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**Reticulate evolution on a mosaic of soils: diversification of the New
Caledonian endemic genus *Codia* (Cunoniaceae)**

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ABSTRACT

We reconstructed the evolutionary history of *Codia*, a plant genus endemic to the New Caledonia biodiversity hotspot in the south-west Pacific, using three single-copy nuclear genes. It seems likely that more than half of *Codia* species have a hybrid origin, but in the absence of cytological information, it is not known whether polyploids occur. Adaptation to ultramafic soils is possibly a plesiomorphic character for the entire genus. We found that species of hybrid origin can have some morphological characters absent in putative parental species, i.e. they exhibit transgressive phenotypes. There is evidence of considerable range alteration post-origin in several species because some likely parental species of hybrid taxa no longer co-occur and are confined to putative rainforest refugia; in some cases, hybrid species do not now co-occur with either of their parental species. These results have implications for the design of conservation strategies, for example prioritization of parental species for ex-situ conservation and preservation of the contact zones between soil types where hybridization is more likely to occur (i.e. conserving the possibility for the process to continue rather than trying to conserve taxa).

INTRODUCTION

Identification of the best strategies for conservation requires not only better knowledge of biodiversity but also better understanding of the evolutionary processes involved in its production (Erwin, 1991; Smith *et al.*, 1993; Cowling & Pressey, 2001; Moritz, 2002). Among factors involved in plant speciation, two may be considered particularly important: hybridization (Rieseberg, 1997) and polyploidization (Ramsey & Schemske, 1998). A large number of model groups have been the subject of studies of hybridization and/or polyploidization, but substantial biases exist in their choice. Many are crops, ornamentals or weeds, and most have one or more of the following characteristics: brief life history, easy cultivation, and temperate climatic preferences (with biases towards native plants of Europe and North America). Although most biodiversity lies in the tropics, comparatively few data are available for tropical plants, particularly woody taxa.

New Caledonia is an archipelago in the south-western Pacific well-known for its rich and unique flora (Jaffré *et al.*, 2001), and considered by Meyers *et al.* (2000) as a global biodiversity hotspot. Its endemic flora is composed of a large number of relict lineages, such as conifers, early diverging angiosperms (including *Amborella*), and magnoliids (Jaffré *et al.*, 2001), as well as major radiations in genera such as *Phyllanthus* (114 species), *Psychotria* (87 species) and *Syzygium* (68 species). A factor often used to explain the peculiarity of the New Caledonian flora is the importance of the ultramafic soils that cover roughly one-third of the main island's surface (Jaffré, 1993). Ultramafic soils, including serpentine in the strict sense, have several characteristics that are limiting for plant growth: low fertility (low N, P, K), high Mg/Ca ratio, high concentrations of heavy metals (Co, Cr, Ni etc.), low water-holding capacity and increased susceptibility to fire (Proctor, 2003). In New Caledonia, large areas of ultramafic substrates are covered by scrubby vegetation termed "maquis" in which levels of species endemism can exceed 90% (Jaffré *et al.*, 2001), especially at high elevation.

Relatively few studies have investigated diversification of particular groups within New Caledonia (but see Eibl *et al.*, 2001; de Kok, 2002; Swenson *et al.*, 2007) compared with areas such as Hawaii (Price & Wagner, 2004) or the Cape region of South Africa (Linder, 2005). Molecular phylogenetics is an ideal tool to evaluate evolutionary processes within endemic groups, but the choice of adequate markers is an important issue. Plastid genes and nuclear ribosomal regions are not the most appropriate genomic regions for tracking hybridization (Small *et al.*, 2004) because the first are generally inherited uniparentally (generally maternally in angiosperms, Corriveau & Coleman, 1988), and the second are subject to concerted evolution (Baldwin *et al.*, 1995), which tends to erase one of the parental copies in species of hybrid origin (Chase *et al.*, 2003). In many cases, concerted evolution in hybrids is strongly biased towards the maternal parent (Pillon *et al.*, 2007), and thus both these categories of markers may indicate congruent evolutionary histories leaving no evidence of hybridization. Single-copy nuclear genes provide better markers for this task, although use of such loci is often difficult due to the lack of universal primers and their often unpredictable modes of evolution.

To investigate processes that have been important in diversification of the New Caledonian flora, and particularly to examine the importance of soil diversity and the possible occurrence of hybridization, we aimed to reconstruct the evolutionary history of the endemic genus *Codia*. This genus belongs to Cunoniaceae, a principally Southern Hemisphere family of 27 genera and ca. 300 species (Bradford *et al.*, 2004) that is well represented in New Caledonia. *Codia* consists of some 14 species (Hopkins, 2005; Hopkins *et al.*, 2007) of often gregarious trees and shrubs that sometimes dominate maquis and rainforests. With species on both ultramafic and non-ultramafic (e.g. sedimentary, basaltic and metamorphic) substrates, *Codia* comprises both widespread and micro-endemic species on the main island (Grande Terre), Yandé and the Belep islands. Species delimitation has been problematic; little

variation is seen in reproductive morphology, but species are often variable in vegetative characters and in some cases hybridization has been suspected (e.g. Hopkins *et al.*, 2007). Through reconstruction of the evolutionary history of this genus using molecular markers, we hoped to gain insights into its morphological and ecological diversification, its biogeography, and the processes that may have triggered this radiation, which would help to demonstrate how this process may be conserved.

MATERIAL & METHODS

All but one species currently recognized in *Codia* according to recent taxonomic works (Hopkins, 2005; Hopkins *et al.*, 2007) were sampled in this study. Material was unavailable for just *C. cinerascens*, known only from the type collection made in the 19th century that indicates a vague locality (“Montagnes d’Ouatendé, Gatope”). In spite of extensive field work, no plant matching this type was found. Although the type of *C. cinerascens* is morphologically distinctive, the broad stipules and minute indumentum on the leaves recall *C. albicans*, and it may be an anomalous form of this species or a cross between it and perhaps *C. spatulata* (Hopkins & Pillon, unpublished). A single accession was used for each species. Because of technical limitations, we chose to multiply the markers rather than the accessions to increase the resolution, since preliminary studies showed that the level of molecular variation within *Codia* was low. Species of *Spiraeanthemum* and *Geissois* were used as outgroups (Pillon *et al.*, 2009; Pillon, unpublished).

