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Conservation genetics and phylogenetics of New Caledonian *Retrophyllum* (Podocarpaceae) species

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Abstract *Retrophyllum* is a genus in the Podocarpaceae consisting of five species with a disjunct distribution in the Southern Hemisphere. Support for the monophyly of the genus, and its sister group relationship to an *Afrocarpus-Nageia* clade, was obtained from *trn*L-F sequences. Within *Retrophyllum*, the Melanesian species (*R. minus*, *R. comptonii*, *R. vitiense*) formed a monophyletic group, sister to the South American species *R. rospigliosii*. This topology is consistent with Gondwanan fragmentation, although the amount of nucleotide substitutions between taxa is surprisingly small for such an ancient disjunction. We were unable to distinguish between historical long distance dispersal or slow substitution rates as the source of this discrepancy. Two of the *Retrophyllum* species are endemic to New Caledonia; one of these (*R. minus*) is endangered with less than 2500 individuals remaining in the wild. The other endemic species (*R. comptonii*) is more widespread. We have used a combination of chloroplast RFLPs and RAPDs to assess the distinctness of these two taxa that can be difficult to distinguish using morphological characters. Both techniques provided taxon-specific markers that allowed the discrimination of the two species and the clarification of uncertain identifications. Within *R. comptonii* we detected evidence for intra-specific genetic differentiation corresponding to geographical isolation of populations.

Keywords *Retrophyllum*; conservation; *trn*L; RAPDs; molecular clock

INTRODUCTION

New Caledonia, lying 1200 km off the Queensland coast of Australia, was recently identified as one of the world's 25 "biodiversity hotspots" (Myers et al. 2000). Over three thousand species of vascular plants, 77% of which are endemic, occur on this island group that has a land area of only 19 000 km^2 (WWF and IUCN 1995). The main island, Grande Terre, is of continental derivation, whereas the other smaller islands in the group such as the Loyalty Islands and the Île des Pins are uplifted coral islands formed over a chain of submarine volcanoes. Habitat heterogeneity associated with patchily distributed ultramafic soil (rich in metal ores, particularly nickel), steep altitudinal and climatic gradients, and the Gondwanan derivation of Grande Terre have all been postulated as contributing factors in the evolution of such high levels of endemism (Jaffré 1992).

Paradoxically, the ultramafic soils that are a potential driving force of evolutionary diversification are also the source of a significant threat to biodiversity on the island. Opencast mining for nickel provides New Caledonia with about 90% of its export income but is a major cause of habitat loss (Mittermeier et al. 1996). Only 526.7 km^2 of land has protected status and not all of this area is covered by mining restrictions (Jaffré et al. 1998). Another

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source of environmental degradation is fire (started accidentally or deliberately).

Habitat destruction on New Caledonia represents a severe threat to the island's remarkable conifer diversity. All of the 43 species of conifer that occur on New Caledonia are endemic; these constitute approximately 7% of the world's total conifer species (Watt 1999). There is no other comparable territory remotely approaching this degree of conifer diversity (Watt 1999). Of the 43 New Caledonian species, 29 species are red-listed by the IUCN and 8 are considered Endangered or Critically Endangered (Farjon & Page 1999). Such a high percentage of the world's conifer species occurring in such a restricted and threatened area is clearly a cause for conservation concern.

Retrophyllum minus (Carrière) C.N.Page (Podocarpaceae) is one such New Caledonian conifer recognised as Endangered by the IUCN. Fossil evidence suggests that this species may once have been more widespread on Grande Terre (Hope & Pask 1998), but it is now recorded from only a small number of sites and is thought to consist of less than 2500 mature individuals (Farjon & Page 1999). A diminutive tree <3 m in height (Carrière 1867; Gray 1962; de Laubenfels 1969, 1972), it is a rheophyte (van Steenis 1981) occurring in or around lowland water courses over ultramafic substrates in the south of the island. The increase of mining activities and the creation of dams are imminent threats to this species. Of particular concern are new mining methods which use large quantities of water to extract metals, as these not only threaten the habitat of *R. minus* but also lower the water table.

A closely related species to *R. minus*, *R. comptonii* (J.Buchholz) C.N.Page, is also endemic to New Caledonia. It is a montane rain forest tree reaching a height of 30 m and is widespread on the island (de Laubenfels 1972); at present it is under no immediate threat of extinction. There is some difficulty in distinguishing these two species in the field, and identification of herbarium specimens and cultivated material is particularly difficult. Clearly, for conservation programmes to be successful, sound identification of the organisms to be protected is essential. The taxonomic characters separating the two species are summarised in Table 1.

When classic examples of these two species are seen, they are unlikely to be mistaken: a 30-m-tall tree in a mountain forest, versus a <3-m-tall tree in a lowland water course. However, there are plants that do not match the species descriptions, and individuals occur in intermediate habitat types. Some individuals growing in the *R. comptonii* habitat of montane rainforest can produce cones when only 3.5 m tall (M. Gardner unpubl. data), whilst individuals growing in typical *R. minus* habitat of lowland watercourses can reach at least 7 m. The midrib character (see Table 1), used by de Laubenfels (1972) in his key to the species, is exceedingly difficult to interpret on both fresh and dried material. It is thus a poor arbiter where there is uncertainty over identifications. Furthermore, material in *ex situ* collections is particularly difficult to identify if detailed notes on the original collection sites are unavailable (M. Gardner unpubl. data).

