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Abstract

The assembly of island plant communities is the result of a number of processes: immigration (dispersal), speciation, and extinction. Using four plastid genes and one low-copy nuclear gene, we investigated the origin of the New Caledonian *Grevillea* (Proteaceae), an otherwise largely Australian genus. In the combined plastid analysis, the species form two distinct clades, the *exul* (four species) and *gillivrayi* groups (six species), within group 3 of *Grevillea*. All New Caledonian *Grevillea* display two distinct copies of *PHYA*, one copy in group 3, and another in group 4. Previously published chromosome counts for *G. meisneri* and two new genome size estimates of *G. gillivrayi* and *G. rubiginosa* suggest that these plants are tetraploids. Altogether, the current data available suggest that New Caledonian *Grevillea* are allotetraploids, resulting from one or two hybridisation events between two or three distinct parents. A possible scenario is that modern *Grevillea* has descended from a hybrid swarm that formed 9-13 Mya between multiple immigrants (now extinct) that reached the island. *Grevillea* can be added to the list of island plant radiations with an early history of hybridisation and polyploidy. The relative importance, location and timing of these two mechanisms remain to be elucidated. The two groups of New Caledonian *Grevillea* may be easily distinguished by their inflorescences, flowers and fruits, with the *gillivrayi* group displaying greater ecological and morphological diversity. The seed characteristics of New Caledonian *Grevillea* are consistent with their pioneer behavior.

Keywords

Adaptive radiation, genome duplication, island biogeography, manganese, seeds, serpentine

Introduction

Islands are widely recognised as unparalled evolutionary laboratories, where the genesis of biodiversity is expected to be easier to investigate. Two central themes in island biology are the importance of arrival history in community assembly and the genomic signature of adaptation and speciation (Warren et al. 2015). The most typical examples of adaptive radiation in island plants include Hawaiian silverswords (Asteraceae) and Hawaiian lobeliads (Campanulaceae), which have in common polyploidy. In the case of the silverswords, the allopolyploidisation event shortly pre-dated their radiation (Barrier et al. 1999). Although it is accepted that Hawaiian lobeliads are all tetraploid (Lammers 1988), it is not clear whether they are auto- or allotetraploids and when the genome duplication event occurred, this being a recurrent trend in Lobelioideae (Antonelli 2008). In another Hawaiian plant radiation, *Cyrtandra* (Gesneriaceae), there is evidence that the island of Hawai'i was colonized by multiples lineages that had uneven success and hybridized (Johnson et al. 2019). Although multiple colonisations, hybridization and polyploidisation may be three important features in the genesis of island plant diversity, their frequency and role may have been underestimated.

It is increasingly accepted that long-distance dispersal is more frequent than was previously thought (de Queiroz 2005; Christenhusz and Chase 2013). Long-distance dispersal through wind, birds and ocean currents is likely the major process involved in patterns of plant disjunct distributions (Gillespie et al. 2012), in particular in the case of some *a priori* unlikely colonisation routes. For example, *Acridocarpus* (Malpighiaceae) reached New Caledonia from Madagascar (Davis et al. 2002), Chamaedoreeae (Arecaceae) and *Weinmannia* (Cunoniaceae) reached the Mascarenes from the Americas (Baker and Couvreur 2013; Pillon et al. 2021), and *Acacia* s.s. (Fabaceae) reached Réunion from the Hawaiian Islands (Le Roux et al. 2014). In spite of being the most isolated archipelagos on Earth, the Hawaiian Islands have been colonized multiple times by several plant genera: *Coprosma* (Rubiaceae) (Cantley et al. 2014), *Santalum* (Santalaceae) (Harbaugh and Baldwin 2007), and *Scaevola* (Goodeniaceae) (Howarth et al. 2003). At least eight dispersals of Hawaiian plants to the distant Marquesas have also been reported (Price and Wagner 2018).

Hybridization is an important process on islands, although "reports of hybridization in insular floras depend on the tendency of systematists to recognize the presence of hybrids

or to neglect them" (Carlquist 1974 p. 534). It therefore varies according to archipelago and taxa (Stuessy et al. 2014). For example, hybridization is generally recognised as a common feature in the Hawaiian flora, e.g. *Cyrtandra* (Smith et al. 1996), *Metrosideros* (Myrtaceae) (Stacy et al. 2016), *Santalum* (Harbaugh 2008), and *Scaevola* (Howarth and Baum 2005) and also potentially important in the Macaronesian flora (Herben et al. 2005). In the flora of New Caledonia, evidence is scarcer, with some reports (mostly recent) in *Araucaria* (Araucariaceae) (Gaudeul et al. 2014), *Corybas* (Orchidaceae) (Faria 2016), *Dacrydium* (Podocarpaceae) (Knopf et al. 2007) and multiple genera of Cunoniaceae (Pillon et al. 2008; Pillon, Munzinger, et al. 2009; Pillon, Hopkins, et al. 2009). Hybridisation may be advantageous to colonise novel habitats (Rieseberg et al. 2007) or promote invasiveness (Schierenbeck and Ellstrand 2009).

Polyploidy may be an important feature of island floras. For instance, polyploids may represent as much as 80% of the native Hawaiian flora (Carr 1998). Polyploidy is also known as an important driver of diversification, as in the case of *Allium* (Amaryllidaceae) (Han et al. 2020). It may facilitate the invasion of introduced plants (Ainouche et al. 2009; te Beest et al. 2012), thereby potentially helping colonisation and settlement on islands. However information on chromosome numbers is uneven between islands, e.g. Hawai'i (50 % with chromosome counts) and New Zealand (86%) (Meudt et al. 2021), and probably much lower for other Pacific islands, including New Caledonia. The timing of polyploidisation on islands, i.e. pre or post-colonisation, is often difficult to determine (Crawford et al. 2009; Meudt et al. 2021) and is a major problem (Johnson et al. 2019).

Dispersal, hybridization and polyploidisation are potentially intimately inter-connected and may be difficult to tease apart. For example, in grasses, polyploids may be more successful at long-distance dispersal than diploids (Linder and Barker 2014). Multiple allopolyploid origins likely increased the ecological amplitude of *Aegilops triuncialis* (Poaceae) during its invasion of California (Meimber et al. 2009). Polyploidy is more likely to follow hybridization when the two parents are distantly related (Paun et al. 2009). The distinction between auto- and allotetraploid is not always straightforward (Ramsey and Schemske 1998), but allopolyploids are expected to be more prevalent in nature than autopolyploids (Soltis and Soltis 2000). There are many examples of endemic species or radiations with a hybrid origin in island plants (Table 6 in Johnson et al. 2019), where it is not

clear which came first: colonisation or hybridization. In the case of *Nicotiana* sect. *Suaveolentes* (Solanaceae), all of which are allotetraploid (Chase et al. 2003), hybridisation in South America preceeded colonisation, with allotetraploid species reaching French Polynesia, New Caledonia, Namibia (Africa) and Australia about six million years ago (Cauz-Santos et al. 2022). However, only in Australia has there been a recent radiation into more than 60 species (Chase et al. 2021). No diploids occur in the range of *N*. sect. *Suaveolentes*, and none of the species of this section occurs within the range of the parental diploids in South America (Chase et al. 2003). The dispersal vector is assumed to be birds (Mummenhoff and Franzke 2007), but the modern wide disjunction between diploid parents and tetraploid offspring is perplexing.

Here, we investigate the origins of New Caledonian *Grevillea* (Proteaceae), which comprises ten endemic species (Majourau and Pillon 2020) among *ca*. 360 species in the genus. They are often dominant in New Caledonian maquis on ultramafic rocks (Jaffré 2023) and are of particular interest because of their tendency to accumulate manganese, which confers to their metal-rich biomass some promising properties for organic chemistry (Losfeld et al. 2015). The only chromosome count available for this group is that of *G. meisneri* (n=22, Carr and McPherson 1986), a much higher count than those typically observed in the family (from n = 10 to n = 14, Stace et al. 1998) and for *Grevillea* in particular (n = 10). With novel molecular phylogenetic data and new genome-size measurements, we sought to determine the number of times *Grevillea* colonised New Caledonia and obtain new lines of evidence for the origins of polyploidy in this group. Their seeds are also characterised and compared in light of their ecology.