Preliminary studies showed that classic markers would be of limited use for the present study. For instance, multiple copies of ITS were recovered in most species of Cunoniaceae (Bradford, 2002; Pillon, unpublished). For plastid genes, little variation was found within the genera of this family (Bradford, 2002; Pillon *et al.*, 2009; Pillon, unpublished), and the level of variation in two candidate genes for plant DNA barcoding

(Kress *et al.*, 2005; Chase *et al.*, 2007), *matK* and *psbA-trnH*, is also low (Pillon, unpublished). This led us to search for suitable single-copy genes. Ideally, the choice of such genes should be based on a number of criteria, including a high-level of variation and the absence of similar orthologous genes, duplication, and recombination (Small *et al.*, 2004). However, when working on non-model organisms, one constraint is the availability of primers that allow amplification of such genes. We thus selected genes that had previously been used in groups that are closely related to Cunoniaceae: *ncpGS* (chloroplast-expressed glutamine synthetase) was used in *Oxalis* (Oxalidaceae, Emshwiller & Doyle, 1999), which belongs to Oxalidales, the same order as Cunoniaceae; *GapC* (glyceraldehyde 3-phosphate dehydrogenase, also known as *G3pdh*) was used in *Manihot* (Euphorbiaceae, Olsen & Schaal, 1999); and the first exon of *PHYC* (phytochrome C) was used in Phyllanthaceae (Samuel *et al.*, 2005); the latter two families both belong to Malpighiales, an order close to Oxalidales (Zhu *et al.*, 2007). In *Populus trichocarpus*, the most closely related model group for which the entire genome has been sequenced (Tuskan *et al.*, 2006), one homolog of *GapC* is found in linkage group I and homologs of *ncpGS* are found in linkage groups VIII and X. These two genes are thus not linked in *Populus*, and *PHYC* is missing in this model species (Howe *et al.*, 1998).

Leaf material was dried in the field in silica gel (Chase & Hills, 1991), and DNA was extracted with a modified CTAB protocol (Csiba & Powell, 2006). A portion of *ncpGS* was amplified using the primers 687F and 994R of Emshwiller & Doyle (1999). The amplification mix included 45µL of 2.5mM MgCl₂ Reddy PCR Master Mix 1.1× (ABgene Ltd), 1µL of 0.4% bovine serum albumin (BSA), 0.5µL of each primer (100ng/µL) and 3µL of template DNAs of unknown concentrations. The following amplification protocol was used: 2 min at 94°C, 38 cycles of 1 min at 94°C, 1 min at 50°C, 1 min 30 s at 72°C and a final extension of 5 min at 72°C. A portion of *PHYC* was amplified using the primer *PHYC-F* and *PHYC-R* from

Samuel *et al.* (2005). The amplification mix included 45µL of 1.5mM MgCl₂ Reddy PCR Master Mix 1.1× (ABgene Ltd), 1.5µL of 0.4% bovine serum albumin (BSA), 0.5 µL of each primer (100ng/µL), 1 µL of DMSO and 2µL of template DNAs of unknown concentrations. The following protocol was used: 3 min at 94°C, 38 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and a final extension of 10 min at 72°C. A portion of *GapC* was amplified using the primer GPDx7F and GPDx9R from Strand *et al.* (1997). The amplification mix included 45µL of 2.5mM MgCl₂ Reddy PCR Master Mix 1.1× (ABgene Ltd), 1.µL of 0.4% bovine serum albumin (BSA), 0.5 µL of each primer (100ng/µL) and 3µL of template DNAs of unknown concentrations. The following protocol was used: 2 min at 94°C, 38 cycles of 1 min at 94°C, 1 min at 53°C, 1 min at 72°C and a final extension of 5 min at 72°C.

In the case of *ncpGS*, the amplification product could generally be sequenced directly. If electrophoregrams showed the presence of more than one allele/copy in an accession, and if these alleles differed in length or by more than one base pair, then the PCR product was cloned. An average of six clones were routinely sequenced. Further clones were sequenced if all polymorphisms observed in the electrophoregrams from the direct sequencing were not recovered. *PHYC* was also able to be sequenced directly. When more than one allele was observed at this locus, sequences of these alleles/copies were deduced by comparison with alleles found in the other accessions that were homozygous. This was necessary for only two accessions. When directly sequenced, *GapC* did not give clean results, and therefore amplification products of all accessions were cloned (up to six clones sequenced per accession in order to account for the heterogeneity observed). For this marker, a consensus sequence was created for each uniquely clustering set of clones, which removed the effects of substitutions induced by the PCR and cloning procedures. For *ncpGS*, the comparison between cloned and direct sequences allowed removal of most such artefacts. DNA sequences

have been submitted to Genbank (accession numbers FJ595023-FJ595081, see supplementary data for full details).

Alignment and phylogenetic analyses were carried out with PAUP*4.01b10 (Swofford, 2002). We searched for putative recombination events using the GARD software (Kosakovsky Pond *et al.*, 2006). A distance matrix between alleles was built with MEGA (Kumar *et al.*, 2004) and used to create a network with TCS (Clement *et al.*, 2000). To represent relationships between the species of *Codia* using all three DNA regions (“total evidence” approach), distances between species were calculated using POAD (Joly & Bruneau, 2006) and used to build a split network (Huson & Bryant, 2006).

RESULTS

The total length of the aligned *ncpGS* matrix was 1062 base pairs (bp); 54 sites were variable, 12 of which were potentially parsimony informative. The aligned matrices of *PHYC* and *GapC* were 579 and 873 bp long, respectively; they displayed 9 and 46 variable sites and provided 8 and 10 potentially parsimony informative sites, respectively. No evidence of recombination in any of the sequences was found with the GARD software.

Networks for alleles of *ncpGS*, *PHYC* and *GapC* found in *Codia* are shown in figure 1, and table 1 shows the distribution of these alleles among species. For *ncpGS* and *GapC*, up to four allele groups could be distinguished in the network, and these alleles were designated A, I, J and N, corresponding to the following species: *C. albicans*, *C. incrassata*, *C. jaffrei* and *C. nitida*. For *PHYC*, no obvious clear-cut groups were found, in contrast to the situation for the two other genes, and two groups (I and J) were tentatively delimited by comparison with these other genes. For none of the loci did we ever recover more than two alleles in a given accession.

Four species had genomes with alleles belonging to single allele-groups. In *Codia jaffrei*, we recovered a single allele (group J) for all three genes; in *C. incrassata*, we also recovered a single allele (group I) for all three genes. For *C. ferruginea*, a single allele was recovered for *ncpGS* and *PHYC*, but two similar alleles (with a difference of two substitutions between them) were found for *GapC*; all these alleles belonged to group I. *Codia nitida* produced two similar but unique alleles (group N) for *ncpGS*; for *PHYC*, it had the J1 allele, and for *GapC*, the N1 allele, which is nested in group I. A phylogenetic tree based on the combined analysis of the three genes for these four species using maximum parsimony showed that *C. nitida* and *C. jaffrei* form a clade (73 bootstrap percentage, BP) that is sister to another clade composed of *C. ferruginea* and *C. incrassata* (99 BP, data not shown).

For each of the other nine species of *Codia*, we recovered in at least one of the three genes two distinct alleles that belonged to different groups. Five species, *C. albifrons*, *C. belepensis*, *C. microphylla*, *C. spatulata* and *C. triverticillata*, displayed a combination of alleles from groups I and J. *Codia albicans* and *C. mackeeana* had a combination of alleles from groups A and I, and *C. montana* a combination of A and J. *Codia discolor* produced alleles of the J group for *PHYC* and *GapC* and alleles from groups I and N for *ncpGS*.

A split-graph showing relationships of the *Codia* species is shown in figure 2. Little structure is observed, but species that combine I and J alleles cluster between *C. incrassata* and *C. jaffrei*.

DISCUSSION

LIMITS OF NUCLEAR SINGLE-COPY GENES

Amplification of all three nuclear single-copy genes was reasonably easy and consistent. Direct sequencing from the PCR products was possible for *ncpGS* and *PHYC*;

when directly sequenced, *GapC* produced electrophoregrams with abundant polymorphisms, so cloning was always necessary.