In addition to the two New Caledonian species of *Retrophyllum*, the genus contains three other species: *R. vitiense* (Seem.) C.N.Page (found in Fiji, New Guinea, and other islands of Indonesia and Melanesia), and *R. piresii* (Silba) C.N.Page and *R. rospigliosii* (Pilg.) C.N.Page, which occur in tropical South America. All *Retrophyllum* species are dioecious evergreen trees with dark red, drupe-like female cones. With the exception of *R. minus* they are emergent rain forest trees, with *R. vitiense* attaining sufficient size to be considered an important timber tree in Fiji (Smith 1979).

Relationships among the species and genera of the Podocarpaceae have been examined by numerous authors and the delimitation of groups has been modified repeatedly (Florin 1931; Buchholz & Gray 1948; Gray 1953, 1962; de Laubenfels 1969; Page 1989). Most recently, Page (1989) gave generic status to the closely related *Retrophyllum*, *Nageia*,

Table 1 Characters differentiating the two New Caledonian species of *Retrophyllum*.

	Character		
Species	Height	Midrib	Habitat
R. minus	to 3 m	Broader than leaf margin	Lowland water courses
R. comptonii	to 30 m	Narrower than leaf margin	Montane rainforest

and *Afrocarpus*, which had previously been treated as sections of a single genus (Bertrand 1874; de Laubenfels 1969, 1987). The distribution of morphological character states amongst the three genera are shown in Table 2.

In our study we have used molecular markers to investigate the population biology and taxonomy of the New Caledonian *Retrophyllum* species. Our aims have been: 1) to place the two New Caledonian species into a phylogenetic context by comparing *trn*L-F sequences from these species with those of other *Retrophyllum* species and related genera; 2) to establish whether field-based identifications of the New Caledonian species are supported by genetic data, and to develop a diagnostic assay to facilitate the identification of material; 3) to examine the partitioning of genetic variation among populations of *R. minus* and *R. comptonii*.

MATERIALS AND METHODS

Plant material

Material representing the two New Caledonian *Retrophyllum* species was collected in 1999 (International Conifer Conservation Programme Expedition) and stored in silica gel at –80°C prior to the start of laboratory work. The collections represent 10 individuals from each of 3 populations of *R. comptonii* (referred to here as Com-A, Com-B, and Com-C) and 3 populations of *R. minus* (referred to here as Min-A, Min-B, and Min-C) (Table 3). The sample populations were taken from throughout the distributional range of the species and with a minimum sample distance of 12 km between populations. A seventh population of uncertain identity was also included which was collected at Rivière Trou Bleu in the south of Grande Terre

Table 2 Distribution of character states among *Afrocarpus*, *Nageia*, and *Retrophyllum* (adapted from Gray 1953, 1962; Hair & Beuzenberg 1958; Zou 1982; Page 1990; Hill & Pole 1992; Kelch 1997).

	Genus		
Character	*Afrocarpus*	*Nageia*	*Retrophyllum*
Chromosome number	24	20, 26, 29	20
Leaf flattening in shoots	Homofacial	Homofacial	Heterofacial (except in *R. minus*)
Leaf arrangement	Often alternate	Opposite	Opposite
Resin canals	Single beneath vascular bundle	Single beneath each vein	Single beneath vascular bundle (numerous in *R. rospigliosii*)
Transfusion tissue	Extending into leaf blade	Absent	Extending into leaf blade
Coloured heartwood	Present	Absent	Present
Epidermal cells	Relatively scattered	Arranged in rows	Arranged in rows
Epidermal cell wall shape	More or less straight	Sinuous	More or less straight
Florin rings	Indistinct	Present	Present
Pollen size	> 44 mm	> 44 mm	< 37 mm
Cap cells	Absent	Absent	Present
Stomatal apparatus shape	Polar subsidiary cells project beyond extensions of lateral subsidiary cells	Polar subsidiary cells project beyond extensions of lateral subsidiary cells	Oval
Polar extensions of guard cells	Narrow, sometimes attached to lateral subsidiary cell wall	Narrow, delicate, never attached to lateral subsidiary cell wall	Very fine, flattened, sometimes attached to lateral subsidiary cell wall

(referred to here as RTB) (Table 3). Our sample from this population consisted of a single mature female tree and a selection of seedlings of varying ages, putative offspring of the adult. This locality is listed in the flora of New Caledonia as a site for *R. comptonii* (de Laubenfels 1972). However, the altitude of this locality, almost at sea level, is more typical of *R. minus*. The single mature tree exceeded the height description for *R. minus* by 4 m, but at 7 m was small for *R. comptonii*.