Material and Methods

Sampling, DNA extraction, amplification, cloning and sequencing

Fresh material was collected for each species of New Caledonian *Grevillea* (*G. deplanchei, G. exul, G. gillivrayi, G. macmillanii, G. meisneri* (both varieties)*, G. mondorensis, G. nepwiensis, G. rubiginosa, G. sinuata* and *G. vuniana*). Two accessions of New Caledonian species of the related genus *Stenocarpus* were also included. All samples were dried in silica-gel (Chase and Hills 1991). Total genomic DNA was extracted with a modified CTAB protocol (Doyle and

Doyle 1987). Five DNA regions were amplified following the protocols of Mast et al. (2008): *(i)* plastid *atpB*, *matK*, *ndhF* and the *rpl16* intron, and *(ii)* nuclear *PHYA* gene.

For each New Caledonian accession, directly sequenced regions of *PHYA* included multiple single base pair polymorphisms that indicated the presence of two distinct alleles differing at a number of bases without insertions/deletions. These bases were coded following the IUPAC nucleotide ambiguity scheme (Cornish-Bowden 1985). Two combinations of alleles were readily distinguishable, and we separated the alleles by cloning for at least one representative (*G. gillivrayi* and *G. exul*) of each combination with pGEM®-T Easy vector (Promega Corp.) according to the manufacturer's instructions. These alleles separated by molecular cloning (five and six clones sequenced) served as guides to reconstruct the two distinct alleles of the other individuals. We kept the IUPAC coding for polymorphisms not found in either cloned accession.

Molecular datasets

The newly generated sequences from the 12 New Caledonian *Grevillea* (and two *Stenocarpus*) specimens were combined with molecular data from the study of Mast et al. (2015). In their study, Mast et al. (2015) assessed the phylogenetic relationships of the subtribe Hakeinae (Proteaceae) with a 171 accession molecular dataset (plastid genes *atpB, matK and ndhF* and the *rpl16* intron, and nuclear PHYA) including 97 specimens (representing 93 species) of *Grevillea* and 55 specimens (one per species) of *Hakea*, and representatives of other genera of Embothrieae and more distantly related Proteaceae. As more distant outgroups, we followed Mast et al. (2015) and included representatives of Platanaceae (*Platanus orientalis*) and Nelumbonaceae (*Nelumbo lutea*). Here, two distinct datasets were assembled: *(i)* a plastid dataset of *atpB, matK, ndhF* and the *rpl16* intron, and *(ii)* a nuclear dataset of *PHYA* sequences. We did not combine the plastid and nuclear datasets because of the presence of distinct alleles in the *PHYA* sequences of New Caledonian *Grevillea*. The *rpl16* intron sequences varied slightly in length, and we used MAFFT v7 (Katoh and Standley 2013) with default option settings to align them. For all exons (*atpB, matK, ndhF* and *PHYA*), we used Mesquite v3.70 (Maddison and Maddison 2021) to check the reading frame for stop codons. The four combined plastid regions for 185

specimens resulted in a matrix of 6,508 aligned characters (2,191 variable characters of which 1,326 are potentially parsimony informative), whereas for *PHYA*, the dataset consisted of 174 sequences (including two sequences for each of 11 New Caledonian accessions) and 1,169 aligned characters (593 variable characters of which 374 are potentially parsimony informative). GenBank accession numbers and voucher information for all corresponding sequences are presented in Table S1.

Phylogenetic analyses

For both datasets, maximum likelihood (ML) analyses included *a priori* division in partitions. The plastid dataset was divided into 10 partitions, with three partitions (one per codon position) for each gene (*atpB, matK and ndhF*) and one for the *rpl16* intron. The nuclear dataset was divided into three partitions, one for each codon position. Following Mast et al. (2015), trees were rooted with *Nelumbo lutea*. Maximum likelihood analyses were conducted with IQ-TREE v2 (Minh et al. 2020). For each dataset, the best-score ML tree was inferred with a heuristic search with 500 random-addition replicates and the following settings: random-starting tree, thorough hill-climbing nearest neighbour interchange (NNI) search (*-allnni* option), perturbation strength of 0.2 (*-pers 0.2* option), partition-resampling strategy (*--sampling GENE* option), and best partition scheme allowing the merging of partitions (*-m MFP+MERGE* option). Support of nodes was assessed with 1000 replicates for both SH-like approximate likelihood ratio tests (SH-aLRT, Guindon et al. 2010) and ultrafast bootstrap (uBV, Minh et al. 2013). Nodes supported by SH-aLRT ≥ 80 and uBV ≥ 95 were considered strongly supported following authors' recommendations.

Dating analyses

Dating analyses were conducted on the plastid dataset using Bayesian relaxed clocks as implemented in BEAST v1.10.4 (Suchard et al. 2018). Here, we used the primary fossil calibrations of Mast et al. (2015) including the same normal distributions to constrain the ages of the most recent common ancestors (MRCA) of: *(i)* Proteaceae and Platanaceae (mean of 112.3 and a standard deviation of 6.48), and *(ii) Embothrium* and *Telopea* (mean of 37.5 and a standard deviation of 1.07). To properly implement the corresponding rates of

evolution, we relied on a calibration strategy of four uncorrelated lognormal clocks, one for each plastid marker. We further used as a guide tree the results of ML plasid analyses. The tree model was set to a birth-death speciation process, which is adequate to analyze datasets with a mixture of inter- and intraspecies sampling (Ritchie et al. 2017). The BEAST analysis consisted of 50 million generations of MCMC with the parameters and trees sampled every 5,000 generations. A burn-in of 25% was applied after checking the log-likelihood curves. The maximum credibility tree, median ages and their 95% highest posterior density (HPD) were generated with TreeAnnotator v1.10.4, which is part of the BEAST software package. Convergence of runs was then assessed graphically by examining the ESS of relevant parameters under Tracer v.1.7 (Rambaut et al. 2018).

Genome-size estimation

Fresh leaves from five individuals of *Grevillea gillivrayi* and *G. rubiginosa* were collected in the field and *G. robusta* from cultivated trees in Noumea. Nuclei were prepared with the following isolation buffer: 45 mM $MgCl_2$, 30 mM sodium citrate, 60 mM MOPS pH 7.0, 1% PVP 10.000, 0.1% Triton X-100 and 10 mM sodium metabisulfite ($S_2O_5Na_2$) (Bourge et al. 2018) and stained with propidium iodide 50 µg/mL. The samples were run on a CytoFLEX S Cytometer (Beckman Coulter), excitation 561nm, 46 mW; emission through a 690/50 nm band-pass filter. The following standards were used: *Solanum lycopersicum* "Roma" (2C = 1.99 pg) and *Petunia hybrida* PxPc6 (2C = 2.85 pg) (Marie and Brown 1993).

Seed morphology

Mature fruits of *Grevillea exul*, *G. rubiginosa* and *G. gillivrayi* were harvested from plants growing in the south of New Caledonia. Seeds were extracted after fruit drying. For each species, 20 seeds were weighed and measured (entire seed and embryo length and width), and average dimensions were determined. Embryo length:seed length (El:Sl) ratio and embryo width:seed width (Ew:Sw) ratio were calculated.

