversity within species. Several species have only two haplotypes detected (*A. nemorosa, A. biramulata, A. schmidii, A. subulata, A. humboltensis*). However, these species were all sampled from 3 or less populations and hence this low diversity may to some extent relate to low sampling of populations. *A. columnaris* has three haplotypes from 6 populations, *A. luxurians* 4 haplotypes from 3 populations, *A. bernieri* 4 haplotypes from 5 populations, *A. scopulorum* 4 haplotypes from 6 populations and *A. laubenfelsii* has 4 haplotypes from 3 populations. Higher diversity is found in *A. muelleri* - 6 haplotypes from 3 populations, *A. montana* - 10 haplotypes from 4 populations, and *A. rulei* - 13 haplotypes from 10 populations. At the within-population level (Tables 6.3 & 6.5) most species have populations with just one or two haplotypes; however, populations of *A. scopulorum, A. montana* and *A. rulei* have >50% of their populations with 3 or more haplotypes. These species also have higher gene diversity estimates (Table 6.5).

6.3.3.2 Diversity within 'species groups'

Table 6.3 shows haplotypic diversity by species according the different species groups, Figs. 6.3, 6.4 and 6.5 shows the geographical distribution of haplotypes by group, and Table 6.5. summarises the intra-population genetic diversity statistics by group. The most striking observation from these data are that the 'big leaved' species have higher levels of intra-specific and intra-population diversity than the other species. The coastal and intermediate species are typified by lower levels of

haplotypic diversity, with the exception of *A. scopulorum*. The big leaved group has a mean of 3.2 haplotypes per population, and a mean gene diversity of $H_E = 0.547$, compared to a mean of 2.08 haplotypes per population and a mean gene diversity of $H_E = 0.362$ in the coastal species, and 1.94 haplotypes per population and mean $H_E = 0.351$ in the intermediate group (Table 6.5).

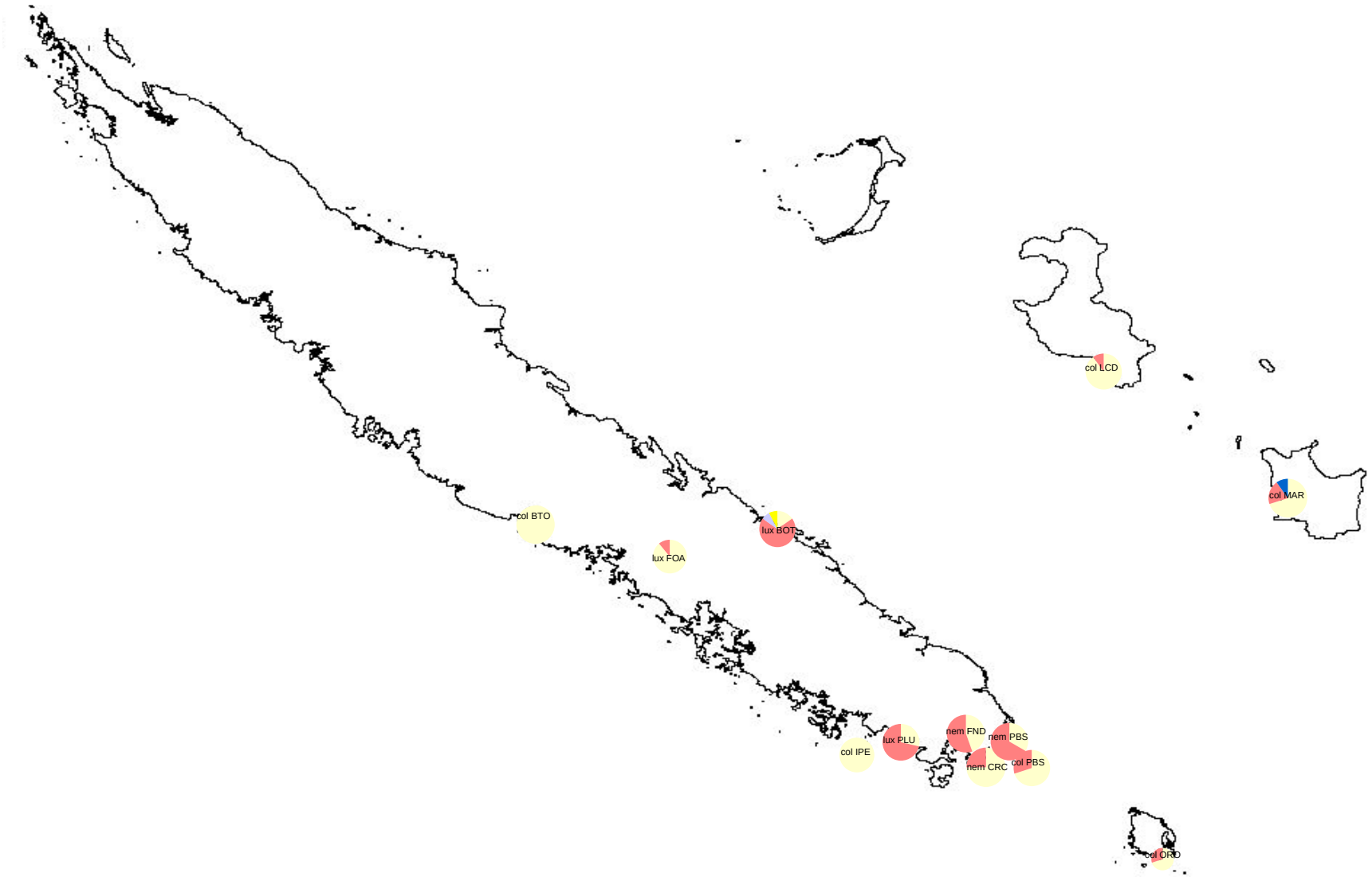


Fig. 6.3: Distribution map of chloroplast haplotypes in the 3 coastal species (*A. columnaris, A. luxurians, A. nemorosa*). For population codes see Table 6.1.

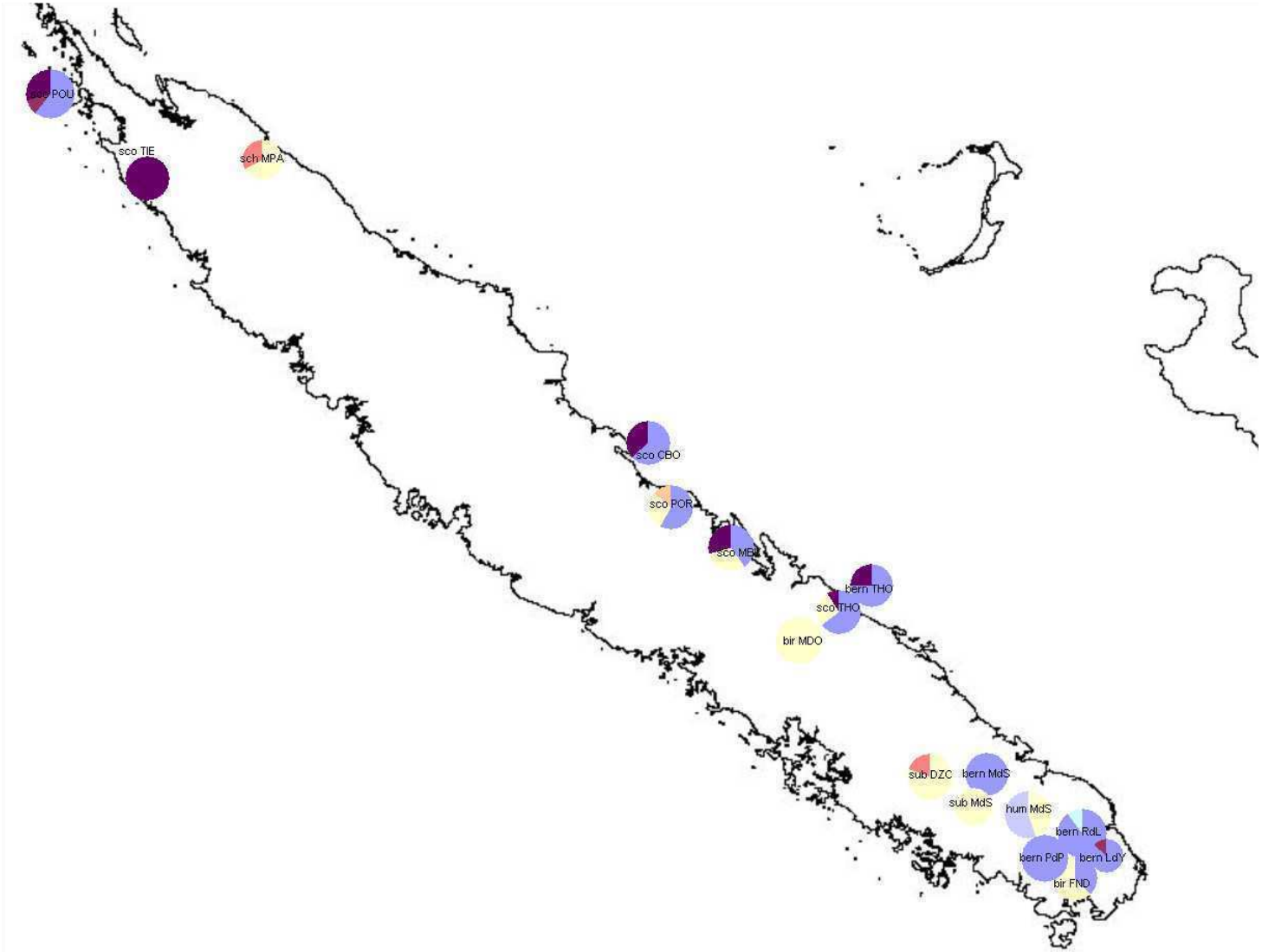


Fig. 6.4: Distribution map of chloroplast haplotypes of the intermediate group of *Araucaria* (*A. bernieri, A. biramulata, A. humboldtensis, A. schmidii, A. scopulorum,*and *A. subulata*). For population codes see Table 6.1.