All populations were screened for RAPD (randomly amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) variation (see below). In addition, single samples from each population, and single samples from the three other species in the genus, were included in the phylogenetic study based on the *trn*L-F region of chloroplast DNA (Table 4). Air-dried material of *R. vitiense* originated from Fiji (elsewhere this species occurs on islands north and west of Fiji to the Moluccas; Smith 1979). Fresh material of *R. rospigliosii*, taken from the Living Collection at the Royal Botanic Garden Edinburgh, originated in Venezuela (elsewhere this species occurs in Colombia, Ecuador, and Peru; de Laubenfels 1982; Torres-Romero 1988; Brako & Zarucchi 1993; Jørgensen & León-Yánez 1999). Permission was granted by the New York Botanical Garden to remove a small amount of leaf material from the isotype specimen of *R. piresii* for analyses; this species has only been recorded from the type locality in the Serra Pacas Novos, Brazil (Silba 1983).

Afrocarpus gracilior (Pilg.) C.N.Page and *Nageia nagi* (Thunb.) Kuntze were included in the phylogenetic analysis as representatives of the two genera thought to be most closely related to *Retrophyllum* (de Laubenfels 1969; Page 1989). Fresh material of *A. gracilior* originated in Kenya. A sequence of *trn*L-F for *N. nagi* (material originating from Japan) was kindly provided by W. Sinclair et al. (unpubl. data), as were sequences for the two taxa chosen as outgroups: *Podocarpus salignus* D.Don (*Podocarpus* subgenus *Podocarpus*) and *P. longefoliolatus* Pilg. (*Podocarpus* subgenus *Foliolatus* de Laub.). Material of *P. salignus* originated from Chile, material of *P. longefoliolatus*

Table 3 Sample localities of *Retrophyllum* on New Caledonia.

Pop. code	Species identification	Location	Comments
Com-A	*R. comptonii*	Mont Ignambi Far north of island at 950–1200 m, 20°26′39″S, 164°36′58″E	Isolated mountain-ridge population of 20+ trees, to approx. 30 m in height
Com-B	*R. comptonii*	Rivière Ouinne South-east of island on margin of river at 800 m, 22°02′54″S, 166°28′47″E	Population of approx.15 trees, to 3.5 m in height
Com-C	*R. comptonii*	Mont Mou (type locality) South-west of island at 800 m, 22°04′S, 166°20′E	Isolated population of approx. 30 trees to 25 m in height
RTB	*Retrophyllum* sp.	Rivière Trou Bleu Far south of island at 50 m on mouth of major river system, 22°20′26″S, 166°57′43″E	One adult tree c. 5 m in height and seedlings sampled at this location
Min-A	*R. minus*	Rivière Bleue South of island at 50 m, 22°18′35″S, 166°49′55″E	Population of approx. 20 trees to 1.5 m in height
Min-B	*R. minus*	Rivière des Lacs South of island at 200 m, 22°10′50″S, 166°50′56″E	Population of 50+ trees to 1 m in height
Min-C	*R. minus*	Grande Lac South of island at 250 m, 22°15′52″S, 166°54′30″E	Regenerating population of 50+ individuals up to 1 m in height

from New Caledonia (Table 4). Our choice of outgroup (which includes both recognised subgenera of *Podocarpus*) was based upon the sister relationship of *Podocarpus* to the ingroup, as resolved in an *rbc*L phylogeny (Conran et al. 2000), and the taxonomic framework of Page (1989). Further support for this choice of outgroup comes from unpublished data based on ITS and *trn*L which confirms the sister relationship of *Podocarpus* to the ingroup (W. Sinclair et al. unpubl. data).

Voucher specimens for all populations/species have been deposited at the herbarium Royal Botanic Garden Edinburgh (E).

Molecular approaches

DNA extraction

DNA was extracted using a method adapted from Doyle & Doyle (1990), modified to include a washing step with ammonium acetate (7.5 *M* NH_4AC) (Weising et al. 1995) to remove impurities co-isolated with the DNA. Persistent impurities in some samples were removed using a modified protocol from the DNEasy Plant Mini Kits for DNA extraction (Qiagen Ltd). This method was successful in obtaining DNA of suitable quality for PCR amplification for most samples. We were unable to isolate amplifiable DNA from the herbarium material of *R. piresii*, which was thus not included in any further analyses.