Results

Phylogenetic analyses

Overall, the plastid ML tree is well supported (Figure 1) with 120 of the 184 nodes supported by both SH-aLRT ≥ 80% and uBV ≥ 95%. *Grevillea* is paraphyletic because representatives of *Hakea* are sister (SH-aLRT 78.7%, uBV 92%; always given in this order below) to 45 *Grevillea* species. New Caledonian *Grevillea* species form two well-supported (≥ 80%, ≥ 95%) clades within group 3 *sensu* Mast et al. (2015): the *exul* group (*G. exul, G. macmillanii, G. rubiginosa* and *G. sinuata*) and the *gillivrayi* group (*G. deplanchei, G. gillivrayi, G. meisneri, G. mondorensis, G. nepwiensis* and *G. vuniana*). Within the *gillivrayi* group, our sample of *G. gillivrayi* fails to pair with the sample of *G. gillivrayi* in Mast et al. (2015). The *exul* group is sister (98.2%, 100%) to a pair of south-western Australian species, *G. infudibularis* and *G. tripartita* (the *oncogyne* group *sensu* Makinson 1999). Sister to the *gillivrayi* group is the Sulawesi endemic *G. elbertii* (98.8%, uBV of 100%) followed by Melanesian *G. chloroxantha* (alternatively placed in the genus *Finschia*) (73.2%, 98%).

In the *PHYA* ML tree (Figure 2), overall support is lower, and only 63 nodes of the 173 nodes are supported by both SH-aLRT ≥ 80% and uBV ≥ 95%. The inferred topology is similar to the plastid tree, with some noticeable differences, i.e., the *Hakea* species form two well-supported clades instead of one (100%, 100%). The direct sequencing of *PHYA* produced many loci with two bases (polymorphisms) and thus indicates that all New Caledonian *Grevillea* have two distinct copies. These alleles form two distinct groups in the *PHYA* tree (Figure 2). The first allele is weakly supported (0%, 83%) as sister (100%, 100%) to most representatives of group 3 *sensu* Mast et al. (2015), whereas the second is embedded (0%, 99%) among representatives of group 4 *sensu* Mast et al. (2015). In both plastid and *PHYA* trees, the two New Caledonian accessions of *Stenocarpus* (*S. milnei* and *S. rubiginosa*) are sister to Australian *S. sinuatus* and *Strangea cynanchicarpa*. Thus, *Stenocarpus* is paraphyletic to *Strangea*.

Dating analyses

Post-burn-in parameters for the BEAST analysis had ESS values ≥ 200. Based on the plastid dataset, the origin of *Grevillea*/*Hakea* occurred during the Eocene with a median age of *ca*. 38.2 million years ago (Mya; 95% HPD: 32.1–44.9 Mya; Figure 3). The estimated ages of the

two New Caledonian clades are largely overlapping. The plastid haplotype of the *exul* group diverged from those of *G. infundibularis* + *G. tripartita* ca. 12.9 Mya (95% HPD: 9.7-16 Mya) with a crown age of *ca*. 10.2 My (95% HPD: 7.3–13.2 My). The plastid haplotype of the *gillivrayi* group diverged from those of *G. elbertii* ca. 13.2 Mya (95% HPD: 7.3-13.2 Mya) with a crown age of *ca*. 9.2 My (95% HPD: 6.5–12.2 My).

Genome-size estimation

We obtained the following genome size estimates: *G. gillivrayi,* mean 1C = 1.84 pg (standard *Petunia hybrida* PxPc6); *G. robusta* with mean 1C = 0.92 pg (standard *Petunia hybrida* PxPc6), and *G. rubiginosa*, mean 1C = 1.60 pg (standard = *Solanum lycopersicum* "Roma").

Seed morphology

The seeds of *Grevillea exul*, *G. rubiginosa* or *G. gillivrayi* present a common structure of a winged testa with the integument surrounding the central embryo (Figure 4). There is some size variation between species in wing size (the largest in *G. exul*) and seed color (bright for *G. gillivrayi*, dark in both *G. exul* and *G. rubiginosa*, the last sometimes black). Sections of seeds revealed that they are all constituted by a large embryo that occupies all space in the central portion. The embryo length:seed length (El:Sl) ratio is greather than 40% in all cases. No endosperm was detected.

In contrast, there are significant differences in seed size, shape and weight between species (Table 1). *Grevillea rubiginosa* and *G. exul* seeds are round, the latter smaller even though it is the heaviest of all studied species. *Grevillea gillivrayi* seeds are more oval than the others with an average weight close to that of *G. rubiginosa*. Finally, considering the space occupied by the embryo, i.e. El:Sl and Ew:Sw ratios, the largest embryo clearly belongs to *G. gillivrayi*, whereas *G. exul* has the smallest.

Discussion

Polyploidy in Proteaceae

Our genome-size estimate for *Grevillea robusta* is larger than that reported previously (1C = 0.92 pg vs. 0.80 pg, Ohri and Kumar 1986). The other *Grevillea* estimate available is for *G. australis*, (1C = 1.21 pg, Jordan et al. 2015). The estimates for the New Caledonian endemics *G. gillivrayi* (1C = 1.84 pg) and *G. rubiginosa* (1C = 1.60 pg) are therefore 2 times and 1.74 times larger than that of *G. robusta,* respectively. Stace (1998) reported n = 10 in ten species of *Grevillea*, whereas New Caledonian *G. meisneri* is n ≈ 22 (Carr and McPherson 1986). These counts and genome-size estimates indicate that the New Caledonian species of *Grevillea* are likely to be tetraploids. Their genome sizes are larger than those otherwise observed in Hakeinae (1C, $\mu = 0.99$; $\sigma = 0.19$) and Grevilleoideae (1C, $\mu = 1.19$; $\sigma = 0.41$) (Pellicer and Leitch 2020).

Genome sizes are comparatively small in Proteaceae, except for Persoonioideae (1C, $\mu = 23.71$) (Jordan et al. 2015; Pellicer and Leitch 2020). Stace (1998) reported only four cases of polyploidy in Proteaceae: *Franklandia triaristata* (Western Australia), *Dilobeia thouarsii* (Madagascar), *Persoonia* (=*Toronia*) *toru* (New Zealand) and *Grevillea meisneri*. Small genome sizes in Proteaceae would be expected considering their notorious ability to cope with low phosphorus soils and multiple unique traits associated with P-economy, including P-scavenging cluster roots, efficient P-remobilization from senescing organs, substitutes to membrane phospholipids etc. (Lambers et al. 2015). The distinctiveness of the larger genomes reported for Persoonioideae (Jordan et al. 2015) and lack of proteoid roots (Lamont 1982) are noteworthy. Polyploidy in the New Caledonian species of *Grevillea*, which are restricted to ultramafic substrates, thus appears counter-intuitive. These soils are particularly P-impoverished, and a previous study suggested than these substrates favoured smaller genomes in the western Balkans (Pustahija et al. 2013). Genome sizes are small in all *Grevillea* species, regardless of their ploidy.

Biogeography and timing of insular allopolyploids

Our phylogenetic results indicate a hybrid origin of New Caledonian *Grevillea* involving two or more, probably three, parental species. All New Caledonian *Grevillea* have a plastid genome similar to that of the *Grevillea* species in group 3 (*sensu* Mast et al. 2015). However, they form two clades: the *exul* group sister to a pair of south-western Australian species (*G.*

infudibularis, *G. tripartita*, the *oncogyne* group *sensu* Makinson (1999) and the *gillivrayi* group sister to *G. elbertii*, described from Sulawesi (Sleumer 1955a). This last species is known from only a few collections and localities, including Kabaena and Malili, where it grows in "dry-bush formation on crystalline slate" (Sleumer 1955b), which is likely to be ultramafic.

All New Caledonian *Grevillea* have two copies of single-copy nuclear *PHYA*. Mast et al. (2015) included a single accession of a New Caledonian species of *Grevillea*, *G. gillivrayi*, and produced a single sequence, which could be explained by the fact that they cloned all their PCR products prior to sequencing. Thus, they likely sequenced just a single clone per accession (and missed the evidence of more than one allele per accession), whereas we directly sequenced our amplification products. They also did not produce a *PHYA* sequence for *G. elbertii*. In general, the *PHYA* tree was weakly supported, but the alleles were separated in two groups, one sister to most representatives of group 3 and another embedded within group 4 (sensu Mast et al. 2015) with the *G. huegelii* and *G. heliosperma* clades and *G. robusta* (all Australian).