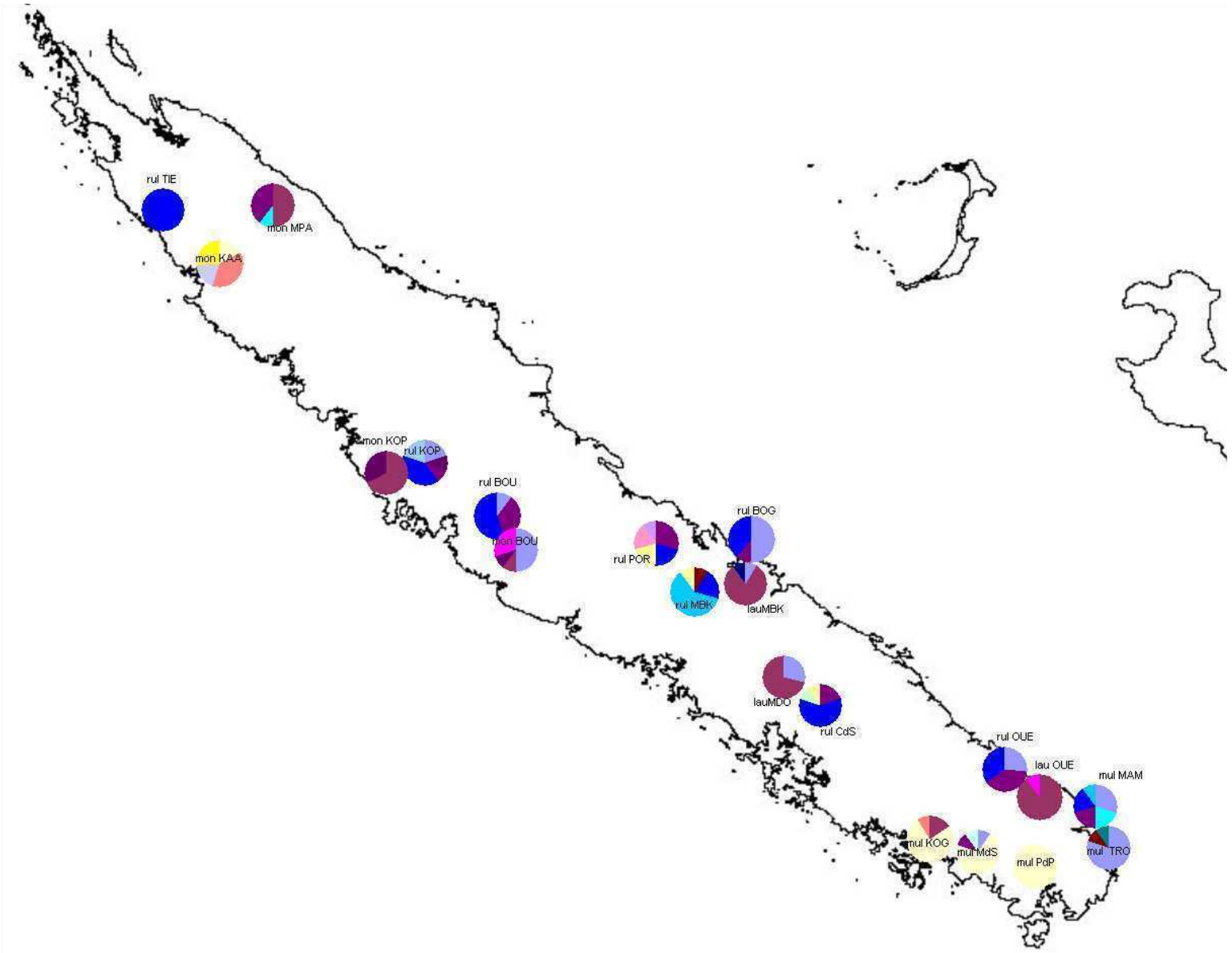


Fig. 6.5: Distribution map of chloroplast haplotypes for the big leaved species of New Caledonian *Araucaria* (*A. laubenfelsii*, *A. montana*, *A. muelleri*, and *A. rulei*). For population codes see Table 6.1.

6.3.3.3 Diversity within regions

Table 6.4 shows haplotypic diversity by species according to the different regions, Figs 6.6, 6.7 and 6.8 show the regional distribution of haplotypes, and Table 6.6 summarises the intra-population genetic diversity statistics by region.

Ten species (*A. bernieri, A. biramulata, A. columnaris, A. humboldtensis, A.laubenfelsii, A. luxurians, A. muelleri, A. nemorosa, A. rulei, A. subulata*) were sampled from the south of New Caledonia from a total of 22 populations. From these populations 14 haplotypes were detected, and 17/22 populations (77%) contained only 1 or 2 haplotypes. The mean number of haplotypes per population was 2.1, and the mean gene diversity was H_E = 0.361 (Tables 6.4 & 6.6).

Eight species (*A. bernieri, A. biramulata, A. columnaris, A.laubenfelsii, A. luxurians, A. montana, A. rulei, A. scopulorum*) were sampled from the central part of New Caledonia, from a total of 19 populations. From these populations 19 haplotypes were detected and only 7/19 populations (37%) contained only 1 or 2 haplotypes. The mean number of haplotypes per population was 2.9, and the mean gene diversity was H_E = 0.528 (Tables 6.4 & 6.6).

Four species (*A. montana, A. rulei A. schmidii, A. scopulorum*) were sampled from the northern part of New Caledonia, from a total of 6 populations. From these populations 10 haplotypes were detected and 50% of the populations contained only 1 or 2 haplotypes. The mean number of haplotypes per population was 2.3, and the mean gene diversity was H_E = 0.424 (Tables 6.4 & 6.6).

Thus populations in the centre of the island are the most diverse, and as mentioned in Section 6.3.2, the central part of the island also has the highest level of private haplotypes (Table 6.4).