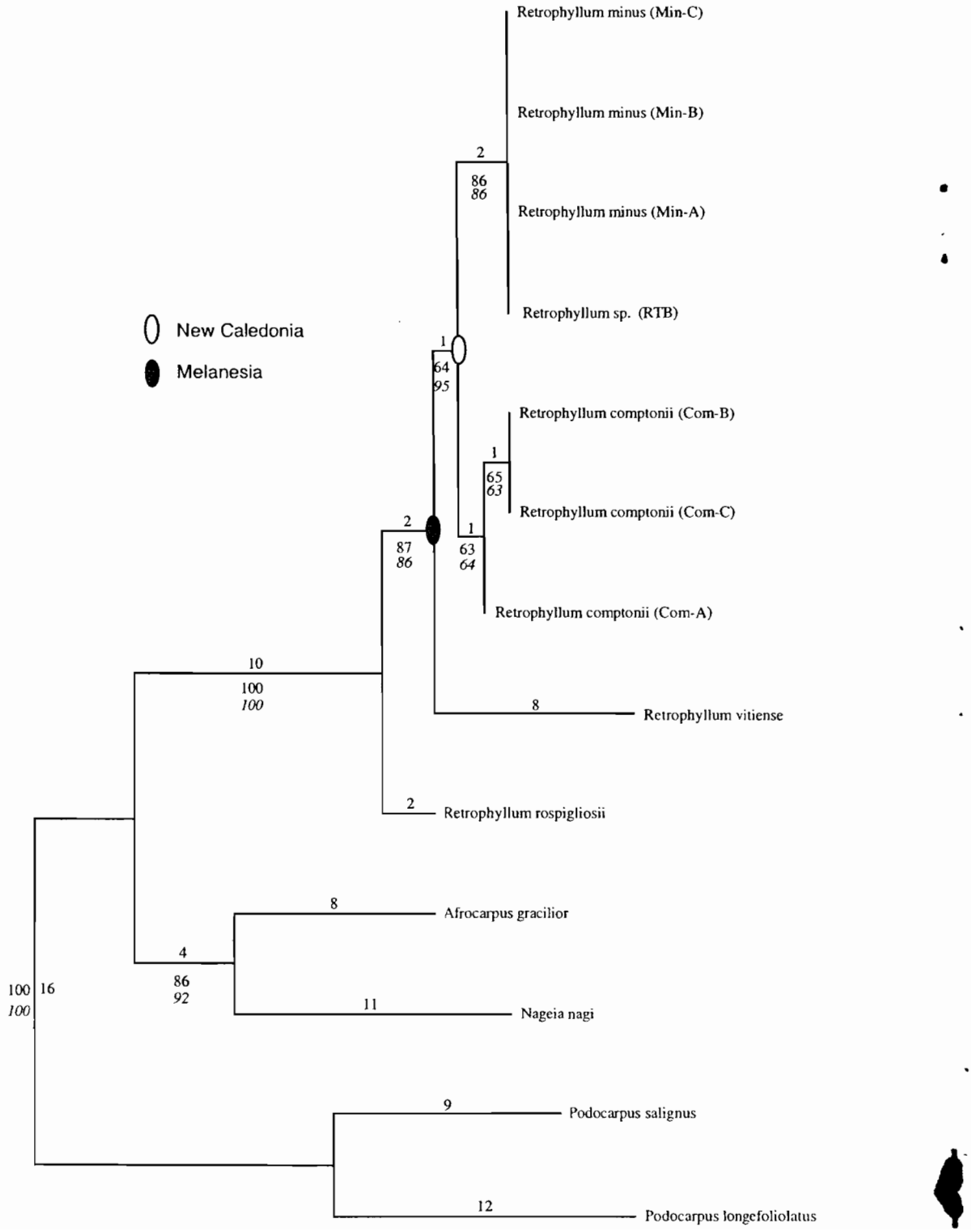
Sequence analysis of the trn*L-F region*

The *trn*L-F region was amplified from an individual of each species of *Retrophyllum* (each population for New Caledonian material) and from an individual of *Afrocarpus gracilior*. Amplification of the *trn*L-F region was carried out using the forward primer "c" and the reverse primer "f" (Taberlet et al. 1991). PCR reactions of 50 µl contained: 2 µl DNA template, 100 µ*M* of each dNTP, 0.3 µ*M* of each primer, 2 units Taq polymerase (Bioline), 2 µ*M* $MgCl_2$, and 5 µl reaction buffer (160 m*M* $(NH_4)_2SO_4$, 670 m*M* Tris HCl, 0.1% Tween 20, pH 8.8). The following PCR profile was used: 1 cycle at 94°C for 4 minutes; 30 cycles at 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 3 minutes; 1 cycle at 72°C for 10 minutes. The resulting PCR products were purified using Quiagen PCR purification kits according to the manufacturer's instructions. Modified dideoxy cycle sequence reactions with dye terminators (using primers "c", "d", "e", and "f" of Taberlet et al. 1991) were run on an ABI 377 automated sequencer and the output files edited using Sequence Navigator and Autoassembler (Applied Biosystems Inc.).

The resulting sequences (and those of *Nageia nagi*, *Podocarpus salignus*, and *P. longefoliolatus*) were aligned manually and their ends were cropped to exclude poor quality sections at the beginning and end of the sequenced region, bringing the total length of analysed sequence to 851 bp. An area of ambiguous alignment consisting of approximately 58 bp within the cropped section was identified. Different alignments of this region were analysed to assess the sensitivity of the resulting phylogenetic hypotheses to such manipulation. The following strategies were employed: (1) an alignment which minimised substitutions; (2) an alignment which minimised substitutions with gaps (insertions/deletions) coded as binary characters; (3) an alignment which minimised gaps; (4) exclusion of the alignment-ambiguous region (aligned matrices are available from the corresponding author on request).

Table 4 Localities of sample taxa used in phylogenetic analyses.

