



Molecular phylogenetics of New Caledonian *Diospyros* (Ebenaceae) using plastid and nuclear markers [☆]

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ABSTRACT

To clarify phylogenetic relationships among New Caledonian species of *Diospyros*, sequences of four plastid markers (*atpB*, *rbcL*, *trnK-matK* and *trnS-trnG*) and two low-copy nuclear markers (*ncpGS* and *PHYA*) were analysed. New Caledonian *Diospyros* species fall into three clades, two of which have only a few members (1 or 5 species); the third has 21 closely related species for which relationships among species have been mostly unresolved in a previous study. Although species of the third group (NC clade III) are morphologically distinct and largely occupy different habitats, they exhibit little molecular variability. *Diospyros vieillardii* is sister to the rest of the NC clade III, followed by *D. umbrosa* and *D. flavocarpa*, which are sister to the rest of this clade. Species from coastal habitats of western Grande Terre (*D. cherrieri* and *D. veillonii*) and some found on coralline substrates (*D. calciphila* and *D. inexplorata*) form two well-supported subgroups. The species of NC clade III have significantly larger genomes than found in diploid species of *Diospyros* from other parts of the world, but they all appear to be diploids. By applying a molecular clock, we infer that the ancestor of the NC clade III arrived in New Caledonia around 9 million years ago. The oldest species are around 7 million years old and the youngest ones probably much less than 1 million years.

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1. Introduction

New Caledonia is an island group located in the southwestern Pacific about 1300 km east of Australia, ranging from around 19° to 23° south with an land area of ca. 19,000 km². It consists of the main island Grande Terre (ca. 16,000 km²), Iles Belep (in the north), Ile des Pins (in the south), Loyalty Islands (in the east) and several other smaller islands. The continental part of New Caledonia (mainly Grande Terre) separated from Gondwanan during late Cretaceous (ca. 80 million years ago, mya; McLoughlin, 2001). During the Palaeocene to late Eocene, this continental sliver was submerged for at least 20 million years (myr), and a thick layer of oceanic mantle accumulated (Pelletier, 2006). After Grande Terre re-emerged in the late Eocene (37 mya), this heavy-metal rich oceanic material covered most of the land. Today, around 1/3 of the

main island is still covered with ultramafic substrates. Because Grande Terre was totally submerged, it is highly unlikely that lineages that were already present in this area before the split from Gondwanan could have survived locally. Current hypotheses suggest that biota present today are derived from elements/ancestors that reached New Caledonia via long distance dispersal (e.g. Morat et al., 2012; Pillon, 2012; Grandcolas et al., 2008) mainly from Australia, New Guinea and Malaysia. Hypotheses of other islands between Australia and New Caledonia having served as stepping stones or refuges for Gondwanan taxa now endemic (e.g. *Amborella*) have been proposed by a few authors (Ladiges and Cantrill, 2007), but there is no consensus of when they existed or how large and numerous they might have been. The New Caledonian climate is tropical to subtropical. The main island is split by a mountain range into a humid eastern portion (2000–4000 mm precipitation per year) and a dry western part (1000 mm precipitation per year) with prevailing winds and rain coming from the south east. New Caledonia is one of the 34 biodiversity hotspots (Mittermeier et al., 2004; Myers et al., 2000), and nearly 75% of the native flora is endemic (Morat et al., 2012), which is the fourth highest for an island (Lowry, 1998). Among these endemic taxa are 98 genera and three families, Amborellaceae, Oncothecaceae and Phellinaceae (Morat et al.,

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2012). One of the reasons hypothesised for the high level of endemism found in New Caledonia is the ultramafic substrates, which have acted as a filter for colonising species that were already pre-adapted to this special soil (Pillon et al., 2010).

Ebenaceae are pantropical and belong to the order Ericales (APG, 2009); the majority of species occur in Africa (incl. Madagascar) and the Indo-Pacific region. Duangjai et al. (2006) divided Ebenaceae into two sub families, Lissocarpoideae and Ebenoideae. Lissocarpoideae are monogeneric (*Lissocarpa*, 8 species in northwestern South America), and Ebenoideae include *Diospyros*, *Euclaea* (18 species in Africa) and *Royena* (17 species in Africa). This classification of Ebenaceae in two subfamilies and four genera has been also supported by palynological data (Geeraerts et al., 2009).