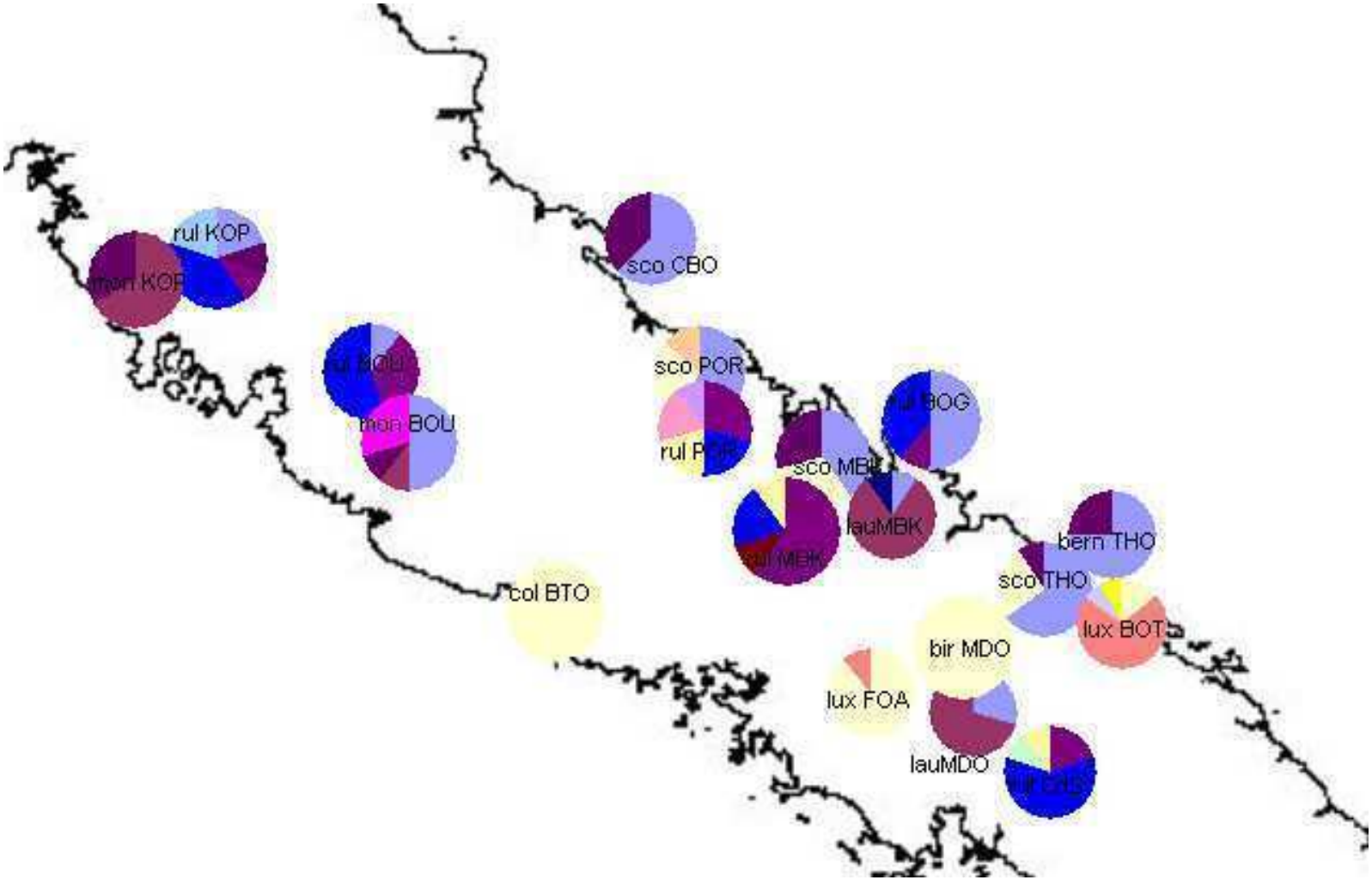


Fig. 6.6: Distribution of chloroplast haplotypes among *Araucaria* populations in the centre of New Caledonia. For population codes see Table 6.1. markers.

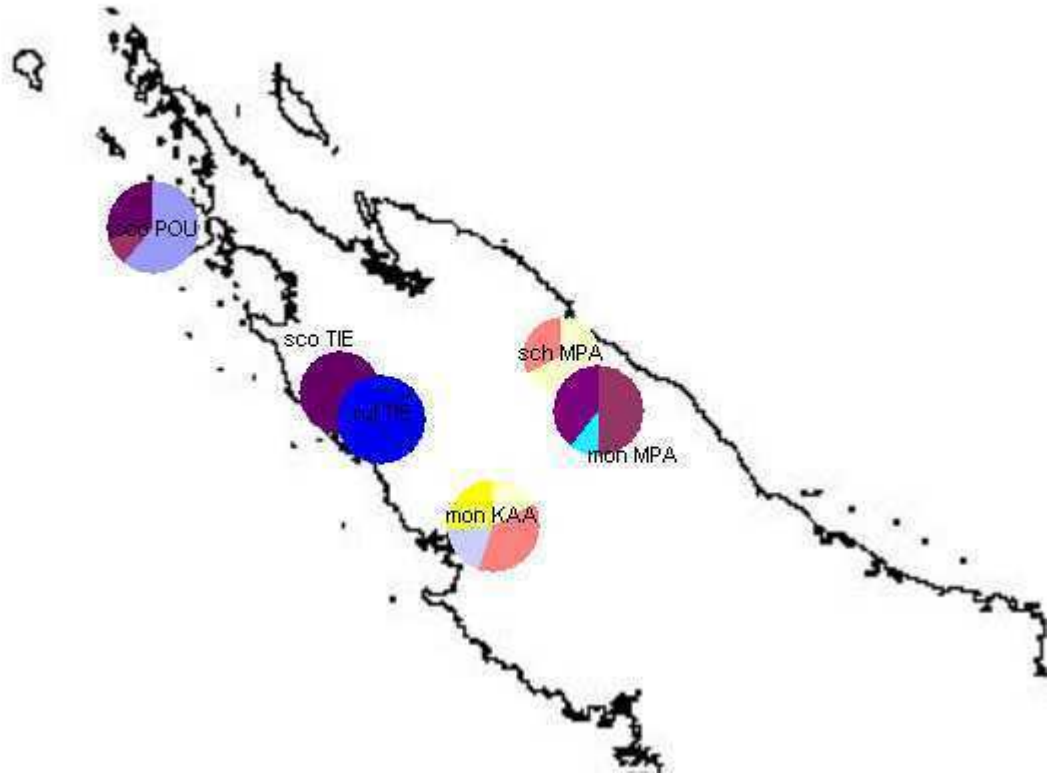


Fig. 6.7. Distribution of chloroplast haplotypes among *Araucaria* populations in the north of New Caledonia. For population codes see Table 6.1.

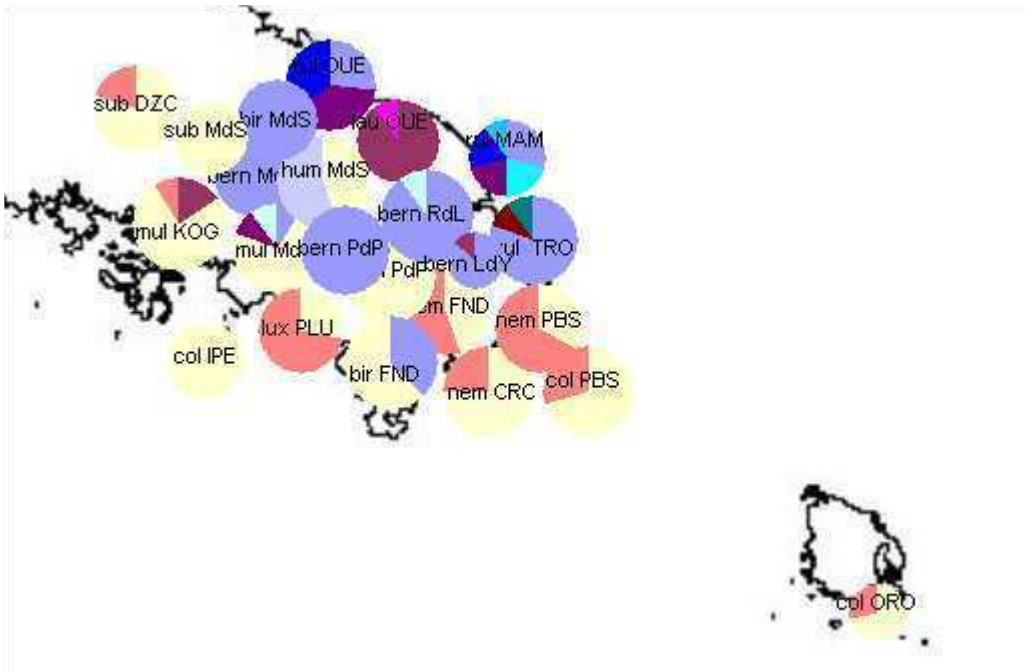


Fig. 6.8. Distribution of chloroplast haplotypes among *Araucaria* populations in the south of New Caledonia. For population codes see Table 6.1.