In the absence of cytological data, we have no way to assess the existence of polyploidy in *Codia*. Although cases of suspected hybridization have been observed in the field, we have no evidence of their fertility and evolutionary potential. Thus, for a given accession, we could not estimate the number of alleles to expect. When direct sequencing is possible, homozygotes can clearly be distinguished from accessions with two or more alleles, but if multiple alleles exist, then cloning is necessary to tease them apart. One issue is the number of alleles to be recovered. If the direct sequence is readable, i.e. the different alleles have the same length (no net indel differences in their sequences), cloned sequences can be compared with the direct sequence, and if all alleles have been recovered, their variability should explain all polymorphisms observed in the directly sequenced electropherograms. If the direct sequence is not readable, then recovery of both alleles with more than a 95% chance of success requires the sequencing of at least six clones.

When direct sequencing is not successful, it is *a priori* not possible to know the number of alleles to expect. However, if one sequences six clones and recovers only a single allele, the chance that the accession is a homozygote is greater than 95% (96.875% exactly); conversely if the accession is a heterozygote, the chance that both alleles will be recovered is also more than a 95%. However, as one increases the number of accessions, the chance of missing an allele increases. When investigating two heterozygous accessions, chances of recovering both alleles from both accessions are $96.875\% \times 96.875\% \approx 93.8\%$. Thus a higher chance exists for a heterozygote to be undetected. For a sample like ours (13 accessions), we would need to sequence nine clones (calculation not shown) to assure a 95% chance of detecting all heterozygotes. Thus, as one increases the number of accessions and genes, sequencing becomes time consuming and costly.

In our study, a high probability exists that some alleles for *GapC* were missed and some heterozygotes not detected. In contrast, we are confident that all homozygotes for *ncpGS* and *PHYC* were detected and that by comparison with the direct sequences, most alleles in heterozygotes were probably recovered. Thus, we have given greater weight to the results obtained with *ncpGS* than to those obtained both with *PHYC*, which exhibits low variability, and with *GapC*, for which some alleles were probably missed.

An ideal nuclear single-copy gene would be one that can be sequenced directly so that both homozygotes and individuals with multiple alleles are readily identified. When no variation in length is found between alleles in accessions with multiple alleles, it is easier to compare the direct sequence and the cloned sequences and thus check that all alleles have been recovered. Recovering all alleles in polyploids with three or more alleles is difficult; to avoid artefacts occurring during PCR or cloning, production of multiples clones for each allele is also advantageous.

EVIDENCE FOR RETICULATE EVOLUTION IN *CODIA*

In networks based on *ncpGS* and *GapC* (Fig. 1), two large groups of alleles could clearly be distinguished, I and J, between which the root was connected. Two smaller allele groups, A and N, could also be distinguished. In the network based on *PHYC* sequences, these groups are not clearly separated due to the low level of variation in this gene, and for most of the following discussion this gene will therefore not be considered.

In our results, we observed two categories of species. *Codia ferruginea*, *C. incrassata*, *C. jaffrei* and *C. nitida* represent the first and were homozygous for all three genes or had alleles that were similar and belong to the same allele group. All other species examined, which represent the second category, exhibit for at least one of these genes two alleles that belong to different groups. A possible explanation of this pattern of variation is reticulate

evolution within *Codia*, in which species of the second category are the result of hybridization events between species belonging to the first category. An alternative hypothesis would be incomplete lineage sorting, where, in an extreme case, most alleles would be present in most species, and homozygotes and heterozygotes would have been randomly sampled. Because a single accession was used for each species, it is not possible to infer whether the same pattern would have been observed if more individuals per species had been sampled. Nevertheless several observations suggest that hybridization, not incomplete lineage sorting, best explains our results.

First, consistent relationships between *Codia ferruginea*, *C. incrassata* and *C. jaffrei* observed for all three genes may be sufficient to rule out alternative hypotheses such as incomplete lineage sorting or an ancestral duplication event predating the radiation of *Codia* that gave rise to the modern species, which was then followed by random loss of gene copies. Sequences of *nbpGS* from one additional accession of *C. spatulata* and of *C. ferruginea*, each from a location different from the one previously sampled, revealed alleles of group I and J for the first one and a single allele of the I group for the second, which is consistent with what we had already observed (Pillon, unpublished).

Heterozygotes make up a large proportion of the sampling and were also recovered in rare species such as *Codia belepensis*. This species is only found on a few islands off the northern coast of New Caledonia, where it is the only species of the genus present. Thus, although one might predict that this species would be fixed for a single allele through genetic drift, two distinct alleles were recovered in the *nbpGS* analysis.

Most heterozygotes sampled had alleles that were far apart in the networks; in the case of incomplete lineage sorting, heterozygotes would be more likely to have alleles that are similar due to more recent divergence. Furthermore, the two alleles found in such

heterozygotes were generally both derived, as suggested by their being placed at the tips of networks, contrary to what would be expected in a case of incomplete lineage sorting.

Several heterozygote accessions such as those of *Codia albicans*, *C. montana* and *C. triverticillata* showed a consistent pattern for both *ncpGS* and *GapC*: they combined two alleles from the I and J groups in each analysis. The species that exhibit different patterns for the two genes are those displaying alleles from two distinct groups in *ncpGS* and a single group in *GapC*. However, as mentioned above, some alleles have probably been missed for this gene, especially when fewer than six clones were sequenced. In spite of this possibility, all species displaying alleles from two groups of *GapC* also showed alleles from two distinct groups in *ncpGS*, with the exception of *C. mackeeana*. Furthermore, in homoploid hybrid species, one parental allele is commonly lost soon after formation (Howarth & Baum, 2005; Friar *et al.*, 2008). Thus we believe that most heterozygotes observed in this study are species of hybrid origin; this seems clear for species such as *C. albicans*, *C. montana* and *C. triverticillata*, which show consistent patterns for two genes, and for *C. spatulata*, which shows the same pattern in two accessions. It is possible that both hybridization and incomplete lineage sorting (and possibly also introgression) explain the pattern observed. The use of multiple accessions in each species would have been useful to clearly demonstrate that some species have a hybrid origin since they would be expected to be fixed heterozygotes. Future studies will require an enlarged sampling, and due to the technical limits associated with nuclear single-copy genes discussed above, alternative markers such as AFLPs would probably be preferable. Such markers are cheaper, more easily applicable to a large sampling and provide a large number of markers spread across the genome. However their analysis is more complex and they do not necessarily allow to clearly identifying the parents of the species of hybrid origin (see e.g. Hedrén *et al.*, 2001).

No chromosome counts are available for *Codia*, and thus it is not known if the species of presumed hybrid origin are homoploid or polyploid. We never found more than two alleles in any accession, although low variability of the markers used and difficulties of recovering multiple alleles in cloning make detection of more than two alleles in putative polyploids difficult. For *GapC*, we detected in some species of putative hybrid origin two distinct alleles corresponding to a single parental group, and thus if one assumes that the other parental allele(s) have been missed in the cloning procedure, such accessions may be polyploids. Currently few cytological data are available for New Caledonian plants, and this study demonstrates the importance of such data. Our results are, nonetheless, more consistent with all species being diploids, but this obviously needs further investigation.