Species	Sample origin	Species range
Retrophyllum vitiense	Fiji	Fiji – Moluccas
R. rospigliosii	Venezuela	Venezuela, Colombia, Ecuador, Peru
R. piresii	Brazil	Brazil
R. comptonii	New Caledonia (3 populations)	New Caledonia
R. minus	New Caledonia (3 populations)	New Caledonia
Retrophyllum sp. (RTB)	New Caledonia (1 population)	New Caledonia
Podocarpus salignus	Chile	Chile
P. longefoliolatus	New Caledonia	New Caledonia
Nageia nagi	Japan	China and Japan
Afrocarpus gracilior	Kenya	East Africa


Retrophyllum minus (Min-C)
Retrophyllum minus (Min-B)
Retrophyllum minus (Min-A)
Retrophyllum sp. (RTB)
Retrophyllum comptonii (Com-B)
Retrophyllum comptonii (Com-C)
Retrophyllum comptonii (Com-A)
Retrophyllum vitiense
Retrophyllum rospigliosii
Afrocarpus gracilior
Nageia nagi
Podocarpus salignus
Podocarpus longefoliolatus
New Caledonia
Melanesia
2
86
86
1
64
95
1
65
63
2
87
86
1
63
64
10
100
100
8
2
8
4
86
92
11
100
100
16
9
12

Sequences were analysed using the parsimony algorithm of the software package PAUP* 4.0b2 (Swofford 1999). Given the small number of taxa (13) in the matrix a Branch and Bound search was possible. Clade support was assessed by Fast-Bootstrap resampling based on 1000 replicates. The following support scheme was followed: bootstrap values of 50–74% = weak support; 75–84% = moderate support; 85–100% = strong support. Standard tree descriptive statistics (CI, RI) were obtained from PAUP*.

RFLP analysis of the trn*L-F region*

The *trn*L-F sequences obtained from the seven New Caledonian populations were analysed using Webcutter (http://www.firstmarket.com/cutter). There was a single cut site for the enzyme *Hind*III in the three individuals of *R. minus* we sequenced (A/AGCTT; bp 209 to 214 in the sequence). This cut site was also present in the individual we sequenced from the RTB population. In contrast, in the three individuals of *R. comptonii* we sequenced, the sequence motif at this position was ATGCTT, which was not cut by *Hind*III. We thus amplified *trn*L-F from five samples from each population of the New Caledonian taxa to establish whether this polymorphism represented a useful marker for taxon differentiation.

RAPD analysis of the New Caledonian populations

RAPD primers (from the Operon kits) OPA-11, OPA-18, OPF-13, OPP-03, and OPP-17 were used to analyse samples of the New Caledonian populations. PCR reactions of 25 µl contained: 2 µl DNA template, 200 µ*M* of each dNTP, 0.5 µ*M* of primer, 1 unit Taq polymerase (Bioline), 2 m*M* $MgCl_2$, 0.5 µl 100% formamide, and 2.5 µl reaction buffer (160 m*M* $(NH_4)_2SO_4$, 670 m*M* Tris HCl, 0.1% Tween 20, pH8.8). The PCR profile was: 1 cycle at 95°C for 2 minutes; 2 cycles at 95°C for 30 seconds, 37°C for 1 minute, 72°C for 2 minutes; 2 cycles at 95°C for 30 seconds, 35°C for 1 minute, 72°C for 2 minutes; 41 cycles at 94°C for 30 seconds, 35°C for 1 minute, 72°C for 2 minutes; 1 cycle at 72°C for 5 minutes. A subset of samples was repeated to test for profile reproducibility.

The presence or absence of bands was scored visually. Only clear and reproducible bands were included. Individuals for which there were incomplete data (e.g., those for which not all RAPD primers worked) were excluded from the analyses to avoid artefacts associated with missing data. Thus, the total number of individuals considered for the analysis was 54. A total of 68 bands was scored from five primers.

The percentage of loci (bands) that were polymorphic within individual populations was calculated. To assess sample interrelationships, individual genotypes were treated as OTUs (operational taxonomic units). A distance matrix was calculated using the Nei & Li coefficient (Nei & Li 1979). A phenetic analysis of relationships was conducted by constructing a Neighbor Joining tree of inter-individual distances (Saitou & Nei 1987) using PAUP* 4.0b2.

RESULTS

Phylogenetic analysis of *Retrophyllum*

The phylogenetic analyses of the *trn*L-F sequences resulted in an identical single most parsimonious tree topology, regardless of the alignment strategy used. The tree shown in Fig. 1 is based on the substitutions-minimised alignment with no gap matrix. There were 39 variable parsimony-uninformative characters and 35 parsimony-informative characters.

The monophyly of *Retrophyllum* was strongly supported with respect to a sister clade containing *Afrocarpus* and *Nageia*. Separate analyses using individual or combinations of *A. gracilior*, *N. nagi*, *P. salignus*, and *P. longefoliolatus* as the outgroup had no effect on the topology of the *Retrophyllum* clade. The relationships inferred are also robust to analytical method. Neighbor Joining and Maximum Likelihood trees both recovered the same topology (not shown).

Fig. 1 Phylogram showing the single most parsimonious tree from a phylogenetic analysis of *Retrophyllum* and related genera. This tree was generated from the "substitutions minimised, no gap matrix, alignment". Values above the branches are branch lengths, values below (in plain text) are levels of bootstrap support. The total number of characters = 851; the number of constant characters = 777; the number of variable parsimony-uninformative characters = 39; the number of parsimony-informative characters = 35. Tree length = 87, Consistency index (CI) = 0.9425, CI excluding uninformative characters = 0.8889, and Retention index (RI) = 0.9286. The italicised figures below the branches are levels of bootstrap support when indels are included in the analysis as binary characters.