Group	Species	Population name	Sample size	Number of haplotypes	Gene diversity (H_E)
Coastal species	*A. columnaris*	col BTO	10	1	0
	A. columnaris	col IPE	10	1	0
	A. columnaris	col LCD	10	2	0.2000 (+/-0.1541)
	A. columnaris	col MAR	10	3	0.5111 (+/-0.1643)
	A. columnaris	col ORO	10	2	0.4667 (+/-0.1318)
	A. columnaris	col PBS	10	2	0.4667 (+/-0.1318)
	A. luxurians	lux BOT	13	4	0.5256 (+/-0.1527)
	A. luxurians	lux FOA	9	2	0.2222 (+/-0.1662)
	A. luxurians	lux PLU	7	2	0.4762 (+/-0.1713)
	A. nemorosa	nem CRC	8	2	0.4286 (+/-0.1687)
	A. nemorosa	nem FND	9	2	0.5556 (+/-0.0902)
	A. nemorosa	nem PBS	12	2	0.4848 (+/-0.1059)
Average			**9.8**	**2.08**	**0.3615**
Intermediate group	*A. bernieri*	bern LdY	7	2	0.2857 (+/-0.1964)
	A. bernieri	bern MdS	10	1	0
	A. bernieri	bern PdP	10	1	0
	A. bernieri	bern RdL	10	2	0.2000 (+/-0.1541)
	A. bernieri	bern THO	8	2	0.4286 (+/-0.1687)
	A. biramulata	bir FND	8	2	0.5357 (+/-0.1232)
	A. biramulata	bir MDO	9	1	0
	A. humboldtensis	hum MdS	9	2	0.5556 (+/-0.0902)
	A. schmidii	sch MPA	9	2	0.5000 (+/-0.1283)
	A. scopulorum	sco CBO	8	2	0.5357 (+/-0.1232)
	A. scopulorum	sco MBK	10	3	0.7333 (+/-0.0764)
	A. scopulorum	sco POR	7	3	0.6667 (+/-0.1598)
	A. scopulorum	sco POU	10	3	0.6000 (+/-0.1305)
	A. scopulorum	sco THO	11	3	0.5636 (+/-0.134)
	A. scopulorum	sco TIE	10	1	0
	A. subulata	sub DZC	10	2	0.3556 (+/-0.1591)
	A. subulata	sub MdS	10	1	0
Average			**9.2**	**1.94**	**0.3506**
Big leaved group	*A. laubenfelsii*	lau MBK	10	3	0.3778 (+/-0.1813)
	A. laubenfelsii	lau MDO	10	2	0.4667 (+/-0.1318)
	A. laubenfelsii	lau OUE	10	2	0.2000 (+/-0.1541)
	A. montana	mon BOU	10	4	0.7111 (+/-0.1175)
	A. montana	mon KAA	11	4	0.8000 (+/-0.0747)
	A. montana	mon KOP	6	2	0.5333 (+/-0.1721)
	A. montana	mon MPA	10	3	0.6444 (+/-0.1012)
	A. muelleri	mul KOG	12	3	0.4394 (+/-0.1581)
	A. muelleri	mul MdS	10	4	0.5333 (+/-0.1801)
	A. muelleri	mul PdP	10	1	0
	A. rulei	rul TRO	10	3	0.3778 (+/-0.1813)
	A. rulei	rul BOG	10	3	0.6444 (+/-0.1012)
	A. rulei	rul BOU	9	3	0.6389 (+/-0.1258)
	A. rulei	rul CdS	10	4	0.6444 (+/-0.1518)
	A. rulei	rul KOP	10	5	0.8222 (+/-0.0969)
	A. rulei	rul MAM	10	5	0.8667 (+/-0.0714)
	A. rulei	rul MBK	10	4	0.6444 (+/-0.1518)
	A. rulei	rul OUE	11	3	0.7273 (+/-0.0679)
	A. rulei	rul POR	10	5	0.8667 (+/-0.0714)
	A. rulei	rul TIE	5	1	0
Average			**9.7**	**3.2**	**0.5469**

Table 6.5. Chloroplast haplotype diversity in New Caledonian *Araucaria* arranged by species group

Location	Species	Population name	Sample size	Number of haplotypes	Gene diversity
Northern	*A. schmidii*	sch MPA	9	2	0.5000 (+/-0.1283)
	A. scopulorum	sco POU	10	3	0.6000 (+/-0.1305)
	A. scopulorum	sco TIE	10	1	0
	A. montana	mon KAA	11	4	0.8000 (+/-0.0747)
	A. montana	mon MPA	10	3	0.6444 (+/-0.1012)
	A. rulei	rul TIE	5	1	0
Average			**9.2**	**2.3**	**0.424**
Central	*A. columnaris*	col BTO	10	1	0
	A. luxurians	lux BOT	13	4	0.5256 (+/-0.1527)
	A. luxurians	lux FOA	9	2	0.2222 (+/-0.1662)
	A. bernieri	bern THO	8	2	0.4286 (+/-0.1687)
	A. biramulata	bir MDO	9	1	0
	A. scopulorum	sco CBO	8	2	0.5357 (+/-0.1232)
	A. scopulorum	sco MBK	10	3	0.7333 (+/-0.0764)
	A. scopulorum	sco POR	7	3	0.6667 (+/-0.1598)
	A. scopulorum	sco THO	11	3	0.5636 (+/-0.134)
	A. laubenfelsii	lau MBK	10	3	0.3778 (+/-0.1813)
	A. laubenfelsii	lau MDO	10	2	0.4667 (+/-0.1318)
	A. montana	mon BOU	10	4	0.7111 (+/-0.1175)
	A. montana	mon KOP	6	2	0.5333 (+/-0.1721)
	A. rulei	rul BOG	10	3	0.6444 (+/-0.1012)
	A. rulei	rul BOU	9	3	0.6389 (+/-0.1258)
	A. rulei	rul CdS	10	4	0.6444 (+/-0.1518)
	A. rulei	rul KOP	10	5	0.8222 (+/-0.0969)
	A. rulei	rul MBK	10	4	0.6444 (+/-0.1518)
	A. rulei	rul POR	10	5	0.8667 (+/-0.0714)
Average			**9.5**	**2.9**	**0.528**
Southern	*A. columnaris*	col IPE	10	1	0
	A. columnaris	col ORO	10	2	0.4667 (+/-0.1318)
	A. columnaris	col PBS	10	2	0.4667 (+/-0.1318)
	A. luxurians	lux PLU	7	2	0.4762 (+/-0.1713)
	A. nemorosa	nem CRC	8	2	0.4286 (+/-0.1687)
	A. nemorosa	nem FND	9	2	0.5556 (+/-0.0902)
	A. nemorosa	nem PBS	12	2	0.4848 (+/-0.1059)
	A. bernieri	bern LdY	7	2	0.2857 (+/-0.1964)
	A. bernieri	bern MdS	10	1	0
	A. bernieri	bern PdP	10	1	0
	A. bernieri	bern RdL	10	2	0.2000 (+/-0.1541)
	A. biramulata	bir FND	8	2	0.5357 (+/-0.1232)
	A. humboldtensis	hum MdS	9	2	0.5556 (+/-0.0902)
	A. subulata	sub DZC	10	2	0.3556 (+/-0.1591)
	A. subulata	sub MdS	10	1	0
	A. laubenfelsii	lau OUE	10	2	0.2000 (+/-0.1541)
	A. muelleri	mul KOG	12	3	0.4394 (+/-0.1581)
	A. muelleri	mul MdS	10	4	0.5333 (+/-0.1801)
	A. muelleri	mul PdP	10	1	0
	A. rulei	rul TRO	10	3	0.3778 (+/-0.1813)
	A. rulei	rul MAM	10	5	0.8667 (+/-0.0714)
	A. rulei	rul OUE	11	3	0.7273 (+/-0.0679)
Average			**9.7**	**2.1**	**0.361**
Loyalty Isles	*A. columnaris*	col LCD	10	2	0.2000 (+/-0.1541)
	A. columnaris	col MAR	10	3	0.5111 (+/-0.1643)
Average			**10**	**2.5**	**0.356**

Table 6.6. Chloroplast haplotype diversity in New Caledonian *Araucaria* arranged by geographical origins of populations

6.3.4 Differentiation among species, species groups and regions

Table 6.7 summarises estimates of differentiation of species, species groups and regions. At the level of the entire data set, 39% of variation was found within populations, 12% of the variation was between populations, and 49% of the variation was between species indicating a strong taxonomic signal in the data. When geographical regions are considered as the unit of analysis, no significant differentiation was detected between the three regions with only 4% of the variance explained by the regional partition. When populations were allocated to groups (coastal, intermediate, big leaved), significant differentiation was detected between the three groups with this difference accounting for 30% of the variation in the data set. Focusing on just the coastal species, 70% of the variation was within populations, with a small (13%) but significant amount of variation between populations within species (19% of the variation was attributable to between species differences in this group, but this was not significant). The intermediate species had a similar amount of variation among populations within species (13%) but a greater degree of differentiation among species (51%). The large leaved species again had c 13% of their variation among populations within species, and a significant difference between species (29%).

At the level of individual species, four of the 11 species for which F_{ST} was estimated, had non-significant estimates of population differentiation (*A. columnaris, A. nemorosa, A. laubenfelsii, A. muelleri*). In contrast, five species had F_{ST} estimates of >0.15 (*A. luxurians, A. biramulata, A. scopulorum, A. montana, A. rulei*). The highest estimate of F_{ST} was from *A. luxurians* (F_{ST} = 0.360).

Taxon	Percentage of variation			F_{ST}	Number of populations
	Among taxa	Among populations within taxa	Within populations		
All species[1]	49.08**	12.33**	38.59	0.614**	49
By region[2]	3.77n.s.	56.43**	39.81	0.602**	47
By group[3]	29.9**	33.77**	36.33	0.637**	49
Coastal species[4]	16.88n.s.	12.92**	70.20	0.298**	12
Intermediate species[5]	50.6**	13**	36.4	0.636**	17
Big leaved species[6]	28.87**	13.14**	57.99	0.420**	20
A. columnaris	-	5.49	94.51	0.055n.s.	6
A. luxurians	-	35.99	64.01	0.360**	3
A. nemorosa	-	7.17	92.83	0.072n.s.	3
A. bernieri	-	7.58	92.42	0.076*	5
A. biramulata	-	30.77	69.23	0.308**	2
A. humboldtensis	-	-	-	-	1
A. schmidii	-	-	-	-	1
A. scopulorum	-	24.56	75.44	0.246**	6
A. subulata	-	11.11	88.89	0.111**	2
A. laubenfelsii	-	1.47	98.53	0.015n.s.	3
A. montana	-	26.51	73.49	0.265**	4
A. muelleri	-	4.43	95.57	0.044n.s.	3
A. rulei	-	17.58	82.42	0.176**	10

Table 6.7. Distribution of genetic variation among populations, species and groups in New Caledonian *Araucaria*.