EVOLUTIONARY HISTORY OF *CODIA*

Four species of *Codia* did not show signs of hybridization in any of the analyses: *C. ferruginea*, *C. incrassata*, *C. jaffrei* and *C. nitida*. All three genes indicate a close relationship between *C. ferruginea* and *C. incrassata* and show that *C. jaffrei* is more distantly related (Fig. 1). The placement of *C. nitida* was somewhat different in the analyses of *ncpGS* and *GapC*. In the *GapC* tree, it is nested in the group containing *C. ferruginea* and *C. incrassata*, whereas in the *ncpGS* network it is clearly distinct from the other two species; its alleles are also different from those of both the *jaffrei* group and the *ferruginea-incrassata* group by three and four substitutions, respectively. The combined analysis of the three genes suggests the following relationships: ((*C. jaffrei*, *C. nitida*)(*C. incrassata*, *C. ferruginea*)). These species are subsequently referred to as “parental species”.

All other species of *Codia* examined exhibited alleles from two groups for at least one gene, and we therefore consider them to be species of hybrid origin (hereafter “hybrid species”). Five of them, *C. albifrons*, *C. belepensis*, *C. microphylla*, *C. spatulata* and *C.*

triverticillata, have combinations from the I and J allele groups and can be considered the result of a cross or crosses between *C. jaffrei* and a member of the *ferruginea-incrassata* group. *Codia albicans* and *C. montana* each produced alleles belonging to two distinct groups, A and I for the former and A and J for the latter. Alleles of group A were not found in any parental lineage and may be the legacy of an extinct element that gave rise to *C. albicans* via a cross with a member of the *ferruginea-incrassata* group; it also is likely to have produced *C. montana* by crossing with *C. jaffrei*. *Codia mackeeana* also has alleles from both the A and I groups according to the *GapC* tree, but for *ncpGS*, no A allele was recovered. It is possible that the history of this species is more complex. *Codia discolor* shows peculiar patterns; for *ncpGS* it has a combination of alleles from the I and N groups, for *PHYC* it has two alleles that are similar to each another and distinct from all others, and for *GapC* it has one allele from the J group. The parentage suggested by *ncpGS* alone would be a combination of the I and N genomes, but the two other genes do not support this hypothesis. Further genes and sampling will be necessary to clarify the origin of this species. A summary of the putative evolutionary history of the genus *Codia* is presented in figure 3.

In the split graph produced by the combined analysis of all three loci presented in figure 2, species of presumed hybrid origin appear in between their putative parents. With the possible exceptions of *Codia montana* and *C. albicans*, it is not possible to distinguish which species are the results of hybridization events. Species with the same parentage do not form clear clusters, and their parentage is not directly interpretable from the graph. A possible weakness of POFAD is that it does not take into account missing alleles, which could be either experimental artefacts or truly the result of natural loss. Older homoploid hybrids would not be expected to exhibit both parental alleles, as demonstrated in *Helianthus* (Rieseberg *et al.*, 2003), *Scaevola* (Howarth & Baum, 2005) and *Dubautia* (Friar *et al.*, 2008), and some of the “missing” alleles in our study may no longer be present in the putative hybrid

species. Thus if one considers two species with the same parentage, one of which has lost one of the parental alleles for a given gene, these two species would not appear in exactly the same place on the tree; one will appear closer to one of the parents. The existence of an extinct parental lineage cannot be detected with this method.

MORPHOLOGY OF *CODIA*

Differences in reproductive characters are few in *Codia*, and most differences between species involve features of the leaves (especially the shape of the blade and the amount and type of indumentum) and stipules (especially size and shape; table 2). Several different patterns of character distribution are observed in hybrid taxa compared with their putative parents.

Our results show that novel characters can occur in hybrid species relative to their parents. For example, most species of *Codia* have opposite leaves, but two consistently have leaves in whorls of three: *C. albifrons* and *C. triverticillata*, both of which have a combination of the I and J genomes and are thus products of hybridization between opposite-leaved parents. Both also share the unique J2 allele for *PHYC*, suggesting they diverged from a common ancestor after the hybridization event or that their parents shared this allele. Thus, it is not clear if the appearance of this novel feature represents a single event or two parallel ones. Whorled leaves can be considered as a transgressive phenotype, comparable to what has been observed in hybrid species of *Helianthus* (Rieseberg *et al.*, 2003; Rieseberg *et al.*, 2007).

Another novel character found only in species of putative hybrid origin is narrowly triangular stipules, which are found in *C. discolor*, *C. montana* and *C. spatulata*; each of these has a different genomic combination (Table 1), suggesting that this character has resulted from several different crosses. Examples of the converse, where characters seen in ancestral species have been lost in hybrids, also occur. For instance, *C. belepensis*, *C. microphylla* and

C. spatulata all lack the pale felty indumentum present in the adult foliage of their putative parents (*C. ferruginea*, *C. incrassata* and *C. jaffrei*).

The occurrence of petals shows a slightly different pattern. Petals occur consistently in *Codia albifrons* (I and J alleles) and *C. montana* (A and J alleles), and they have been observed occasionally or rarely in six other species, including the parental species *C. incrassata* and *C. nitida*. Importantly, both of the other genera in the tribe Codieae, *Callicoma* and *Pullea* (Bradford & Barnes, 2001), have flowers that lack petals, suggesting that this could therefore be the ancestral condition for the group.

ECOLOGY OF *CODIA*

The preferred soil types (ultramafic or non-ultramafic) of *Codia* species are summarised in table 2, together with an indication of habitat, which can include open scrub and maquis, other disturbed situations such as roadsides and along tracks, and forest edges and interiors. Although some species have restricted ecological preferences, several show a degree of plasticity.

The four parental species with no evidence of hybridization are each restricted to a single soil type: *Codia ferruginea*, *C. jaffrei* and *C. nitida* only grow on ultramafic soils, and *C. incrassata* occurs only on non-ultramafic substrates. Thus, adaptation to ultramafic soils appears either to be ancestral for the genus and to have been lost in *C. incrassata*, or it may have evolved twice, in the *C. jaffrei*-*C. nitida* clade and in *C. ferruginea*.

Most hybrid species are restricted to one type of soil, but three occur on both (*Codia albicans*, *C. montana* and *C. spatulata*). The lack of sub-grouping within the I group of alleles does not allow us to distinguish whether *C. ferruginea* or *C. incrassata* is the parent of the putative hybrid species with an allele of the I group. It is also possible that these two species diverged subsequent to the hybridization events. Furthermore the ecology of the putative

extinct parental lineage is not known. However, with the exception of *C. albicans* and *C. mackeeana*, at least one of the parents of each hybrid species grows on ultramafic soils. This implies that adaptation to these distinctive soils can be transmitted from parent to daughter species and can be lost, even if both parents grow on ultramafic soils.