The phylogenetic results together with the cytogenetic data indicate an allotetraploid origin for the species of New Caledonian *Grevillea*, involving Australian diploid species in clades 3 and 4 (*sensu* Mast et al. 2015). Although it has apparently not been investigated specifically in Proteaceae, plastid genomes in *Grevillea* and related genera are likely to be maternally inherited, as in most angiosperms (Corriveau and Coleman 1988). Since the New Caledonian species have the plastid genome of clade 3 (*sensu* Mast et al. 2015), a species of clade 3 likely was the maternal parent. The fact that *G. exul* and *G. gillivrayi* groups occur in two separate clades in the plastid tree suggests that two different maternal parents were involved, but this seems to be contradicted by the *PHYA* tree in which these two alleles fell in a single clade. However, in *PHYA* variation is lower, and the accession of *G. elbertii* is missing, so this result could be an artefact due to these two factors.

Phylogenetic and cytogenetic results suggest one or two allotetraploid origins of the New Caledonian *Grevillea* species. Their parents were one or two lineages of clade 3 (from Australia and possibly Sulawesi) and one from clade 4 (Australia). New Caledonian *Grevillea* can therefore be added to the list of island endemics with an hybrid origin involving non-native parents (Johnson et al. 2019). Two scenarios are possible. One implies two or three

dispersal events from Australia (or Sulawesi) to New Caledonia, followed by hybridisation/polyploidization and local extinction of the parental diploids, whereas the other scenario would include hybridization/polyploidization in Australia and their dispersal to New Caledonia followed by extinction of the tetraploid progenitors in Australia.

The prime ecosystem for both New Caledonian *Grevillea* clades is maquis on ultramafic substrates. Their crown ages (c. 10.2 and 9.2 My) is older than that of New Caledonian *Dracophyllum* (5.2 Ma, Wagstaff et al. 2010), another group mostly confined to this open ecosystem. Considering their important botanical diversity (Isnard et al. 2016), maquis probably have had a long history before human arrival on the island.

Contrast between the two New Caledonian lineages

The two clades of New Caledonian *Grevillea* species are readily separable morphologically. The four species of the *exul* group all have unilateral inflorescences, white flowers and more or less spherical fruits, whereas the six species of the *gillivrayi* group have cylindrical (to spherical) inflorescences, non-white flowers (generally reddish, but sometimes pale pink, orange or yellow), and laterally flattened fruits. Our study on seed shapes also revealed a common trait between *G. exul* and *G. rubiginosa*, both rounded, in contrasts with the oval form of *G. gillivrayi*, which is like that of *G. meisneri* (1.8 × 0.3 mm) according to Jaffré and Pelletier (1992). Most species are maquis shrubs or less commonly small trees in low forest on ultramafic rocks (peridotites and serpentinites). The species of the *exul* group have a stronger pioneer behavior, being the first ligneous species capable of growing on ancient nickel mines (Jaffré 1977). They all have simple entire leaves, similar to those of *G. elbertii* (Sleumer 1955b), unlike the pinnatifid leaves of *G. robusta*.

All New Caledonian *Grevillea* species are manganese accumulators (Jaffré 1979), with some populations of *G. meisneri* from extreme northern New Caledonia (Massif of Tiébaghi) qualifying as manganese hyperaccumulators (Losfeld et al. 2015, Pillon et al. unpublished). This means that these plants (hyper-)accumulate high quantities of manganese in their leaves (ranging from several thousands to more than 10,000 μg g^{-1}). In both *G. meisneri* and *G. exul s.l.*, manganese was found to be most highly concentrated in their epidermal cells (Fernando et al. 2008; Bihanic et al. 2021), mostly on the lower epidermis in *G. meisneri*,

whereas it is in the upper epidermis in *G. exul*. Contrary to what has been found in several Mn accumulators or hyperaccumulators, including species in some other genera of Proteaceae (Fernando et al. 2006), Mn was not primarily concentrated in the palisade mesophyll in the *Grevillea* species studied so far. In *G. meisneri*, Mn was also found in other tissues, including spongy mesophyll and vascular strands, but in much lower concentration. Overall, localisation of Mn in *G. exul* and *G. meisneri*, representing the two groups of New Caledonian *Grevillea*, appears similar.

Variation with each group

The *exul* and *gillivrayi* groups also differ in their taxonomic, morphological and ecological diversity. The four species of the *exul* group are relatively similar to one another, differing in inflorescence and tepal pubescence (from glabrous in *G. sinuata* to pale pubescent in *G. exul* and *G. macmillanii* and red in *G. rubiginosa*) and inflorescence structure (mostly simple racemes except in *G. rubiginosa* in which they may be ramified and composed of up to five racemes). The leaves of *G. macmillanii* are long, narrow and almost needle-like, whereas those of *G. rubiginosa* are relatively broad and often covered with reddish hairs underneath. All have a similar ecology in relatively disturbed maquis on ultramafic substrates, with only *G. rubiginosa* also at high elevation, up to 1600 m. The four species have largely non-overlapping distributions, and allopatry has likely been the main driver of divergence.

The six species of the *G. gillivrayi* group display greater diversity. *Grevillea meisneri* and *G. vuniana* have pendent almost spherical inflorescences. *Grevillea meisneri* is a specialist of relatively dry maquis and low forest mostly on serpentinites in the north-west of the main island, whereas *G. vuniana* is a rare species known only from low, wet forest at a single location (Yaté) in the south-east. Other species have cylindrical and ± horizontal inflorescences and are found in disturbed to pristine maquis on peridotites. *Grevillea gillivrayi* has a peculiar architecture, with a drastic decrease in branching vigour and intensity along the trunk and inflorescences born on spindly erect axes (Salmon 2019), similar to *G. deplanchei*. The two species have broadly overlaping distributions in the southern half of Grande Terre and the Isle of Pines, but G*. deplanchei* is a riparian species with typical stenophyllous leaves. *Grevillea nepwiensis* is isolated in the north-western region with

inflorescences covered by red pubescence, whereas *G. mondorensis* is known from a single location (Mont Dore) and has glabrous inflorescences borne on older wood, sometimes on trunks (cauliflory). Therefore, in addition to allopatry, factors such as pollination syndromes (inflorescence orientation and position) and ecology (river, substrate), probably triggered diversification of this group. It should also be expected that interspecific hybridization/allopolyploidization and environmental stress (e.g., ultramafic substrates) would activate (retro)transposons (Grandbastien et al. 2005; Parisod et al. 2010), which could also be generating interspecific reproductive barriers in these species of *Grevillea* through genomic structural changes making hybrids less fertile or inviable.

Seed morphology

Seed morphology of *G. exul, G. rubiginosa* and *G. gillivrayi* revealed common traits such as a wing, recognized for several New Caledonian *Geissois* species (Cunoniaceae) as essential for wind dispersal. Olde (1997) classified these species as exhibiting the winged seed type, in which a membranous outgrowth of the testa forms a wing that completely surrounds the seed, and the two others are of the oat-style seed, which lacks the membranous wing but instead has revolute margins and ellipsoidal/hemispherical seeds with a convex outer surface and a flat inner surface. Dispersal by anemochory can provide a real advantage in an open, nutrient-poor environment (Fenner and Thompson 2005). Indeed, in such an environment, the distance of the seeds from the mother plant limits competition for mineral resources. From this point of view, *G. exul* with its larger wing has an advantage, which is reinforced by the non-dormant seed type of our studied species (sensu Baskin and Baskin 2004) Seeds of these species all germinate in less than 30 days, the first appearing roughly 10 days in favorable conditions (L'Huillier et al. 2010). This characteristic also occurs in the southwestern Australian *G. synaphae* (Sweedman and Merritt 2006), a member of group 3 (Mast et al. 2015), and could be associated with winged seed type (Olde 1997), indicating affinities with these three New Caledonian species, and also with *G. meisneri* (personal observation). In contrast, the seeds of *G. sericea* and *G. buxifolia* (both group 4, Mast et al. 2015), defined as oat-style seed (Olde 1997), both have physical dormant seed (Briggs et al. 2005). The fact that New Caledonian species germinate easily is due in a large part to the fact that they have exalbuminate seeds with a large embryo. Several authors have studied

the embryo morphology (Forbis et al. 2002; Baskin and Baskin 2014), and all agree that underdeveloped embryos are primitive and mature embryos advanced because germination does not alone guarantee plantlet establishment. To optimize this, embryo size and reserve type are critical factors. *Grevillea exul* and *G. rubiginosa* are known to possess oleaginous seeds (Bombarda et al. 2010), and lipids produce twice as much energy as carbohydrates for the same mass, which confers to both these species the ability to colonize open areas quickly and efficiently. Moreover, *G. exul* seeds, with an average weight 1.6 times that of *G. rubiginosa* despite being almost the same size, seems to be even better adapted.