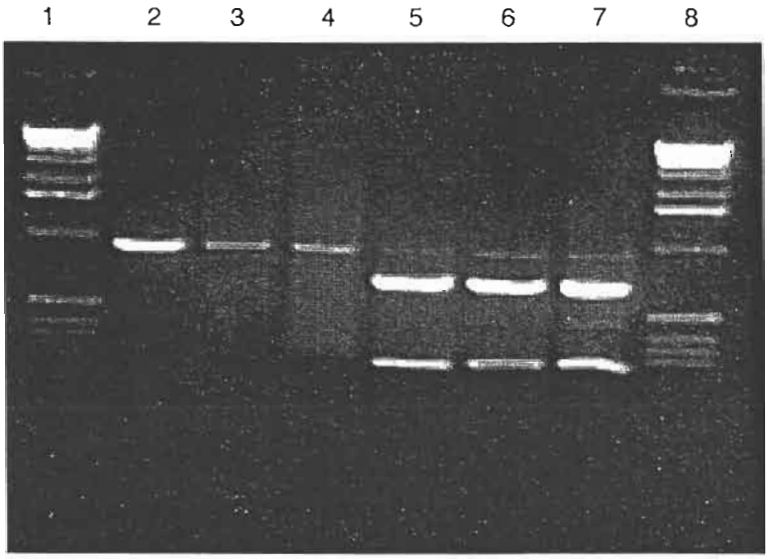


Fig. 2 Restriction digest of *trn*L-F sequences for New Caledonian *Retrophyllum* species using *Hind*III. Lanes 1 and 8 = 1 Kb ladder; lanes 2–4 = *R. comptonii*; lanes 5–7 = *R. minus*.

Table 5 *trn*L-F mutations among New Caledonian samples.

Population	Base 56	Base 210	Base 545	Base 546	Base 612	Base 619
Com-A	T	T	G	A	–	–
Com-B	A	T	G	A	–	–
Com-C	A	T	G	A	–	–
RTB	T	A	T	C	–	–
Min-A	T	A	T	C	–	–
Min-B	T	A	T	C	T	G
Min-C	T	A	T	C	–	–

The South American *R. rospigliosii* was sister to a strongly supported clade containing the Melanesian species (i.e., *R. minus*, *R. comptonii*, and *R. vitiense*). Within this clade, the samples from New Caledonia formed a monophyletic group that was sister to the Fijian *R. vitiense*. Fixed base pair differences (see Table 5) between samples of *R. minus* and *R. comptonii* resulted in their separation into two clades. The unidentified population, RTB, shared a haplotype with the *R. minus* populations.

RFLP results

The *Hind*III restriction digest of *trn*L-F in the New Caledonian samples produced the banding patterns shown in Fig. 2. All samples of *R. minus* and the RTB population produced haplotype (A), consistent with the presence of the *Hind*III cut site; all samples of *R. comptonii* produced haplotype (B), consistent with the absence of the *Hind*III cut site.

RAPD results

The Neighbor Joining phylogram (Fig. 3) produced from the RAPD data of New Caledonian *Retrophyllum* shows a clustering of the individuals into two groups. These correspond with their species designations with the RTB population grouping with *R. minus*. Identical results were obtained with UPGMA (unweighted pair group method analysis) and PCO (principal coordinates) analyses (not shown). Within the *R. comptonii* cluster, population Com-A is clustered and separate from the rest of the individuals of populations Com-B and Com-C which group together.