Adaptation to ultramafic soils in *Codia* is possibly plesiomorphic, as de Kok (2002) suggested for five other genera in New Caledonia. However, he used cladistic analyses based on morphological characters, which cannot detect reticulate evolution. A plesiomorphic adaptation to ultramafic soils would not be surprising if, as suggested by Pelletier (2006), New Caledonia was entirely covered by ophiolite as it re-emerged from a period of deep-sea submersion in the late-middle Eocene (around -35My). Patterson & Givnish (2003) found that adaptation to serpentine substrates in Californian *Calochortus* (Liliaceae) evolved at least seven times and was thus highly homoplasious, but again, because they used only plastid markers, cases of hybridization may have been missed.

It is clear that soil type is a significant factor in the current distribution of *Codia* since 10 of 13 species are restricted to either ultramafic or non-ultramafic soils. The importance of soil type in constraining distribution is strengthened by evidence of past range movements (see below), which suggests that dispersal is not a limiting factor. Fruits of *Codia* are small, indehiscent and woolly, and appear to be wind dispersed.

The three *Codia* species with broad ecological preferences, *C. albicans*, *C. montana* and *C. spatulata* (the last two being the most widespread members of the genus) are all putatively of hybrid origin. *Codia albicans* is polymorphic, and the limits between *C. montana* and *C. spatulata* are not clear in parts of their range, especially in north-western New Caledonia. It is possible that these taxa have had multiple origins, as is commonly observed for hybrid species (Soltis *et al.*, 1999), and this could help to explain their polymorphic morphology. Alternatively, these species could each have had a single origin and

intermediates between them might be the result of recent hybridization in zones where they come into contact with one another, or their variation could be due to a combination of these processes.

Codia microphylla is rare and known only from a few localities in northern New Caledonia. Although herbarium specimens provide few data on its ecology, field observations suggest that this species may be restricted to phtanite, a type of siliceous sedimentary rock, that gives rise to dry, nutrient poor soils that support a short shrubby vegetation. The only other species of *Codia* that is sometimes found in this environment is *C. montana*, another hybrid species. Thus, adaptation to this novel habitat, unoccupied by any of the parental species, may have been facilitated by hybridization, as was observed in similar situations in *Helianthus* (Rieseberg *et al.*, 2003; Rieseberg *et al.*, 2007).

BIOGEOGRAPHY OF *CODIA*

Each of the four parental species has a relatively small geographical range. *Codia incrassata* is restricted to the area of mica-schist and sediments of north-eastern New Caledonia, and *C. ferruginea* is restricted to the ultramafic strip along the central-eastern part of the island (Fig. 4). *Codia jaffrei* is only found in the far south, and *C. nitida* also occurs in this region, as well as in a few isolated localities in the north-west. The southern part of Grande Terre and much of the eastern seaboard have higher rainfall than other parts of New Caledonia (Service d'Hydrologie de l'ORSTOM, 1981; Caudmont & Maitrepierre, 2007), and three of the parental species are largely restricted to the wettest areas; the part of the east coast where *C. ferruginea* occurs is somewhat drier. Studies on palms (Pintaud & Jaffré, 2001; Pintaud *et al.*, 2001) have suggested that the wet areas in the south and east of New Caledonia may have been refugia for rainforest taxa during the drier episodes of the Pleistocene, and the biased occurrence of diploid parental species in glacial refugia is a common feature in several

polyploid complexes in Europe (Trewick *et al.*, 2002; Abbott & Brochmann, 2003; Pillon *et al.*, 2007). All the species of *Codia* occurring on the markedly drier western coast of New Caledonia have a hybrid origin, except *C. nitida* (see above), which is nevertheless rare there. Further work is needed to confirm the importance of rainforest refugia in New Caledonia and to locate them precisely, and population genetics of relatively widespread species (Taberlet *et al.*, 1998) could potentially shed some lights on this issue.

Another striking distributional feature of *Codia* is that the main species involved in the production of hybrids, *C. jaffrei* on one side and the *ferruginea-incrassata* group on the other, do not have overlapping ranges (Fig. 4). However, hybridization between these taxa probably took place when the parental species (the ancestors of the modern taxa) occurred in sympatry; since the origin of the hybrids, the ranges of the parental taxa must have shifted, leaving their modern descendents in allopatry. Similar shifts in ranges of the parents of hybrids are thought to have occurred in *Nicotiana* (Chase *et al.*, 2003). Transgressive hybrids are well known for their altered ranges relative to their parental taxa. Hybridization events that have given rise to most species of *Codia* were thus probably relatively ancient, and their current distributions may not be representative of earlier points in their history. We have little information about alterations in climate and vegetation types between the emergence of New Caledonia in the late Eocene (Pelletier, 2006) and today, but changes in Quaternary vegetation have been charted by palynological studies (Stevenson *et al.*, 2001; Stevenson & Hope, 2005; Cabioch *et al.*, 2008).

Further evidence of alterations in distribution comes from hybrid species that have restricted distributions and do not co-occur with their parents; these include *Codia belepensis*, *C. microphylla* and *C. triverticillata*. *Codia belepensis* is an extreme case as it is restricted to three islands off the north of Grande Terre, Yandé, Art and Pott, where it is the only member of the genus present. The two species with whorled leaves, *C. albifrons* and *C. triverticillata*,

which are possibly descended from the same hybrid ancestor, do not co-occur at present; the former is restricted to the south of New Caledonia, and the other is found on only three mountains near the north-western coast: Kopeto, Boulinda and Paéoua.

Considerable range alterations, such as those we have hypothesised to have occurred in *Codia*, have been detected in other genera such as *Gossypium*, in which Cronn *et al.* (2003) found evidence of introgression in the Mexican cotton *G. gossypioides* from an African species. Similarly, all species of *Nicotiana* sect. *Suaveolentes* (Chase *et al.*, 2003), restricted to Africa (Namibia) and Oceania and comprising ca. 25 species, are the result of a single hybridisation involving parents from two lineages that are now confined to South America. Barraclough and Vogler (2000) suggested using the level of range overlap between sister species to determine the dominant mode of speciation: sympatric or allopatric, but when applied to South African *Protea*, Barraclough and Reeves (2005) found evidence for extensive range movements of species, preventing them from determining the mode of speciation in this genus. Hybrid speciation has been important in *Codia*, despite what contemporary species distributions suggest. Range changes could be a common and important phenomenon that obscures both biogeographic patterns and the historical diversification of a clade.

IMPLICATIONS FOR CONSERVATION

New Caledonian biodiversity is currently facing multiple threats, including fires, nickel mining, invasive species and climate change. The small size of the Archipelago (18,500 km²) and importance of micro-endemism almost certainly mean that a large proportion of New Caledonian species are susceptible to extinction and should be included in the IUCN Red List, the updating of which is urgently needed for New Caledonia (Munzinger

et al., 2008). Prioritisation of species and areas to preserve is needed, especially as few ex-situ conservation programs currently exist for threatened species in New Caledonia.

Vane-Wright *et al.* (1991) suggested that some species have higher conservation value than others, i.e. they have no close relatives. For example, the two recently described species, *Codia jaffrei* and *C. triverticillata* (Hopkins *et al.*, 2007), are both directly threatened by mining activities and have been assigned the provisional IUCN status of vulnerable (VU) and endangered (EN), respectively. Based on the level of threat, conservation priority might be given to the more threatened species, *C. triverticillata*. However, *C. jaffrei* represents a distinct parental lineage, whereas *C. triverticillata* is a hybrid species with a genomic content similar to that of several other species, e.g. *C. albifrons* and *C. belepensis*. The evolutionary potential of *C. jaffrei* may therefore be considered higher as it might hybridize again with other parental species to give rise to new hybrid taxa, and thus, it could be argued that *C. jaffrei* is of greater conservation importance than *C. triverticillata*.