Conclusions

The ten species of New Caledonian *Grevillea* may descend from one or two hybridization events involving two or three parental diploid taxa that colonized the island independently but are now extinct. They may represent the remnants of a hybrid swarm that formed 9-13 Mya, when these diploids came into contact in a new, challenging environment. It is possible that allotetraploidy helped these plants adapt and diversify on the ultramafic substrates of New Caledonia via the lineage-specific ohnologue resolution (LORe) model (Robertson et al. 2017), in which the redundant, modular structure of duplicated gene regulatory networks offers polyploid species increased possibilities for evolutionary innovation and adaptation via novel substitutions in duplicated genes. Multiple colonizations and hybridisations may therefore be under-reported processes that could play an important role in the genesis of island radiations. Reticulate evolution will have to be taken into account when considering taxonomic concepts in subtribe Hakeineae.

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Data availability statement

All DNA sequences are available in GenBank. Voucher information, accession numbers, complete trees and and scripts are available in supplementary material online.

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Table 1. Seed characteristics of *Grevillea exul*, *G. rubiginosa* and *G. gillivrayi* (n=20)

	G. exul	*G. rubiginosa*	*G. gillivrayi*
Seed average weight (mg ± s.d.)	**24.5 ± 1.7**	**15.7 ± 1.2**	**16.9 ± 1.9**
Seed average length (mm ± s.d.)	12.9 ± 1.4	11.6 ± 1.1	15.6 ± 1.8
Embryo average length (mm ± s.d.)	5.2 ± 0.6	5.9 ± 0.7	9.4 ± 0.9
Embryo length:seed length (El:Sl) ratio	**40.3%**	**50.9%**	**60.2%**
Seed average width (mm ± s.d.)	9.6 ± 1.3	8.3 ± 0.8	7.0 ± 1.0
Embryo average width (mm ± s.d.)	3.9 ± 0.5	3.6 ± 0.6	4.1 ± 0.5
Embryo width:seed width (Ew:Sw) ratio	**40.6%**	**43.4%**	**58.6%**

Figure 1. Plastid tree. Maximum likelihood tree from analysis of the plastid dataset. To increase the prominence of the New Caledonian taxa, only a fraction of the outgroup taxa is figured and all *Hakea* species are lumped. Clade support is indicated by circles on nodes. New Caledonian *Grevillea* species are highlighted in blue (*exul* group) or red (*gillivrayi* group). On the left, photographs of inflorescences from species belonging to the *exul* group (*G. macmillanii*, *G. rubiginosa* and *G. sinuata*, from the top left and then down) and the *gillivrayi* group (*G. deplanchei* and *G. gillivrayi*, from top to bottom, in that order), respectively, are shown for illustration (all photographs by Y. Pillon).

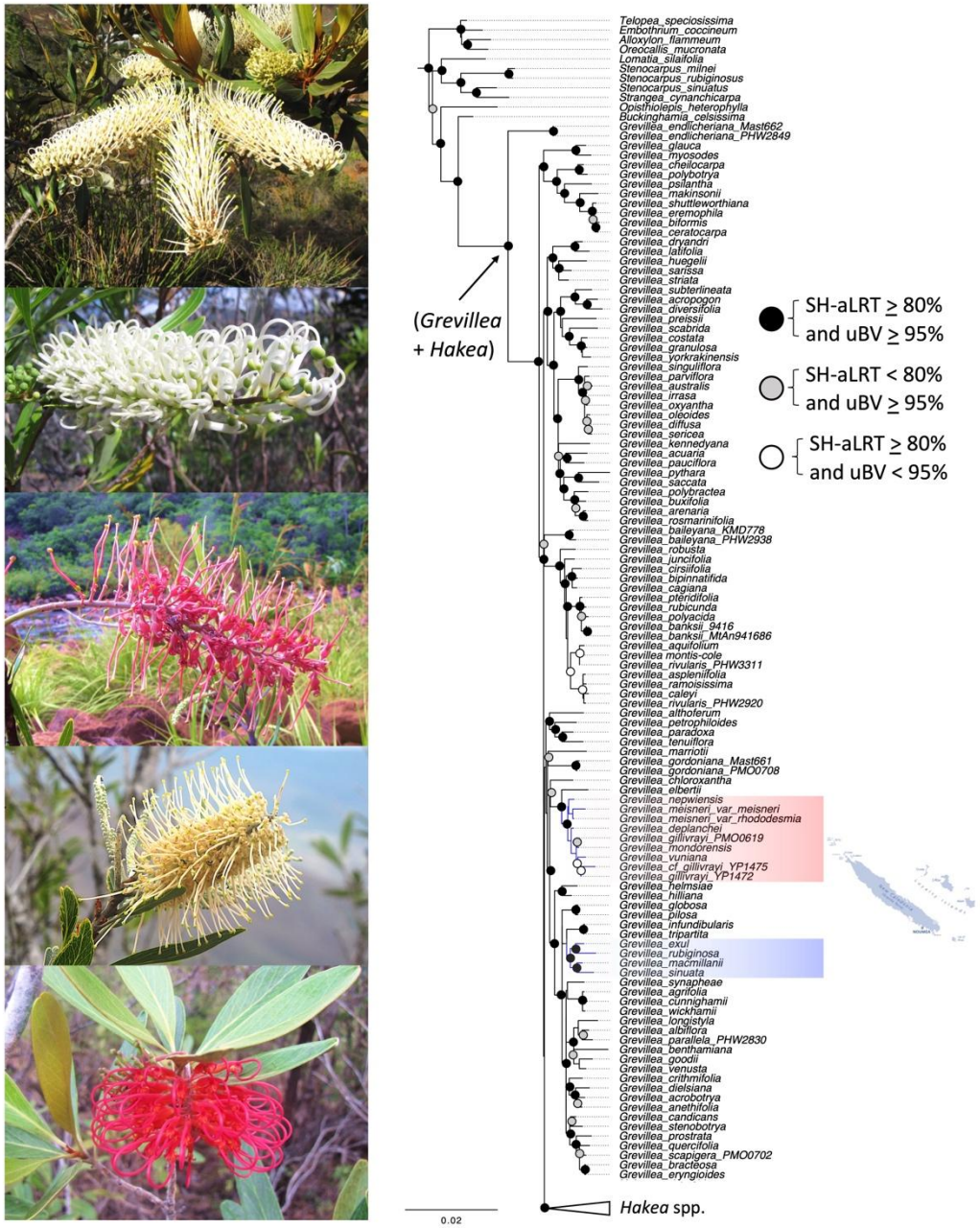


Figure 2. *PHYA* tree. Maximum likelihood tree from analysis of the nuclear gene *PHYA*. To increase the prominence of the New Caledonian taxa, only a fraction of the outgroup taxa is figured and *Hakea* species are lumped. Clade support is indicated by circles on nodes. New Caledonian *Grevillea* species are highlighted in blue (*exul* group) or red (*gillivrayi* group). On the left, photographs of whole specimens of species belonging to the *exul* group (*G. macmillanii*, *G. rubiginosa* and *G. sinuata*, from the top left and then down) and the *gillivrayi* group (*G. deplanchei* and *G. gillivrayi*, from top to bottom, in that order), respectively, are shown (all photographs by Y. Pillon).