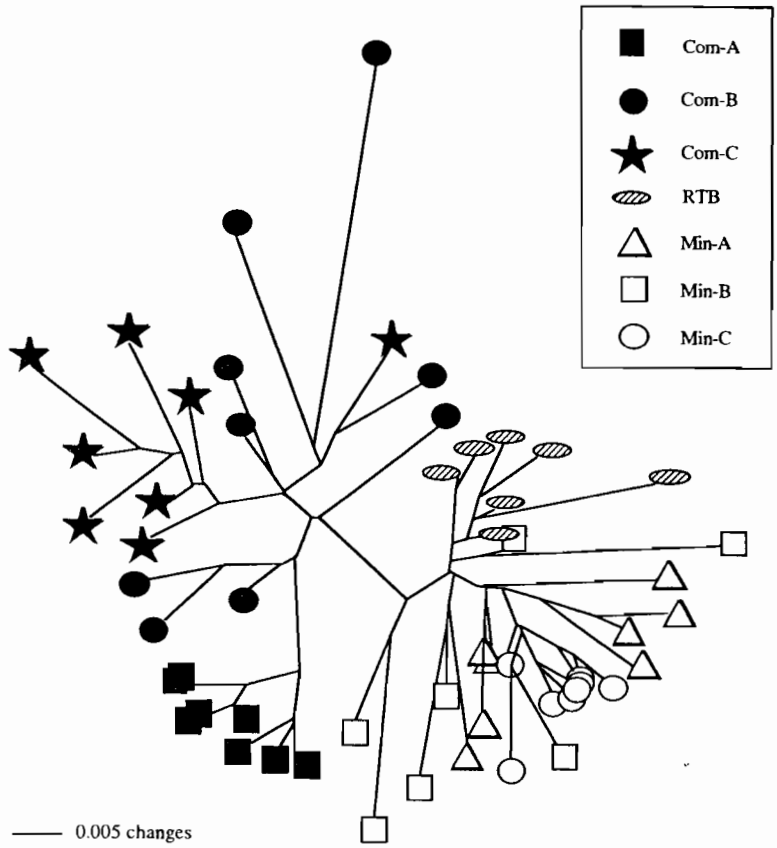


Fig. 3 Neighbor Joining tree of RAPD data for New Caledonian *Retrophyllum* species.

Table 6 Percentage polymorphic RAPD bands in New Caledonian *Retrophyllum* populations.

Population	% Polymorphic bands
Com-A	18.3
Com-B	61.6
Com-C	41.6
RTB	28.3
Min-A	41.6
Min-B	50.0
Min-C	23.3

The percentage of RAPD bands that were polymorphic are shown in Table 6. Within species, 83.3% of bands were polymorphic in *R. comptonii* and 70% were polymorphic in *R. minus* (including the RTB population). The lowest level of polymorphism is found in the geographically isolated northern population Com-A.

DISCUSSION

Phylogenetics

The phylogenetic study showed *Retrophyllum* to be a strongly supported monophyletic group with respect to *A. gracilior* and *N. nagi*. This supports the classifications of earlier authors (e.g., Bertrand 1874; de Laubenfels 1969; Page 1989) who recognised three separate groups of species, now referred to as *Retrophyllum*, *Afrocarpus*, and *Nageia*. The data also support the recognition that these three genera are more closely related to each other than any is to *Podocarpus*.

The generic level topology conflicts with two recent studies. The first was a morphological classification based on cone morphology that placed *Afrocarpus* together with *Podocarpus*, in a separate order from *Nageia* and *Retrophyllum* (Melikian & Bobrov 2000). The second was a morphological cladistic analysis that placed *Retrophyllum* and

Afrocarpus together, sister to *Nageia*, in turn sister to a paraphyletic *Podocarpus* (Kelch 1997). However, the former of these was not based on any formal analyses, and in the latter, there is poor bootstrap support for the nodes that conflict with our topology. Support for our topology comes from the *rbc*L phylogeny of the Podocarpaceae that places *Retrophyllum* as a sister clade to *Afrocarpus* and *Nageia,* which in turn form a group sister to *Podocarpus* (Conran et al. 2000). Furthermore, an expanded *trn*L-F phylogeny of the Podocarpaceae, rooted on two different Araucariaceae species and *Taxus baccata* L. (Taxaceae), recovered an identical topology with respect to the taxa included in our study, with all nodes having >97% bootstrap support at the generic level (Sinclair et al. unpubl. data).

The tree topology within *Retrophyllum* is (*R. rospigliosii* (*R. vitiense* (*R. minus*, *R. comptonii*))) (Fig. 1). A small number of morphological characters support these relationships but none is unambiguous and discrete. For instance, the number of scale leaves subtending the female cone appears to be fixed at 7 pairs in *R. rospigliosii* whilst it varies from 4 to 6 in the Melanesian group (Herbert 2000). There are some characters that are shared between the South American *R. rospigliosii* and the Fijian *R. vitiense* that, in the light of the phylogeny, might be interpreted as plesiomorphic. These include: leaf shape, predominantly lanceolate in *R. vitiense* and *R. rospigliosii* versus elliptic or elliptic-lanceolate in the New Caledonian taxa; and midrib definition, which is clear on both leaf surfaces in *R. rospigliosii*, clear on the upper leaf surface in *R. vitiense*, but indistinct in *R. minus* and *R. comptonii* (Herbert 2000).

The *Retrophyllum trn*L-F tree topology, coupled with the Melanesian/South American disjunction, is consistent with a Gondwanan fragmentation event. There are fossils identified as *Retrophyllum*-like from Chile dated at c. 54 million years ago (mya) (Gray & Buchholz 1948); from Australia dated at c. 38 mya (Hill & Merrifield 1993); and from New Zealand dated at c. 26 mya (Pole 1992). This is consistent with the current disjunction being ancient. The break-up of Gondwana began in the early Jurassic (about 180 mya); the separation of Tasmantia (the continental block incorporating New Caledonia) from Australia and West Antarctica occurred c. 80 and 84 mya, respectively, with South America and Antarctica breaking apart some 30 mya (McLoughlin 2001). The number of substitutions between the Melanesian and South American species are fewer than would be expected if the distribution of *Retrophyllum* is Gondwanan, based on published rate estimates. Average divergence rates for cpDNA are generally given as 0.1–0.3% per million years (Zurawski et al. 1984; Wolfe et al. 1987). The slowest rate we are aware of is that for *Phylica* (Rhamnaceae) at 0.048% per million years (Richardson et al. 2001). We corrected for rate heterogeneity within our data by producing an ultrametric tree in TreeEdit (Rambaut & Charleston 2000) from maximum likelihood branch lengths (Non Parametric Rate Smoothing; Sanderson 1997). Using the date of the isolation of Tasmantia from West Antarctica (84 mya) to calibrate this tree, we calculated a rate of sequence divergence of 0.031% per million years. This comparatively lethargic rate could be due to factors such as generation time effects and gene-specific and lineage-specific variation (Li et al. 1987; Gillespie 1991; Baldwin et al. 1995). Alternatively, the minimal observed sequence divergence could reflect historical long distance dispersal. Possible scenarios for this include either transoceanic dispersal, or stepping stone dispersal via intermediate landmasses (Australia and/or pre-glacial West Antarctica) (McLoughlin 2001).