The results of this study support the idea of Pintaud *et al.* (2001) and Pintaud & Jaffré (2001) that the southern and eastern parts of New Caledonia may have served as rainforest refugia during the climatic oscillations of the Pleistocene. Southern New Caledonia is the most species-rich region for Cunoniaceae; in addition, of the five families endemic to the Archipelago (Amborellaceae, Oncothecaceae, Paracryphiaceae, Phellinaceae and Strasburgeriaceae), all but Amborellaceae occur there, and two (Oncothecaceae and Strasburgeriaceae) are restricted to this area. Refugia are also expected to host greater genetic diversity (Taberlet *et al.*, 1998) and more rare genotypes (Petit *et al.*, 2003) within species and to represent important areas for the long-term persistence of biodiversity (Taberlet & Cheddadi, 2002). Thus, these possible refugia should be given greater conservation attention, the network of southern reserves should be re-enforced and new reserves should be designated on the eastern coast where there are currently almost none (Jaffré *et al.*, 1998).

It has been argued that it is important to preserve the conditions required for the persistence of evolutionary processes that generate biodiversity (Smith *et al.*, 1993; Cowling & Pressey, 2001; Moritz, 2002). This could be a particularly appropriate strategy in the case of species complexes that are evolving rapidly and/or where species delimitations are controversial (Ennos *et al.*, 2005; Pillon *et al.*, 2006). In New Caledonia, most plant species are restricted to one type of substrate, either ultramafic or non-ultramafic, and zones between these two soil types that still have intact vegetation are not numerous, but they provide evolutionarily important opportunities for some species to co-occur. One such site is Mont Koghis, close to the capital city of Nouméa, where rainforest covers a complex matrix of soils derived from peridotite, serpentine, and sedimentary rocks, and species that are not normally sympatric due to divergent ecological preferences are found in close proximity. It appears likely that hybridization between species with different substrate preferences has given rise to *Cunonia koghicola* on this mountain (Pillon *et al.*, 2008). Such sites should be preserved to maintain one of the evolutionary processes that have contributed to the remarkable biodiversity of New Caledonia.

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Table 1. List of alleles found in each *Codia* accession and their supposed genomic composition. Uncertain genomic compositions are in parenthesis. For *GapC*, the number of clones successfully sequenced is indicated in parenthesis.

Species	<i>ncpGS</i>	<i>PhyC</i>	<i>GapC</i>	Genome
<i>C. albicans</i>	A2,I10	A1,J1	A1,I10 (4)	A,I
<i>C. albifrons</i>	I3,J3	J2	I1,I8 (5)	I,J
<i>C. belepensis</i>	I8,J6	J3	I2,I4 (4)	I,J
<i>C. discolor</i>	I5,N3	J4,J5	J2 (5)	(I,N)
<i>C. ferruginea</i>	I4	I2	I9,I11 (3)	I
<i>C. incrassata</i>	I2	I3	I7 (3)	I
<i>C. jaffrei</i>	J1	J1	J1 (6)	J
<i>C. mackeeana</i>	I1,I9	I1,J1	A3,I3 (6)	A,I
<i>C. microphylla</i>	I6,J4	-	J4,J5 (6)	I,J
<i>C. montana</i>	A1,J7	A1	A2,J6 (4)	A,J
<i>C. nitida</i>	N1,N2	J1	N1 (4)	N
<i>C. spatulata</i>	I7,J5	I1	I5 (1)	I,J
<i>C. triverticillata</i>	I1,J2	J2	I6,J3 (5)	I,J

Table 2. Ecology and morphology of *Codia* species.

Species	substrate ¹		habitat ²		# leaves per node	pale, indumentum on lower leaf surface	felty on leaves	arachnoid on leaves	hairs	stipules ³	petals
<i>C. albicans</i>	UM	NUM	(F)	M	2	+		--		A	--
<i>C. albifrons</i>	UM		(F)	M	3	+		++		A	++
<i>C. belepensis</i>	UM			M	2	--		--		A	--
<i>C. discolor</i>	UM		(F)	M	2	+		--		B	(+)
<i>C. ferruginea</i>	UM			M	2	+		+		A	(+)
<i>C. incrassata</i>		NUM	F	(M)	2	+		--		A	--
<i>C. jaffrei</i>	UM		F		2	+		--		A	(+)
<i>C. mackeeana</i>		NUM	F		2	--		--		A	--
<i>C. microphylla</i>		NUM		M	2	--		(+)		A-B	(+)
<i>C. montana</i>	UM	NUM	(F)	M	2	--		--		B	++
<i>C. nitida</i>	UM		(F)	M	2	--		--		C	(+)
<i>C. spatulata</i>	UM	NUM	F	M	2	--		--		B	(+)
<i>C. triverticillata</i>	UM			M	3	+		--		A	--

Rare occurrences are in parentheses.

¹ UM = ultramafic, NUM = non-ultramafic.

² F = forest, M = maquis and/or other types of scrub.

³ A = triangular, broadly ovate or cordate ; B = narrowly triangular ; C = elliptic.

Figure 1. Network of alleles obtained with *ncpGS*, *PHYC* and *GapC*.