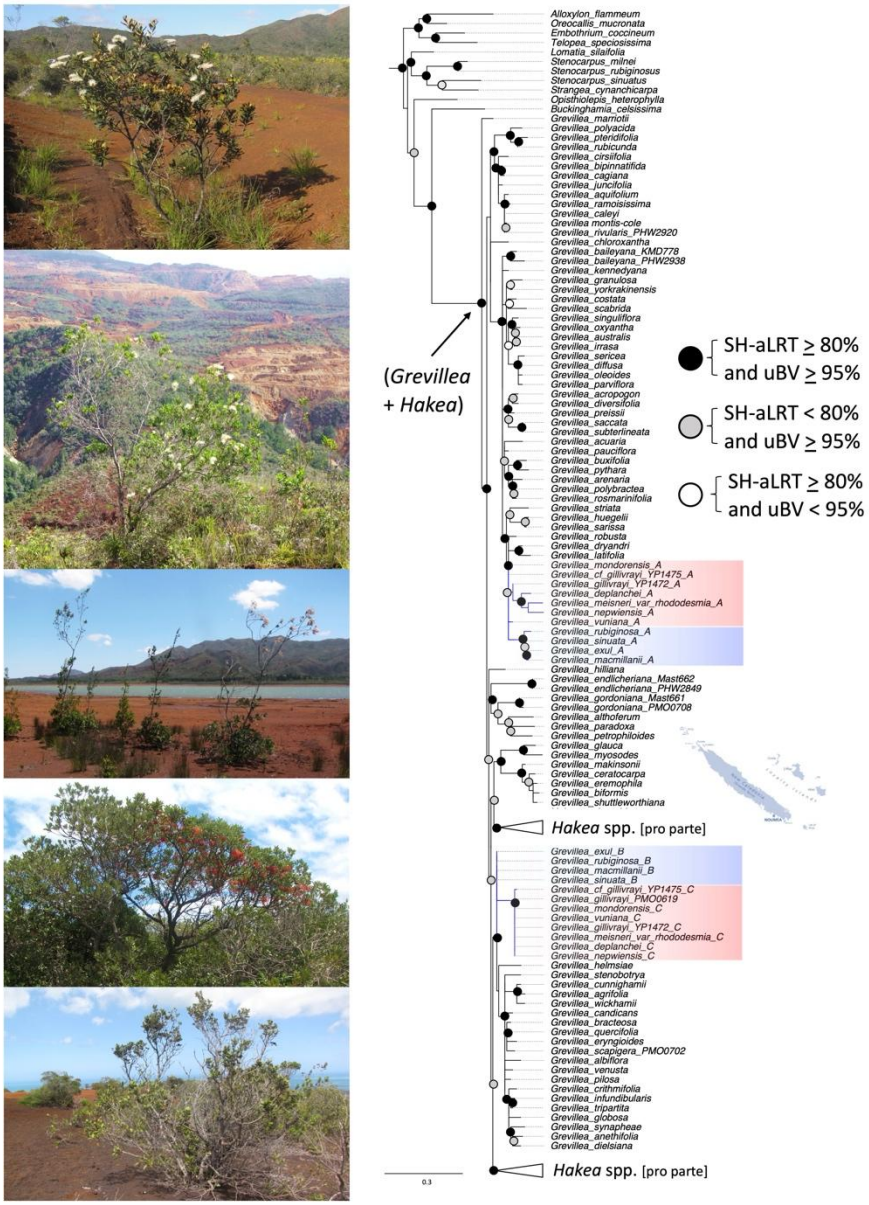


Figure 3. Dated tree. Dated phylogenetic tree resulting from the BEAST analyses of the plastid dataset. To increase the prominence of the New Caledonian taxa, outgroup taxa are not figured and *Hakea* species are lumped. Horizontal bars on nodes represent 95% HPD of age estimates. New Caledonian *Grevillea* species are highlighted in blue (*exul* group) or red (*gillivrayi* group), with arrow pointing to the crown nodes. Time-scale (horizontal) in Mya (Plio.: Pliocene, Plei.: Pleistocene). At the bottom, photographs displaying fruits from species belonging to the *exul* group (*G. rubiginosa*, first picture on the left) and the *gillivrayi* group (*G. deplanchei* and *G. nepwiensis*, from left to right), respectively, are shown (all photographs by Y. Pillon).

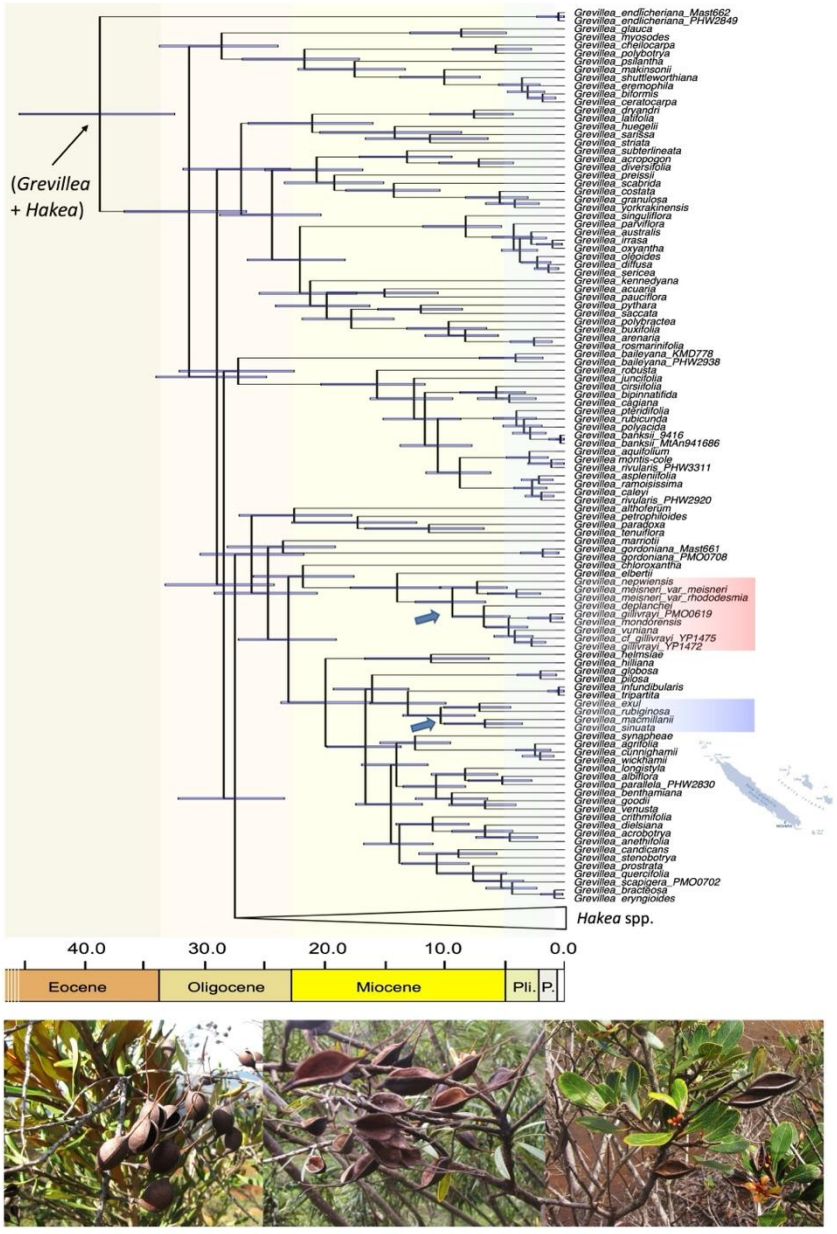
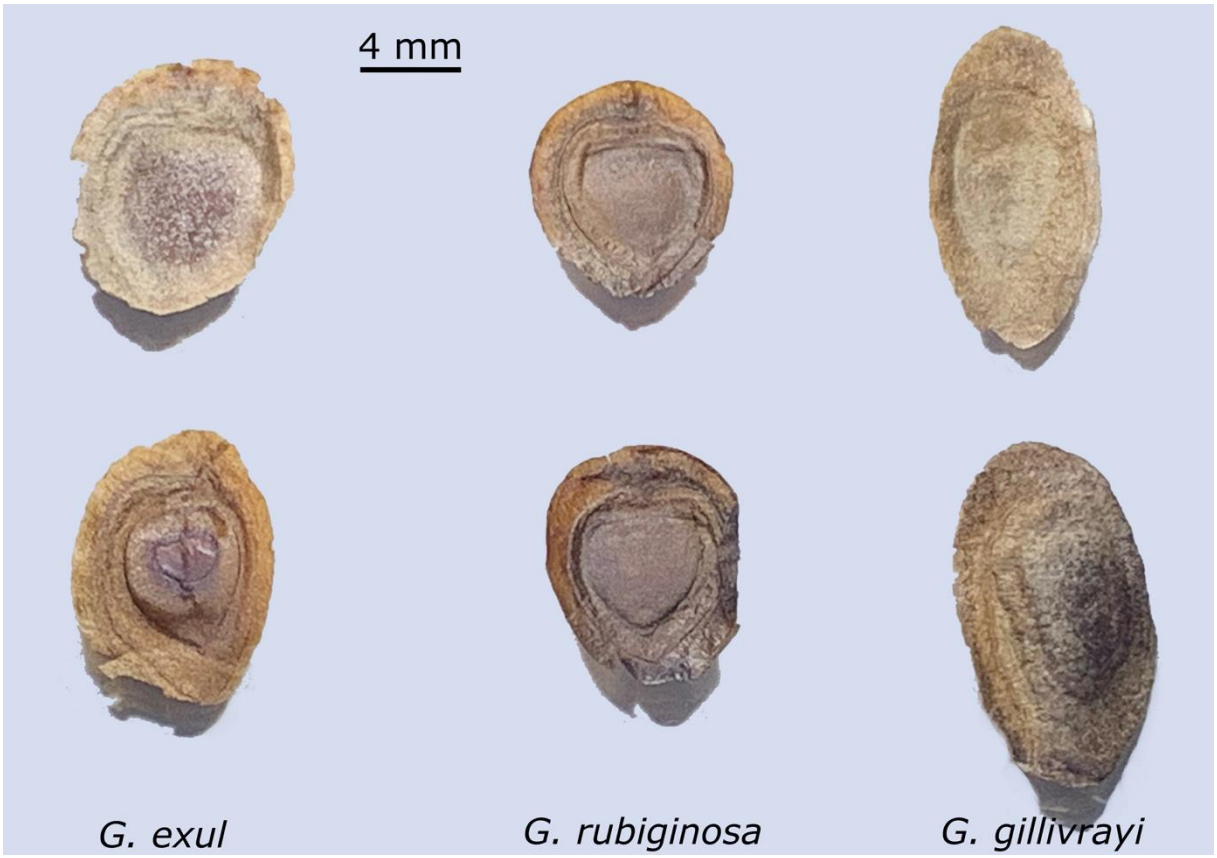