R. minus and *R. comptonii*: species differentiation

Both the RAPD data and the *trn*L-F data support the differentiation of the New Caledonian populations into separate groups corresponding to the two species, with the RTB population grouping with *R. minus* (Fig. 2, 3). Chloroplast sequences give three apparently taxon specific bases (Table 5). More widespread screening for one of these markers by RFLP analysis (Fig. 2) suggests that the difference is representative of the different species. Accepting the RTB population as *R. minus*, there is one taxon-specific RAPD marker and three that show strong frequency differences (differentially present or absent at frequencies of >90%) between the two taxa (data matrix available on request from corresponding author).

The molecular results strongly suggest that the RTB population is *R. minus*. However, this population included a tree that, at over 7 m tall, far exceeds the maximum height of 3 m given in previously published species descriptions (Carrière 1867; Gray 1962; de Laubenfels 1969, 1972). The potential for individuals of *R. minus* to grow to more than 3 m has been highlighted recently by Jaffré (1995) who described the species as up to 6 m in height. However, the fact that some individuals of *R. comptonii* we examined were sexually mature

(producing cones) at less than 4 m tall suggests that height alone cannot be used to distinguish the two species. At present it seems that habitat appears to be the most reliable method for identification of these species. This breaks down, however, in populations where habitat classification is not so easy, particularly by watercourses at intermediate altitudes.

Implementing conservation policy requires two decisions: 1) what to conserve, and 2) how to conserve it. The first of these is a fundamentally important step in any conservation programme and is particularly pertinent in groups with difficult or complex taxonomies. If a species cannot be defined or recognised, it is not possible to assess its current distribution and assess the threats to its populations. For *R. minus* on New Caledonia, unambiguous identification of material can be difficult. The results of this study suggest that molecular markers can be useful in differentiating between the *Retrophyllum* species on New Caledonia. The *Hind*III restriction enzyme assay represents a quick and efficient screening technique that avoids the problems posed by the ambiguous morphological characters. Given that the current estimate of total population size of *R. minus* falls into the IUCN category of <2500 individuals in the wild (Farjon & Page 1999), this screen could relatively quickly be applied to broad-scale population samples to enhance our knowledge of the taxonomic status of these populations. Although there are no reported cases of hybridisation between the New Caledonian species, the use of a nuclear marker assay may also be prudent to guard against any misleading inferences. Such use of molecular markers for taxonomic clarification can be a powerful method of facilitating the conservation of endangered species (Hollingsworth 2001).

Intra-specific variability

Inferring population genetic structure from limited sampling based on dominant data is problematic. Population genetic theory is well established for comparing gene frequency estimates from co-dominant markers (Hartl & Clark 1997). However, while dominant marker technologies have paved the way for easy access to large numbers of physically scattered markers, their analyses are complicated. In the absence of progeny tests, the frequency of heterozygous individuals remains unknown. In the current study, not only is the dominance of the data an issue, but also the sample sizes of individuals per population is low. In this respect, "individual" level analyses are perhaps most appropriate as the data are effectively treated as phenotypic characters of individuals, and not reduced to averages which may be misrepresentative of their populations.

In the Neighbor Joining tree (Fig. 3) it can be seen that all individuals of the geographically isolated northern population of *R. comptonii* (Com-A) cluster together (this result was also obtained from UPGMA and PCO analyses; not shown). The single individual sequenced for *trn*L-F from the Com-A population had a different haplotype to the other *R. comptonii* samples (Table 5). This population is also the least genetically variable (albeit based on an undesirably small sample) with $P = 18.3\%$. Its genetic differentiation from the other populations of *R. comptonii* for both cpDNA and RAPDs is consistent with its spatial isolation.

Individuals from the remaining populations are either intermingled with those from conspecific populations or, in the case of RTB, separated on a very short branch (Fig. 3). When alternative methods of exploring the data were used (UPGMA, PCO; not shown), the RTB grouping broke down and these individuals were intermingled with other *R. minus* populations. Thus, Com-A was the only population to form a discrete cluster in all methods of analysis.

The mixing of individuals among different populations may indicate low levels of population genetic differentiation, possibly due to gene flow. However, mixed clustering of populations does not necessarily indicate that they are directly exchanging genes, since intermediate populations may maintain the connections between more distant populations. It should also be noted that genetic similarity in these markers does not necessarily indicate contemporary gene flow, and historical communication can leave a deep "footprint" in genetic marker patterns (Whitlock & Macauley 1999).

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