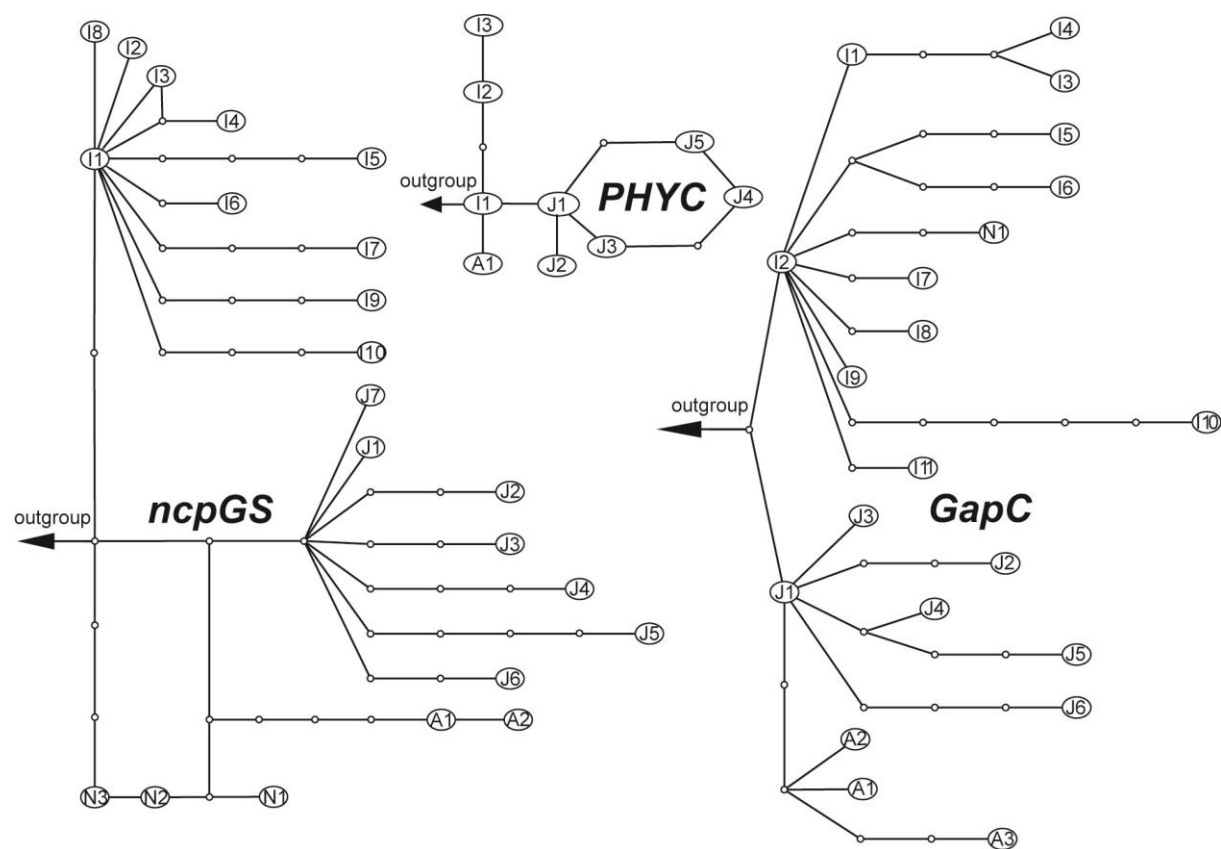


Figure 2. Split network of the species of *Codia* based on all three genes (*ncpGS*, *PHYC* and *GapC*). The predicted placement of the putative extinct parental lineage is shown by dashed lines.

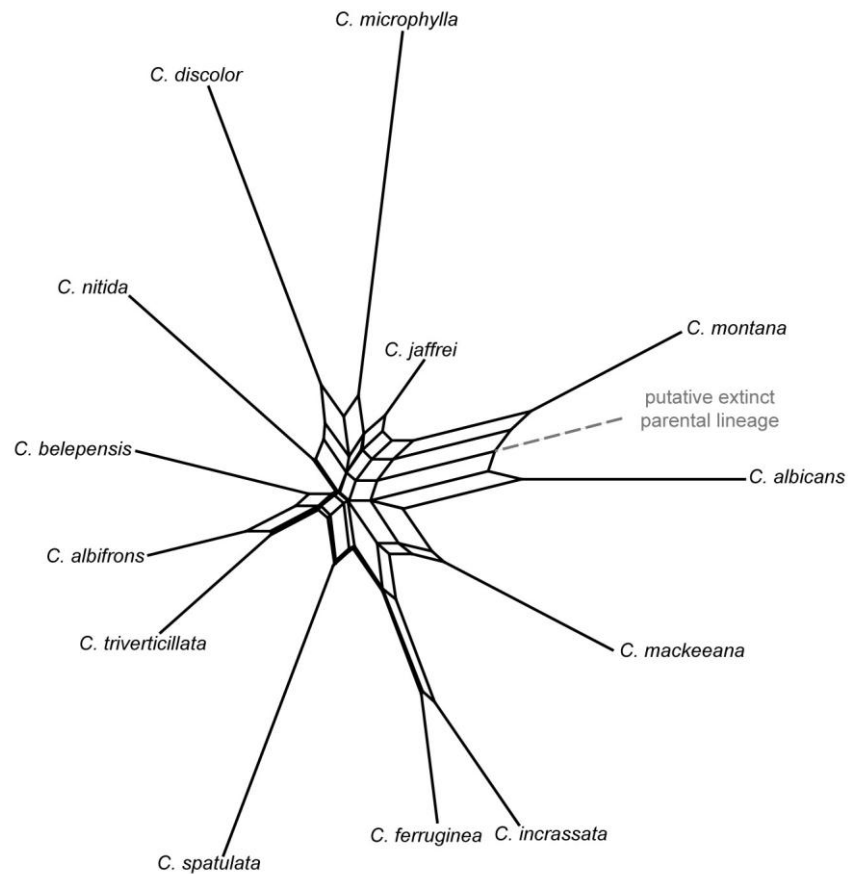


Figure 3. Summary diagram depicting the hypothetical relationships between the species of *Codia*. The cross indicates a putatively extinct lineage. Species with uncertain parentage are shown in brackets. Species growing on ultramafic soils are shown in red, species growing on non-ultramafic soils in blue, and species growing on both types of soils in purple. The dashed rectangle indicates the two species with whorled leaves.