The different hierarchical levels in the AMOVA (among taxa, among populations within taxa, within populations) were defined as follows in the separate analyses starting with the lowest level moving to the highest level (e.g. effectively moving from right to left in the table):

[1]All species = variation within populations, variation among populations within species, and variation between species.

[2]By region = variation within populations, variation among populations within regions (all species within regions pooled), variation between regions.

[3]By group = variation within populations, variation among populations within group (all species within each group pooled), variation between groups. (The groups correspond to coastal species, intermediate species and big-leaved species).

[4]Coastal species = variation within populations, variation among populations within species, variation between the species, all in the coastal group.

[5]Intermediate species = variation within populations, variation among populations within species, variation between the species, all in the intermediate group.

[6]Big leaved species = variation within populations, variation among populations within species, variation between the species, all in the big leaved group.

* = $p < 0.05$, ** $p < 0.01$, n.s. = not significant.

6.4 Discussion

6.4.1 General patterns of genetic diversity among species

Although levels of sequence divergence among these species are low (Chapters 2 and 3), a large number of chloroplast haplotypes were recovered based on the three microsatellite loci and one minisatellite locus employed here. These haplotypes were not randomly distributed among taxa, and the strongest single partition in the data set is the between-species component when all species are analysed together. This indicates that unlike some of assemblages of tree species (e.g. the European oaks; Petit *et al.* 2002), New Caledonian *Araucaria* do not have a species independent distribution of chloroplast diversity. Where species are sampled from the same locality they typically show clear and marked differences in chloroplast haplotypes frequencies (see Fig. 6.3).

The extent of inter-specific differences in the data set present here is likely to be an underestimate. Thus sequence data generated in Chapter 2 showed, for instance, fixed nucleotide substitutions between the coastal species and the other New Caledonian taxa. As these differences were not included in the large scale population screens that we employed here, micro/mini-satellite haplotypes appear shared between the coastal and other species, when infact they are separable via sequencing.

Further interpretation of levels of differences between species in the different groups (c.f. Table 6.7) is confounded both by numbers of species in each group, and the variability of the markers. Thus the phylogenetically derived coastal group shows the lowest between-species differences. However, the markers used here show little variability within this group, and hence the power to detect frequency differences is low. The intermediate group showed the largest differences between its species, but it is also noteworthy that this is the group with the most species included, and hence

the greatest opportunity for there to be large taxon differences between some taxon pairs.

6.4.2 Do species groups or geographical regions best explain the data?

The chloroplast haplotypes among the 13 species were examined in the context of three informal groups (coastal, intermediate and big leaved) and also three geographical regions (southern, central and northern). In AMOVA analyses (Table 6.7), with informal groups specified, they account for 30% of the variation in the data set. In contrast, when regions are specified, they only account for 4% of the variation in the data. Given the strong taxonomic signal in the data at the species level, it is not surprising that lumping populations by geographical regions results in lower variance partitions.

The big leaved species show strong evidence for higher levels of diversity than the other two groups, regardless of whether this is measured as total number of haplotypes per group, total number of haplotypes per species, highest numbers of haplotypes found within a population, or highest mean within population genetic diversity measures. The reasons for this difference are not immediately obvious. One possible explanation relates to population sizes, as the large leaved species frequently occur in large mountain top populations that are likely to be efficient at maintaining genetic variation. However, this alone is an inadequate explanation. Populations of *A. columnaris* for instance, can consist of many thousands of trees, and yet this species has relatively limited amounts of haplotypic variation. Likewise at Montagne de Source, populations of either *A. bernieri* or *A. subulata* are extremely abundant, and yet samples from both of these species were monomorphic at this site. Another possible explanation is one of species age. If the more derived species in the genus have evolved relatively recently, they may have had limited time to generate new haplotypes. Certainly the lowest haplotypic variation of all is associated with the coastal group which is phylogenetically the most derived. However, this must be qualified with the fact that this group also contains the lowest number of species which acts as a confounding variable. A final possibility relates to possible responses to glacial cycles. The paleoclimatic data of New Caledonia suggest a decrease in the

temperatures during glacial maxima as well as a decrease in the rainfall (Pintaud *et al.*, 2001). If the island became cooler during glacial cycles, one might expect that the range of mountain top species (e.g. the big leaved group) would expand. In contrast, the range of the lower altitude species, which include many of the intermediate and coastal groups, might be expected to contract as they typically grow in warmer conditions. A historically larger population size in the large leaved species is one possible explanation for the differences in diversity levels seen today.

The presence of a high density of the big-leaved species populations (particularly *A. rulei*) in the centre of the island acts as something of a confounding variable to assess whether there are any geographical hotspots of genetic diversity (there is more diversity in the centre of the island, but this can be explain by the fact that there is more populations of *A. rulei* in the centre of the island, rather than by the high 'within population' diversity in the region per se). Variable populations of *A. rulei* occur in the south of the island at e.g. Mamié and Ouinée, and there is no marked evidence for a hotspot of diversity within this species in the central region (or a cold spot in the south). Rather the differences seem to stem from the fact that the coastal species and the intermediate species are typically less variable, and four of the six 'intermediate' group species have a southern distribution. The large leaved species are typically more variable, and more of their populations are located in the central and northern regions. However, one counterpoint to this is the observation that the one intermediate species that has a strong distribution outside of the south of the island (*A. scopulorum*) is also the most variable of the intermediate species. Thus one should perhaps keep open for discussion the option that the central/northern regions have maintained larger population sizes than the southern region at some point in history.

6.4.3 Over what spatial scales and conditions do populations of *Araucaria* become genetically isolated on New Caledonia?

One of the driving forces behind this study was evaluating whether there was evidence for population differentiation in any of the New Caledonian *Araucaria,* or whether the species were essentially panmictic. The data obtained here have

provided strong and clear evidence for genetic differentiation among populations of these species. This is perhaps most clearly evident in Fig. 6.6. This shows clear differences in cpDNA haplotypes between populations of *A. rulei* and also *A. montana*. The populations of *A. montana* on the west and east of the north of New Caledonia is one example of this. The population at Kaala Gomen has four haplotypes, and that at Mont Panié has three, but no haplotypes are shared between these populations which are less than 100km apart. A more extreme example is present in *A. rulei* between the population of Tiébagui in the north of the island, and Le Tro in the south of the island. The northern population is fixed for one haplotype, and the southern population contains three completely different haplotypes. These frequency differences have stood up to increased sampling of 30 plants per population where they have been tested further in *A. rulei* (A. Clark, unpublished data).

The current data set does not allow more precise insights into the conditions which might promote divergence, or allow an assessment of whether some species might be more prone than others to show population differentiation. At the outset of this project, it was hypothesized that the mountain top species (which grown in open habitat and are exposed to strong winds) might show lower population differentiation than the species like *A. subulata* and *A. bernieri* which typically grown in valleys and experience some degree of enclosure from the surrounding mountains and ridges. However, our sparse sampling of these valley-dwelling species does not yet allow this question to be tackled. The tall height of these canopy emergents, their usual absence of branches within 20m of the ground, and the dense vegetation in which they grow presents a massive logistical and physical challenge for sampling, and even obtaining 10 samples from a population can take one or more days. However, the fact that averaged over species within each informal group, the amount of variation among populations within species was 13% in each case, suggests that a simple pattern relating divergence to habitat is unlikely to be forthcoming.

6.4.4 Conclusions

Two simple points emerge from this study. Firstly, there is strong evidence for population differentiation among the New Caledonian *Araucaria*. That this was detected using paternally inherited markers suggests it is likely to reflect genome wide differences as paternally and bi-parentally inherited markers typically show good correlation in estimates of population genetic structure (Petit *et al.,* 2005). This contrast with the generalisations that conifers typically show low levels of population differentiation (c.f. Hamrick *et al.* 1992), and instead supports hypotheses that differentiation and speciation could have occurred *in situ,* particularly given the strong environmental gradients on New Caledonia.