Figure 4. Seeds of *Grevillea exul*, *G. rubiginosa* and *G. gillivrayi*.



Supplementary material.

Figure S1. Plastid tree. Maximum Likelihood tree (with all analyzed taxa) resulting from the analysis of the plastid dataset. To increase the visibility of the tree, a cladogram representation is used. Clade support is provided on nodes with SH-aLRT values on the left and uBV values on the right. New Caledonian *Grevillea* species are also highlighted using blue branches.

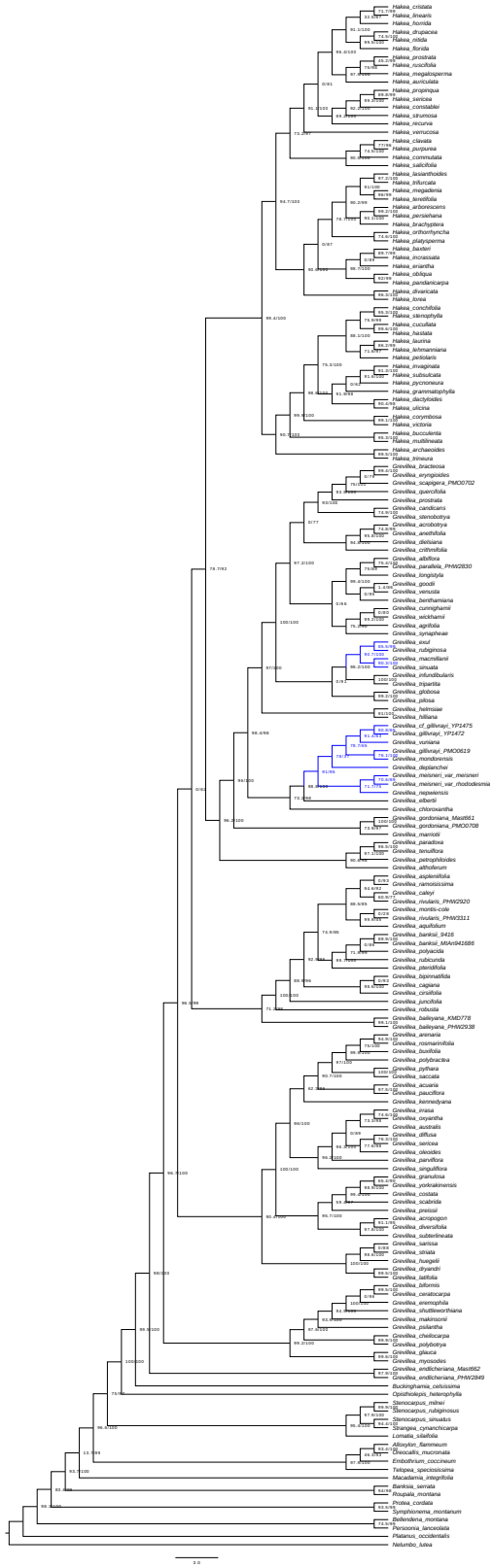


Figure S2. *PHYA* tree. Maximum Likelihood tree (with all analyzed taxa) resulting from the analysis of the nuclear gene *PHYA*. To increase the visibility of the tree, a cladogram representation is used. Clade support is provided on nodes with SH-aLRT values on the left and uBV values on the right. New Caledonian *Grevillea* species are also highlighted using blue branches.