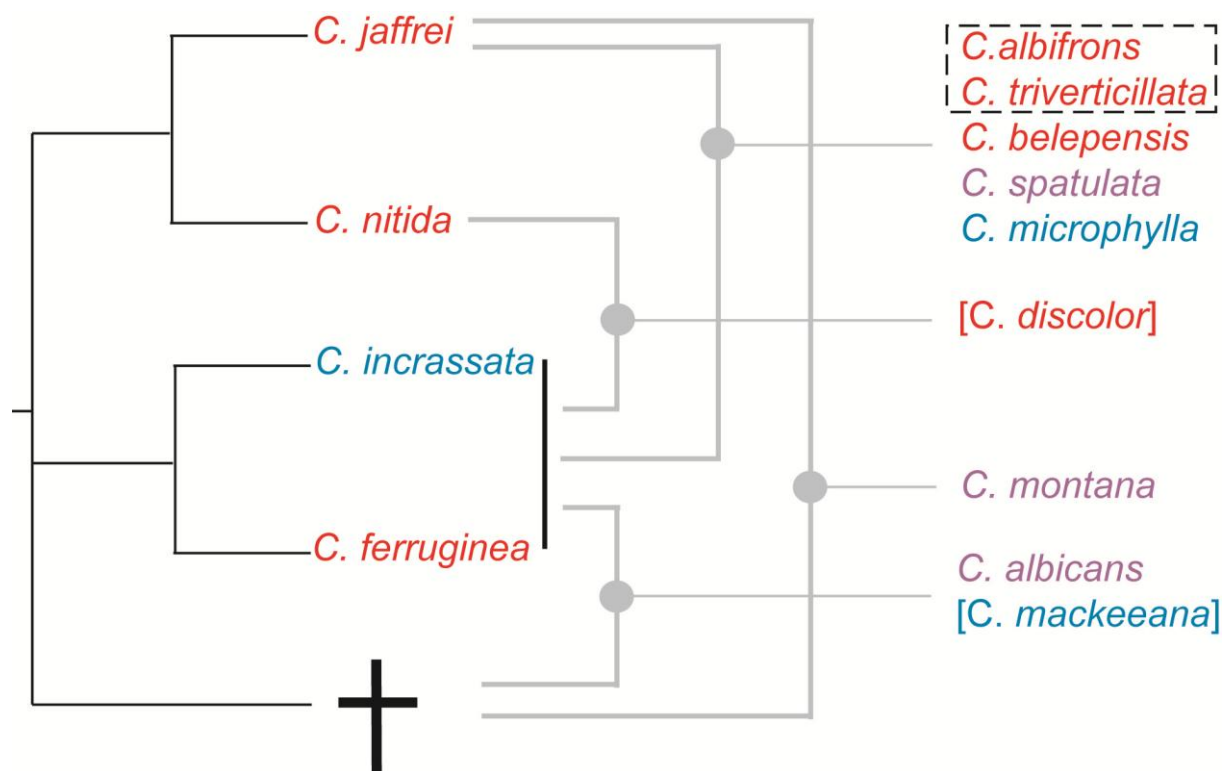
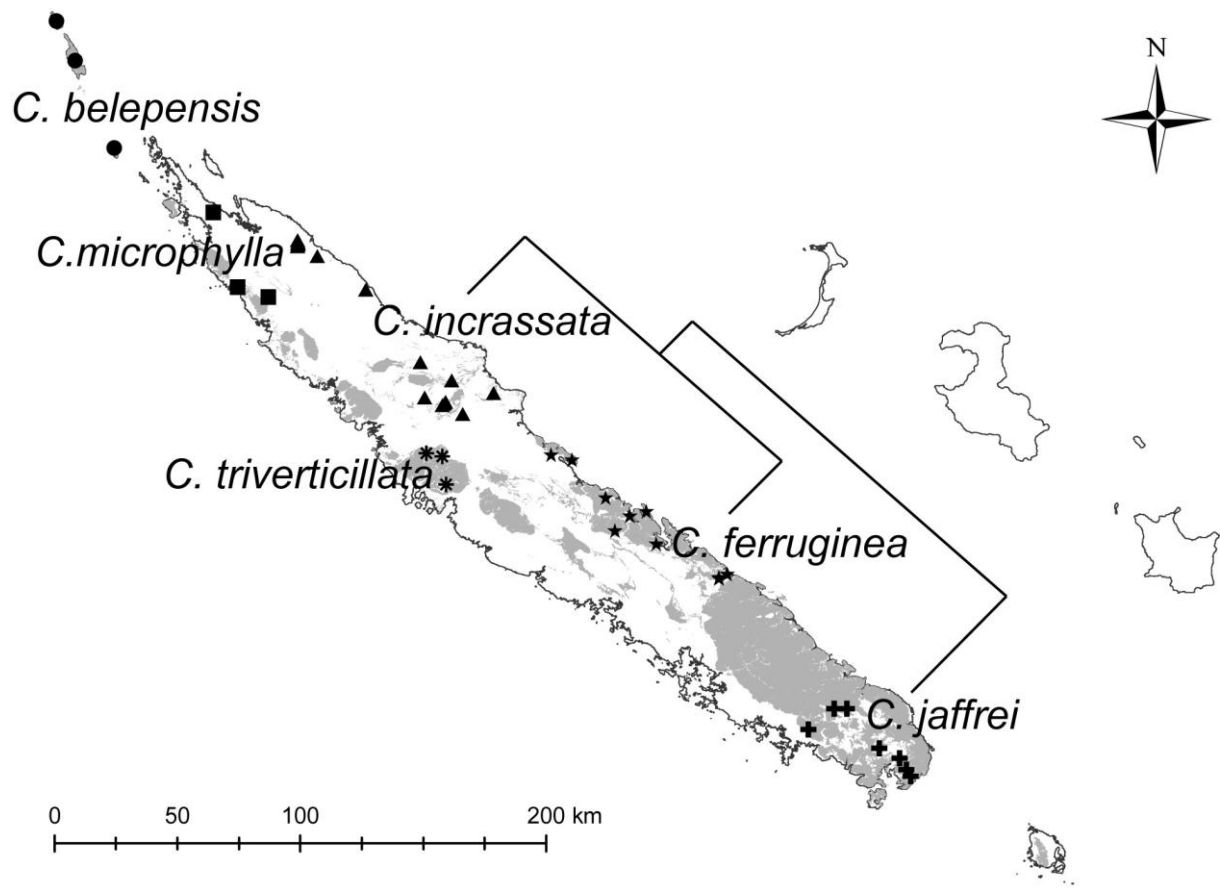


Figure 4. Distributions of the three parental species *C. ferruginea*, *C. incrassata* and *C. jaffrei* and three species that are suspected to be the result of hybridisation between them: *C. belepensis*, *C. microphylla* and *C. triverticillata*. Areas covered with ultramafic soils are shown in grey.



Supplementary data. List of *Codia* accessions included in the study with voucher information and GenBank accessions number for DNA sequences.

Species	Voucher	Herbarium	Locality	<i>ncpGS</i>	<i>PhyC</i>	<i>GapC</i>
<i>Codia albicans</i> Vieill. ex Pamp.	Pillon et al. 478	NOU	Hienghène	FJ595023 FJ595024	FJ595046 FJ595047	FJ595061 FJ595062
<i>Codia albifrons</i> (Brongn. ex Schinz & Guillaumin) Baker f.	Bradford & Hopkins 1144	MO,NOU	Koghis	FJ595025 FJ595026	FJ595048	FJ595063 FJ595064
<i>Codia belepensis</i> H.C.Hopkins	Munzinger & Swenson 3031	NOU	Yandé	FJ595027 FJ595028	FJ595049	FJ595065 FJ595066
<i>Codia discolor</i> (Brongn. & Gris) Guillaumin	Bradford et al. 1193	MO,NOU	Thio	FJ595029 FJ595030	FJ595050 FJ595051	FJ595067
<i>Codia ferruginea</i> Brongn. & Gris	Pillon & Barrabé 330	NOU	Cap Bocage	FJ595031	FJ595052	FJ595068 FJ595069
<i>Codia incrassata</i> Pamp.	Pillon & Munzinger 80	K,P,NOU	Mandjélia	FJ595032	FJ595053	FJ595070
<i>Codia jaffrei</i> H.C.Hopkins & Fogliani	Pillon 38	NOU	Grand Kaori	FJ595033	FJ595054	FJ595071
<i>Codia mackeeana</i> H.C.Hopkins & Fogliani	Fogliani & Hopkins 120 [Type]	K,P,NOU	Col d'Amieu	FJ595034 FJ595035	FJ595055 FJ595056	FJ595072 FJ595073
<i>Codia microphylla</i> Vieill. ex Guillaumin	Munzinger 2956	NOU	Col d'Arama	FJ595036 FJ595037	-	FJ595074 FJ595075
<i>Codia montana</i> J.R.Forst. & G.Forst.	Pillon & Vergnes 225	NOU	Tao	FJ595038 FJ595039	FJ595057	FJ595076 FJ595077
<i>Codia nitida</i> Schltr.	Bradford & Hopkins 1149	MO,NOU	Koghis	FJ595040 FJ595041	FJ595058	FJ595078
<i>Codia spatulata</i> Brongn. & Gris	Pillon 158	NOU	Lac de Yaté	FJ595042 FJ595043	FJ595059	FJ595079
<i>Codia triverticillata</i> H.C.Hopkins & Pillon	Pillon et al. 137	K,P,NOU	Kopeto	FJ595044 FJ595045	FJ595060	FJ595080 FJ595081