Secondly, while evidence for genetic biodiversity hotspots in this study was somewhat equivocal, it is clear from a conservation perspective that different populations of *Araucaria* should not be considered as genetically equivalent. Mining companies often use the defense that 'population X can be lost, as other populations of the species occur elsewhere' (T. Jaffré pers comm., 2001). This study provides empirical evidence to support what was otherwise an unsupported defense: these populations are not genetically equivalent and instead reflect different allelic variants and combinations of genetic biodiversity, and some of these populations may have been diverging for considerable periods of time.

CHAPTER 7 - CONCLUDING REMARKS AND FURTHER WORK

This study has used a combination of molecular and morphological approaches to investigate the taxonomy and evolution of New Caledonian *Araucaria*. Although the study has perhaps raised as many questions as answers, some clear findings have emerged.

7.1 Phylogeny

The New Caledonian *Araucaria* are a monophyletic group of species, and their sister species is Norfolk Island pine (*A. heterophylla*). Although dating the colonisation of *Araucaria* on New Caledonia is difficult, long distance dispersal, rather than a Gondwanan origin cannot currently be ruled out. What can be said with more confidence, however, is that the species have been on New Caledonia longer than the 3 million years that would be implied using the age of Norfolk Island as an upper constraint. The New Caledonian species appear to have radiated within the last 45-16 *mya*. Although a diffuse estimate, this encompasses the time frame of the deposition of ultramafic soils and their subsequent erosion. And while the date estimates for this diversification are diffuse, they do at least fall outwith times for more recent (e.g. < 10 millions years) radiations that have been recorded in other species rich groups such as *Inga* (Richardson *et al.* 2001).

Within New Caledonia two main nested clades were identified. The vast majority of the small leaved species share molecular synapomorphies, and nested within this clade, the coastal species share synapomorphies. The large leaved species are concentrated instead in a basal polytomy in the genus. While not fully resolved, this does provide the first estimate of phylogenetic relationships within the New Caledonian *Araucaria*.

7.2 Taxonomy and distribution

In this thesis I have provided an overview of the current taxonomic status of the New Caledonian species. It is clear that many outstanding questions remain, particularly whether *A. laubenfelsii* is distinct from *A. montanta.* However, the work carried out here has also clarified the taxonomy/identity of several populations. Perhaps most importantly it has led to a revision of the distribution status of *A. rulei* and *A. muelleri*. Morphological (particularly stomatal) and molecular (cpDNA haplotypes) characters allowed clear groupings of populations. Based on this work it appears that *A. muelleri* is rarer than previously thought and *A. rulei* has a somewhat wider distribution that previously thought. This clarification of taxonomy and distribution will be useful for conservation.

7.3 Phylogeography and genetic hotspots

A multi-species phylogeographic study detected strong taxonomic signal in the distribution of cpDNA haplotypes. There was also a marked difference in levels of diversity between the large leaved species and the smaller leaved species. Particularly populations of *A. rulei* and *A. montana* showed atypically high levels of diversity, and it was hypothesised that these large leaved species may have been more common during glacial maxima compared to the smaller leaved species.

7.4 Molecular work on New Caledonian *Araucaria* in a broader context of the history of the New Caledonian flora.

Placing the studies of New Caledonian *Araucaria* into a broader context of the New Caledonian flora is difficult given the paucity of other studies on the island's biota. There is thus little comparative information, but that available from other molecular studies is described below and set in the context of the hypothesised history of the island.

The history of the New Caledonian biota has always been linked to Gondwanan history and hypotheses concerning vicariance vs. long distance dispersal (e.g. Sanmartin and Ronquist, 2004; Davis *et al*., 2002; Swenson *et al*., 2001, Linder

et al., 1995). The lack of fossil evidence in New Caledonia is a restraint in obtaining firm evidence on this question. Moreover, the geological history of the region is complex and uncertainties remain concerning the fact that landmasses in the region (e.g. New Zealand or New Caledonia) might have been entirely submerged at some point of their history (Pole, 1994; Picard, 1999; Hill and Brodribb, 1999). This is coupled with a shortage of empirical studies attempting to investigate the age of the arrival of the biota of the island. Morat *et al.* (1994) compiled information that suggested that the New Caledonian flora had more than 26% of floristic affinities with the Australian flora and suggested it was linked to a Gondwanan vicariance event, which would make the colonization more than 80 *mya*. Balgooy (1996) argued that the levels of endemism of New Caledonia indicated "continuous speciation in isolation" and also that New Caledonia has always remained above sea level. Considering the radiation of the Sapindaceae in South Pacific, he suggested that the distribution of the genera *Arytera, Cupaniopsis* and *Guioa* (Sapindaceae) showed that a major vicariance event seems to have taken place around 80 *mya* between Australia and New Caledonia. However, Sanmartin and Ronquist (2004) questioned this hypothesis in their work on southern hemisphere biogeography. By computing an optimal area cladogram obtained from a plant dataset from several studies on South Pacific phylogenies, they retrieved a sister-group relationship in the geological area cladogram between New Guinea and New Caledonia, instead of a New Caledonia/ New Zealand relationship that would be expected in a vicariance scenario, as New Zealand was the last Gondwanan landmass to have had contact with New Caledonia. Pole (1994) suggested that these two landmasses had been submerged during the Paleogene and the biotic similarities between them were due to post Eocene long distance dispersal. Swenson *et al.* (2001) also retrieved a strong biogeographic signal between New Caledonia and New Guinea while studying the arrival of *Nothofagus* on both islands. However they refuted the hypothesis of a biotic interchange between the two landmasses as it failed to predict any other observation of extinct fossils, and suggested that the migration route to New Caledonia involved ancient land links between New Caledonia and New Zealand. A similar hypothesis was also suggested in Cracraft (2001) and is supported by the assumption from Herzer *et al.* (1997) that the Norfolk ridge might have been uplifted

during Miocene. The current work made on *Araucaria* does not contradict this hypothesis, and the sister relationship retrieved between the New Caledonian *Araucaria* and the Norfolk Island species would fit such a scenario.

7.5 Future work

The lack of empirical evolutionary and systematic studies carried out on the global biodiversity hotspot of New Caledonia is a major obstacle to understanding more about the processes that underlie such high levels of species richness on the island.

7.5.1 Phylogeny future work

The inclusion of New Caledonian species in phylogenetic studies of widely distributed plant groups is a pre-requisite to enhancing understanding of the assemblage of the flora and fauna. Until further studies are available, the ratio of speculation to data will be uncomfortably high. In addition, a much stronger understanding is required as to the extent to which substitution rates vary from species to species. Research effort needs to be applied to obtaining and compiling estimates of substitutions rates based on good calibrations from a wide range of taxonomic groups. As it stands, molecular clock estimates of node dates often have so much variance associated with them, that virtually any biogeographic scenario can be invoked for any topology by varying the calibration points and rates used. What is critical, is the evaluation of whether there is any form of predictability as to which groups / lineages are likely to have fast rates, and which are likely to have slow rates.

7.5.2 Phylogeography future work

New Caledonia is a remarkably heterogeneous island given its small size. Its steep environmental gradients, coupled with the uneven distribution of ultramafic soils are likely to lead to spatial heterogeneity in the partitioning of genetic variation. That high levels of population differentiation can occur was evident from the study of *Araucaria* in Chapter 6. However, few of the *Araucaria* species sampled are sufficiently widespread on the island to provide phylogeographic insights that are not

confounded by taxonomy. A high priority is to undertake multi-species comparative phylogeography studies to investigate whether there are genetic diversity hotspots on the island. The current study found very limited evidence for higher diversity in the centre of the island (but this was confounded by uneven species distributions). To test this, sampling 10 or so unrelated species whose distributions encompass the length of the island would give good insights into the distribution of genetic biodiversity.

7.5.3 Further work on New Caledonian *Araucaria*

Concerning the biology of the New Caledonian *Araucaria*, a high priority is to utilise nuclear DNA markers to improve understanding of relationships and species limits. AFLPs were tried in this project, but adequate results were not obtained. Likewise, little success has been obtained from sequencing the internal transcribed spacers of nuclear ribosomal DNA. When ITS and AFLPs don't work, there are few other satisfactory DNA approaches to studying the nuclear genome other than the labour intensive challenge of working with single and low copy nuclear protein encoding regions. One additional possibility is to assess whether the flanking regions of the nuclear microsatellite markers developed for these species by Robertson *et al.* (2004) contain any useful markers. It is also possible that the microsatellites themselves will be useful, although the small number of loci that are available (7 including two unpublished loci) and the high variation at each locus, makes them poor candidates for taxonomic/phylogenetic markers, despite their usefulness for population genetics.

A change of conservation status for the different species should be envisaged in light of the re-identification of some populations in this study. Manauté *et al.* (2003) suggested the urgent definition of protected areas for *A. rulei, A. montana, A. muelleri* and *A. nemorosa* and to some lesser degree *A. scopulorum*. I also suggest that the status of *A. muelleri* and *A. bernieri* to be changed to VU in the light of recent population modifications. Before reviewing the status of *A. montana,* a clear assessment of its boundaries with *A. laubenfelsii* should be defined, and relevant molecular markers should be isolated as morphological characters are misleading when dealing with the two species.