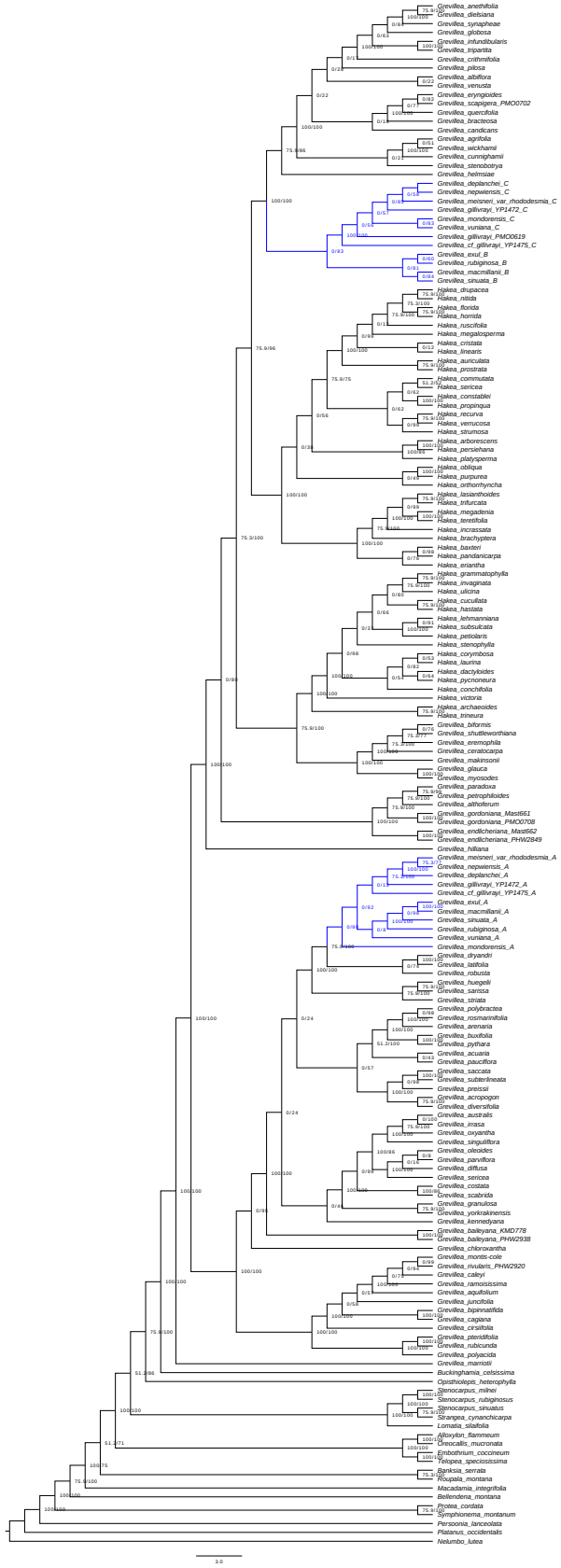


Figure S3. Dated tree. Dated phylogeny (with all analyzed taxa) resulting from the BEAST analyses of the plastid dataset. Median ages are provided on nodes. Horizontal bars on nodes represent 95% HPD of age estimates. New Caledonian *Grevillea* species are also highlighted using blue branches. Two stars indicate the nodes that are constrained with fossil calibrations.

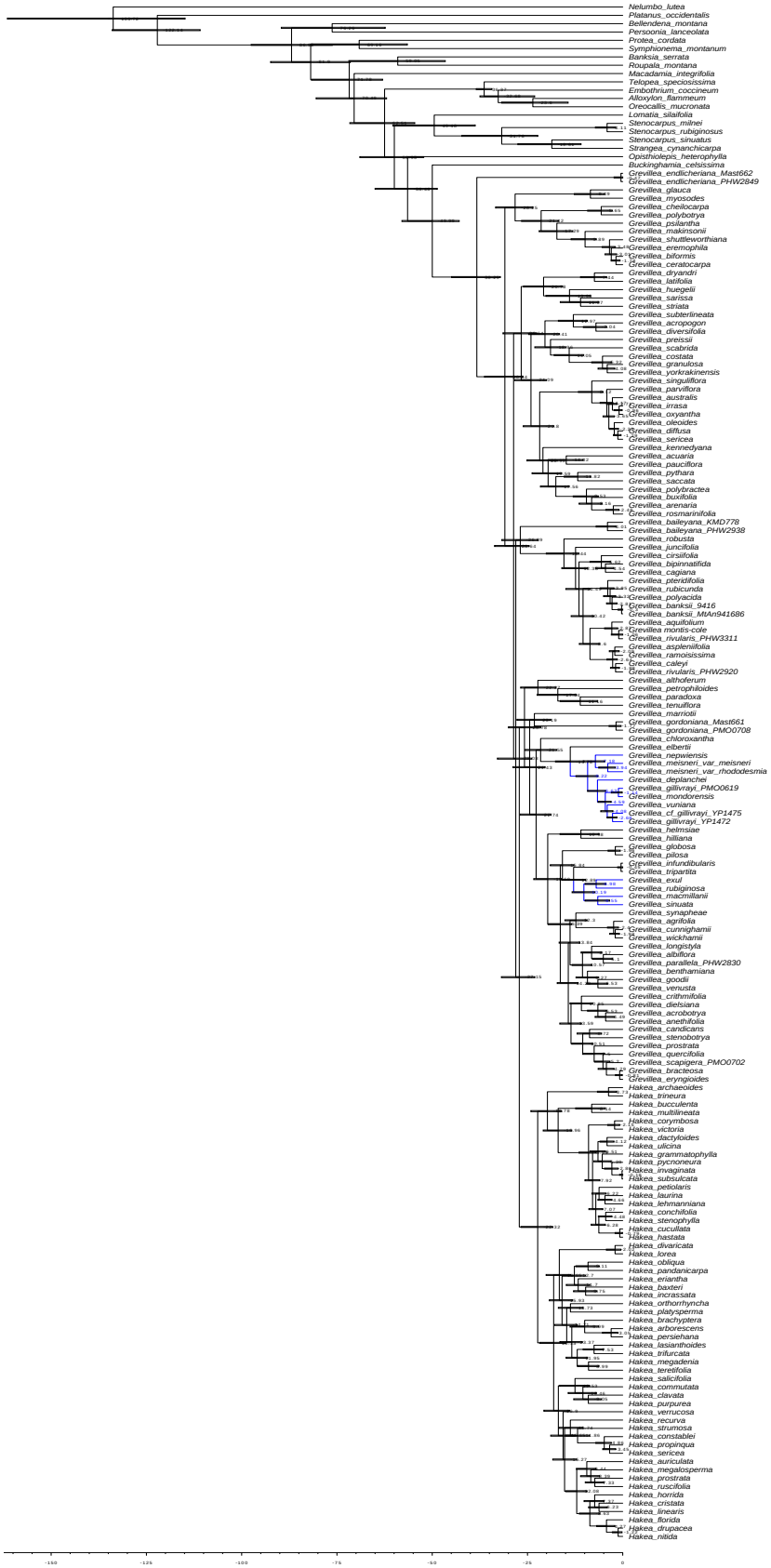


Table S1. List of DNA sequences produced for this study with voucher information and GenBank accession numbers.

species	specimen_voucher	GenBank accession number				
		atpB	*mat K*	*ndhF*	*PHYA*	*rpl16*
Grevillea cf *gillivrayi* YP1475	Pillon & Bruy 1475	OP589202	OP589215	OP619967	OP619975&OP619976	OP675548
Grevillea deplanchei	Pilllon, Desnues & Isnard 1471	OP589203	OP589216	OP619963	OP619977&OP619978	OP675549
Grevillea exul	Pillon & Nigote 1477	OP589204	OP589217	OP619968	OP619979&OP619980	OP675550
Grevillea gillivrayi YP1472	Pillon 1472	OP589205	OP589218	OP619964	OP619981&OP619982	OP675551
Grevillea macmillanii	Pillon 1486	OP589206	OP589219	OP619969	OP619983&OP619984	OP675552
Grevillea meisneri var meisneri	Pillon 1469	OP589207	OP589220	OP619970	-	OP675553
Grevillea meisneri var rhododesmia	Pillon & Poullain 1463	OP589208	OP589221	OP619971	OP619985&OP619986	OP675554
Grevillea mondorensis	Pillon 1460	OP589209	-	OP619965	OP619987&OP619988	OP675555
Grevillea nepwiensis	Pillon, Poullain & Fleurot 1464	OP589210	OP589222	OP619966	OP619989&OP619990	OP675556
Grevillea rubiginosa	Pillon & Isnard 1481	OP589211	OP589223	OP619962	OP619991&OP619992	OP675557
Grevillea sinuata	Pillon & Bruy 1474	-	-	-	OP619993&OP619994	OP675558
Grevillea vuniana	Pillon & al 1511	OP589212	OP589224	OP619972	OP619995&OP619996	OP675559
Stenocarpus milnei	Pillon 1488	OP589213	OP589225	OP619973	OP619997	OP675560
Stenocarpus rubiginosus	Pillon 1491	OP589214	OP589226	OP619974	OP619998	OP675561