For the future, it remains unclear whether DNA barcoding approaches will provide the set of tools which some proponents of the approach claim (Herbert *et al.* 2004). However, if such approaches can be utilised to provide large scale techniques for rapid identification and delimitation of biodiversity, they will be a welcome set of tools for problems like those of the taxonomy and conservation of New Caledonian *Araucaria* which are common to other species rich regions of the world.

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http://www.adonline.id.au/plantevol/tour/tour12.htm

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http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/W/Welcome.html

A. bernieri

Lac de Yate
361

L1	W1	L2	W2
1	1	2	1
1	1	2	1
1	1	3	1.5
1	1	2	1.5
1	1	2	1.5
1	1	3	1.5
1.5	1	3	1.5
1.5	1	4	1.5
1	1	2	1.5
1.5	1	3	1.5

Pic des Pins
669

L1	W1	L2	W2
1.5	1	3	1
1	1	4	1
1	1	5	1
1	1	5	1
1	1	4	1
1	1	4	1
1.5	1	4	1
1	1	4	1
1	1	3	1
1	1	4	1

Riviere des lacs
4001

L1	W1	L2	W2
1	1	3	1.5
1	1	3	1
1	1	4	1.5
1	1	3	1
1	1	5	1
1	1	3	2
1	1	5	1
1	1	3	1.5
1	1	3	2
1	1	4	1.5

Riviere des lacs
15393

L1	W1	L2	W2
1	1	2	1.5
1	1	2	1.5
1	1.5	2	1.5
1	1	2	2
1	1.5	3	1.5
1	1	2	1.5
1.5	1	3	1.5
1	1	2	1.5
1	1.5	2	1
1.5	1.5	2	1.5

Lac de Yate
362

L1	W1	L2	W2
1	1	2	1.5
1	1	3	1
1	1	2	1
1	1	4	1
1	1	3	1.5
1	1	4	1
1	1	4	1
1	1	4	1
1	1	3	1.5
1	1	3	1

A. subulata

Dzumac
680

L1	W1	L2	W2
4	1,5	4	2
4	1	4	1,5
3	1	3	1,5
3	1,5	4	2
4	1	3	1,5
4	1,5	4	2
4	1	4	2
3	1,5	4	2
3	1,5	3	1,5
4	1,5	4	2

Dzumac
679

L1	W1	L2	W2
4	1	5	1
4	1	5	1
4	1	5	1
4	1	5	1
4	1	5	1
4	1	5	1
4	1	5	1
4	1	4	1
3	1	5	1
4	1	5	1

Dzumac
5038

L1	W1	L2	W2
6	1.5	2	1
4	1.5	3	1.5
5	1.5	2	1.5
5	1.5	3	1.5
6	1.5	3	2
4	1.5	2	1.5
5	1.5	3	1.5
6	1.5	2	1
6	1.5	3	1.5
6	1.5	2	1.5

Dzumac
5039

L1	W1	L2	W2
3	1	4	1
4	1.5	3	2
3	1	3	2
4	1	3	2
3	1	5	1.5
3	1	5	1.5
4	1.5	5	1
3	1.5	5	1
3	1	5	1.5
3	1	4	1

Specimens from dubious populations of Montagne des Sources

Population S
4258

L1	W1	L2	W2
1	1	4	1
1	1	4	1
1	1	3	1
1	1	3	1.5
1	1	4	1
1	1	2	1
1	1	2	1.5
1	1	4	1
1	1	3	1
1	1	2	1

Population S
4261

L1	W1	L2	W2
1	1	3	1
1	1	4	1
1	1	4	1.5
1.5	1	4	1
1.5	1	4	1
1	1	4	1
1.5	1	4	1.5
1	1	3	1
1	1	4	1
1	1	2	1

Population B
4272

L1	W1	L2	W2
2	1	2	1
2	1	2	1
2	1	3	1
2	1	4	1
2	1	4	1
3	1	4	1
2	1	3	1
3	1	4	1
2	1	3	1
2	1	3	1

ANNEXE 4.1: Measures of the *Araucaria* leaves for the morphological study. L1 and W1: Length and width of the leaves on the branches bearing twigs. L2 and W2: Length and width of the leaves on the twigs. Measures are given in mm. 10 measures of different leaves were taken for the same specimen

A. muelleri

Koghis
1024

L1	W1	L2	W2
N/A	N/A	25	15
N/A	N/A	30	15
N/A	N/A	30	15
N/A	N/A	30	15
N/A	N/A	25	10
N/A	N/A	35	15
N/A	N/A	35	15
N/A	N/A	35	15
N/A	N/A	35	15
N/A	N/A	30	15

Montagne des Sources
3019

L1	W1	L2	W2
N/A	N/A	20	13
N/A	N/A	23	13
N/A	N/A	25	15
N/A	N/A	25	15
N/A	N/A	26	15
N/A	N/A	30	16
N/A	N/A	30	15
N/A	N/A	25	14
N/A	N/A	30	16
N/A	N/A	23	13

Montagne des Sources
3007

L1	W1	L2	W2
20	6	15	5
20	6	17	5
25	7	15	5
24	7	20	7
30	10	25	8
29	8	25	8
25	6	20	7
30	8	17	6
30	8	20	7
35	10	25	7

Montagne des Sources
3006

L1	W1	L2	W2
N/A	N/A	25	13
N/A	N/A	25	13
N/A	N/A	30	15
N/A	N/A	30	15
N/A	N/A	30	15
N/A	N/A	20	12
N/A	N/A	25	14
N/A	N/A	25	14
N/A	N/A	30	15
N/A	N/A	27	14

Koghis
919

L1	W1	L2	W2
17	6	20	7
17	6	20	7
20	7	20	7
20	7	17	6
20	7	25	8
16	5	24	8
18	6	20	7
17	6	24	8
20	7	23	8
20	7	19	7

Pic des Pins
653

L1	W1	L2	W2
17	8	20	12
20	9	20	12
17	8	24	14
25	10	25	14
24	10	20	13
25	10	20	12
25	9	25	14
20	8	25	14
25	9	25	14
25	10	25	13

Koghis
7

L1	W1	L2	W2
25	8	25	10
25	8	30	14
20	7	30	14
25	8	30	14
20	7	32	14
24	8	30	13
25	7	25	12
28	9	25	10
26	5	30	14
20	5	33	14

Mont Mou
3554

L1	W1	L2	W2
N/A	N/A	30	15
N/A	N/A	30	15
N/A	N/A	30	15
N/A	N/A	30	15
N/A	N/A	30	15
N/A	N/A	26	13
N/A	N/A	29	14
N/A	N/A	25	15
N/A	N/A	30	15
N/A	N/A	30	15

A. rulei

Bogota
241

L1	W1	L2	W2
N/A	N/A	12	4
N/A	N/A	12	4
N/A	N/A	15	5
N/A	N/A	15	5
N/A	N/A	12	4
N/A	N/A	14	5
N/A	N/A	15	5
N/A	N/A	12	4
N/A	N/A	15	4
N/A	N/A	15	5

Camps des Sapins
312

L1	W1	L2	W2
N/A	N/A	20	6
N/A	N/A	20	6
N/A	N/A	17	5
N/A	N/A	18	6
N/A	N/A	20	6
N/A	N/A	19	6
N/A	N/A	17	5
N/A	N/A	17	6
N/A	N/A	17	5
N/A	N/A	20	6

Camps des Sapins
311

L1	W1	L2	W2
N/A	N/A	20	6
N/A	N/A	20	6
N/A	N/A	17	5
N/A	N/A	18	6
N/A	N/A	17	5
N/A	N/A	19	6
N/A	N/A	17	5
N/A	N/A	17	6
N/A	N/A	16	5
N/A	N/A	20	6

ANNEXE 4.1(continued): Measures of the *Araucaria* leaves for the morphological study. L1 and W1: Length and width of the leaves on the branches bearing twigs. L2 and W2: Length and width of the leaves on the twigs. Measures are given in mm. 10 measures of different leaves were taken for the same specimen (N/A: non available)

A. rulei (continued)

Riviere des Lacs
5006

L1	W1	L2	W2
N/A	N/A	20	11
N/A	N/A	20	11
N/A	N/A	20	11
N/A	N/A	20	11
N/A	N/A	25	11
N/A	N/A	25	11
N/A	N/A	20	11
N/A	N/A	18	10
N/A	N/A	22	11
N/A	N/A	20	11

Bogota
1040

L1	W1	L2	W2
N/A	N/A	17	7
N/A	N/A	17	7
N/A	N/A	17	7
N/A	N/A	20	8
N/A	N/A	20	8
N/A	N/A	17	7
N/A	N/A	13	5
N/A	N/A	18	7
N/A	N/A	20	7
N/A	N/A	17	7

Bogota
242

L1	W1	L2	W2
N/A	N/A	17	6
N/A	N/A	15	5
N/A	N/A	17	6
N/A	N/A	17	6
N/A	N/A	13	4
N/A	N/A	16	5
N/A	N/A	17	6
N/A	N/A	15	5
N/A	N/A	12	4
N/A	N/A	15	4

Poro
86

L1	W1	L2	W2
N/A	N/A	18	7
N/A	N/A	15	5
N/A	N/A	17	7
N/A	N/A	17	7
N/A	N/A	15	4
N/A	N/A	16	5
N/A	N/A	15	6
N/A	N/A	15	6
N/A	N/A	12	4
N/A	N/A	15	6

Poro
194

L1	W1	L2	W2
N/A	N/A	15	7
N/A	N/A	18	8
N/A	N/A	15	5
N/A	N/A	15	5
N/A	N/A	14	4
N/A	N/A	20	8
N/A	N/A	15	5
N/A	N/A	17	6
N/A	N/A	15	5
N/A	N/A	15	5

Thiebagui
38

L1	W1	L2	W2
N/A	N/A	13	6
N/A	N/A	15	7
N/A	N/A	15	6
N/A	N/A	15	6
N/A	N/A	15	7
N/A	N/A	13	5
N/A	N/A	17	6
N/A	N/A	13	5
N/A	N/A	12	4
N/A	N/A	15	6

Specimens from dubious populations

Population 1 : Le Trou
870

L1	W1	L2	W2
N/A	N/A	25	11
N/A	N/A	28	14
N/A	N/A	30	14
N/A	N/A	30	14
N/A	N/A	25	12
N/A	N/A	30	14
N/A	N/A	30	14
N/A	N/A	25	13
N/A	N/A	20	11
N/A	N/A	23	11

Population 2 : Mamie
331

L1	W1	L2	W2
N/A	N/A	17	6
N/A	N/A	17	6
N/A	N/A	18	6
N/A	N/A	17	6
N/A	N/A	17	6
N/A	N/A	16	6
N/A	N/A	20	7
N/A	N/A	17	6
N/A	N/A	18	6
N/A	N/A	17	5

Population 2 : Mamie
333

L1	W1	L2	W2
N/A	N/A	25	10
N/A	N/A	25	11
N/A	N/A	25	11
N/A	N/A	25	11
N/A	N/A	23	10
N/A	N/A	23	10
N/A	N/A	22	10
N/A	N/A	25	11
N/A	N/A	25	11
N/A	N/A	25	11

Population 3: Ouinee
2233

L1	W1	L2	W2
N/A	N/A	20	9
N/A	N/A	20	9
N/A	N/A	20	12
N/A	N/A	21	12
N/A	N/A	22	10
N/A	N/A	20	9
N/A	N/A	19	9
N/A	N/A	17	9
N/A	N/A	20	10
N/A	N/A	20	10

Population 3: Ouinee
2251

L1	W1	L2	W2
N/A	N/A	18	8
N/A	N/A	19	8
N/A	N/A	20	9
N/A	N/A	20	9
N/A	N/A	10	5
N/A	N/A	13	5
N/A	N/A	15	6
N/A	N/A	15	6
N/A	N/A	18	8
N/A	N/A	15	6

Population 3: Ouinee
2237

L1	W1	L2	W2
N/A	N/A	13	6
N/A	N/A	15	7
N/A	N/A	15	7
N/A	N/A	15	7
N/A	N/A	17	7
N/A	N/A	15	7
N/A	N/A	15	6
N/A	N/A	13	5
N/A	N/A	15	5
N/A	N/A	12	4

Population 4 : Bwa Meyu
4111

L1	W1	L2	W2
N/A	N/A	20	5
N/A	N/A	21	6
N/A	N/A	20	4
N/A	N/A	20	5
N/A	N/A	19	4
N/A	N/A	19	6
N/A	N/A	20	7
N/A	N/A	23	5
N/A	N/A	20	4
N/A	N/A	25	7

Population 4 : Bwa Meyu
4099

L1	W1	L2	W2
N/A	N/A	17	7
N/A	N/A	15	5
N/A	N/A	18	6
N/A	N/A	15	5
N/A	N/A	17	5
N/A	N/A	20	7
N/A	N/A	17	5
N/A	N/A	17	5
N/A	N/A	18	6
N/A	N/A	17	7

Population 4 : Bwa Meyu
4106

L1	W1	L2	W2
N/A	N/A	30	12
N/A	N/A	30	12
N/A	N/A	35	13
N/A	N/A	25	8
N/A	N/A	29	10
N/A	N/A	28	11
N/A	N/A	30	11
N/A	N/A	32	13
N/A	N/A	28	11
N/A	N/A	29	12

ANNEXE 4.1(continued): Measures of the *Araucaria* leaves for the morphological study. L1 and W1: Length and width of the leaves on the branches bearing twigs. L2 and W2: Length and width of the leaves on the twigs. Measures are given in mm. 10 measures of different leaves were taken for the same specimen (N/A: non available)

TRNS-TRNFM

	SEQUENCES		MICROSATELLITES		
	3	185	AP1	AP2	AP3
'Araucaria bernieri'	C	C	**AAAAAAAA (A)**	**CCCCCCCCC (C) (C)**	**TATATATATATA -**
'Araucaria biramulata'	C	C	**AAAAAAAA -**	**CCCCCCCCC -**	**TATATATATATA -**
'Araucaria columnaris'	A	T	**AAAAAAAA -**	**CCCCCCCCC (C)**	**TATATATATATA -**
'Araucaria humboldtensis'	C	C	**AAAAAAAA (A)**	**CCCCCCCCC -**	**TATATATATATA -**
'Araucaria laubenfelsii '	C	C	**AAAAAAA - (A) (A)**	**CCCCCCCCC (C)**	**TATATATATATA (TA)**
'Araucaria luxurians '	A	T	**AAAAAAAA (A)**	**CCCCCCCCC (C)**	**TATATATATATA -**
'Araucaria montana'	C	C	**AAAAAAAA (A) (A)**	**CCCCCCCCC (C)**	**TATATATATATA -**
'Araucaria muelleri'	C	C	**AAAAAAAA (A)**	**CCCCCCCCC (C)**	**TATATATATATA (TA)**
'Araucaria nemorosa'	A	T	**AAAAAAAA -**	**CCCCCCCCC (C)**	**TATATATATATA -**
'Araucaria rulei'	C	C	**AAAAAAAA (A) (A) (A)**	**CCCCCCCC - (C) (C) (C)**	**TATATATATA - (TA)**
'Araucaria schmidii'	C	C	**AAAAAAAA -**	**CCCCCCCCC (C)**	**TATATATATATA -**
'Araucaria scopulorum'	C	C	**AAAAAAA - (A) (A)**	**CCCCCCCCC (C)**	**TATATATATATA (TA)**
'Araucaria subulata'	C	C	**AAAAAAAA -**	**CCCCCCCCC (C)**	**TATATATATATA -**

PSBA-TRNH

	SEQUENCES					MINISATELLITE
	156	412	473	560	568	ML13
'Araucaria bernieri'	C	G	G	G	T	**CTAAATCTAGACT CTAAATCTAGACT** ----------
'Araucaria biramulata'	C	G	G	G	T	**CTAAATCTAGACT (CTAAATCTAGACT)** ----------
'Araucaria columnaris'	C	G	G	G	T	**CTAAATCTAGACT** ---------- ----------
'Araucaria humboldtensis'	C	A	G	G	T	**CTAAATCTAGACT** ---------- ----------
'Araucaria laubenfelsii '	C	A	G	G	T	**CTAAATCTAGACT CTAAATCTAGACT** ----------
'Araucaria luxurians '	C	G	G	G	T	**CTAAATCTAGACT** ---------- ----------
'Araucaria montana"	C	A	G	A/G	T	**CTAAATCTAGACT (CTAAATCTAGACT) (CTAAATCTAGACT)**
'Araucaria muelleri'	C	A	G	A/G	T	**CTAAATCTAGACT (CTAAATCTAGACT) (CTAAATCTAGACT)**
'Araucaria nemorosa'	C	G	G	G	T	**CTAAATCTAGACT** ---------- ----------
'Araucaria rulei'	C	A/G	G	A/G	T	**CTAAATCTAGACT CTAAATCTAGACT (CTAAATCTAGACT)**
'Araucaria schmidii'	C	G	G	G	C	**CTAAATCTAGACT** ---------- ----------
'Araucaria scopulorum'	C	G	G	G	T	**CTAAATCTAGACT (CTAAATCTAGACT)** ----------
'Araucaria subulata'	T	G	A	G	T	**CTAAATCTAGACT** ---------- ----------

ANNEXE 5.1: Details of the different markers that were used in the study. SSRs sites are in bold. In bracket is shown the different variations encountered