In this paper, we use the circumscription of *Diospyros* as proposed by Duangjai et al. (2006). *Diospyros* is the largest genus of Ebenaceae with more than 500 species, making it also one of the largest angiosperm genera. The greatest species of diversity is in Asia and the Pacific region (~300 species). Fruits of some species (persimmons; e.g. *D. kaki*, *D. lotus* and *D. virginiana*) are edible, and ebony wood (e.g. *D. ebenum*) is one of the most expensive timbers. Species of *Diospyros* are shrubs or trees that occur in most tropical and subtropical habitats, where they are often important and characteristic elements. Duangjai et al. (2009) found 11 mostly well-resolved clades within *Diospyros*. In New Caledonia, there are 31 described *Diospyros* species, of which all but one are endemic, and they belong to three clades (Duangjai et al., 2009; Fig. 4, clades II, III and XI). The first clade (clade II) contains five species from New Caledonia that are related to Australian species of *Diospyros*. The second clade (clade III) includes species from Hawai'i, Indian Ocean islands and 24 taxa from New Caledonia, within which the species from New Caledonia form a subclade, here termed NC clade III. Although Duangjai et al. (2009) analysed more than 8000 base pairs of plastid DNA, low variability and little resolution was found among these endemic New Caledonian species. The third clade (clade XI), consisting of taxa from Asia, America, Pacific Islands and New Caledonia, includes two *Diospyros* species from New Caledonia, one endemic and the other found throughout the southern Pacific. These two species are not sister species, accounting for two more colonisations of New Caledonia (i.e. four in total). Similar, multiple colonisation events are also found among other organisms in New Caledonia (e.g. Murienne et al., 2005). *Diospyros* is observed in all types of New Caledonian vegetation except mangrove; the species range from sea level up to ca. 1250 m (New Caledonia's highest point is 1628 m). There are several micro-endemics restricted to just a small area (White, 1992). Most New Caledonian *Diospyros* species from clade III are morphologically clearly defined and restricted by edaphic factors and occur on just one substrate type. For example, *D. labillardierei* (Fig. 1D) is distinctive with its long narrow leaves and *Salix*-like habit; it is a rheophyte on non-ultramafic substrates. *Diospyros veillonii* (Fig. 1F) is a remarkable species with coralloid inflorescence axes (unique among New Caledonian *Diospyros*) and large leaves, but is known from only a single locality in dry forest on black clay soil. Other species have broader distributions and ecologies, such as *D. parviflora* (Fig. 1J), which grows on both ultramafic and non-ultramafic substrates and is widespread throughout Grande Terre and Balabio Island in dense humid forests as well as in more open and dry vegetation. Some species can have similar ecological requirements, but are morphologically well differentiated; for example *D. vieillardii* (Fig. 1A) has a calyx narrower than its prune-like fruit, whereas *D. glans* (Fig. 1N) has a thick calyx much wider than its fruit, but both grow in maquis vegetation and co-occur at some sites.

For establishing phylogenetic relationships, sequences of low-copy nuclear genes are not as often used as regions from the plastid genome, often due to methodological difficulties. Low-copy genes are present in one or few copies in the genome, and primers are of-

ten highly specific for individual groups, requiring them therefore to be newly designed for each study. On the other hand, low-copy nuclear markers are normally highly informative and as they are biparentally inherited they may also help detect recent hybridization (e.g. Moody and Rieseberg, 2012). However, in a study of Hawaiian endemics in two unrelated genera, Pillon et al. (2013) found that although two low-copy nuclear loci displayed a high level of variability, they also exhibited heterozygosity, intraspecific variation, and retention of ancient alleles; allele coalescence was older than the species under study. Nonetheless, we hoped that inclusion of low-copy nuclear genes might provide additional insight into species relationships and thus included two such loci. Phytochrome A (*PHYA*) belongs to the gene family of the phytochromes, which has eight members across the seed plants (*PHYA*–*PHYE* in angiosperms and *PHYN*–*PHYP* in gymnosperms); *PHYN*/*PHYA*, *PHYO*/*PHYC* and *PHYP*/*PHYBDE* are orthologs, the rest being paralogs of the others (Mathews et al., 2010). Genes of this family encode photoreceptor proteins that mediate developmental responses to red and far red light. The three main paralogs (*PHYA*, *PHYB* and *PHYC*) are different enough to be amplified with specific primers (Zimmer and Wen, 2012). Sequences of *phy* genes have been used successfully across the flowering plants (e.g. Mathews et al., 2010; Nie et al., 2008; Bennett and Mathews, 2006) for phylogenetic reconstruction. The gene *PHYA* used in this study consists of four exons and three introns. Glutamine synthetase (*GS*), codes for a protein involved in nitrogen assimilation. There are two main types of *GS* genes, cytosolic- and chloroplast-expressed. Chloroplast-expressed glutamine synthetase (*npcGS*) consists of 12 exons and 11 introns and has been shown to be a single-copy gene in plants (Emshwiller and Doyle, 1999). This combination of coding and non-coding regions has been shown to be highly informative for inferring phylogenetic trees of various groups (e.g. Oxalidaceae, Emshwiller and Doyle, 1999; *Passiflora*, Yockteng and Nadot, 2004; *Spiraeanthemum*, Pillon et al., 2009a; *Codia*, Pillon et al., 2009b; *Achillea millefolium*, Guo et al., 2012).

Beside phylogenetic relationships, the age of clades is of interest. In many cases, there are no fossils available for direct dating of a group of interest in a particular region, which is often the case for islands and is certainly true for New Caledonia (the few fossils recorded to date are older than the last emergence of the island and are not certain to be angiosperms; Salard and Avias, 1968). Rates of DNA divergence are generally consistent with a molecular clock (Zuckermandl and Pauling, 1965), and therefore DNA data contain information about the relative ages of taxa. When substitution rates (e.g. Silvestro et al., 2011; Alba et al., 2000) or fossils belonging to defined clades (e.g. Pirie and Doyle, 2012; Magallón, 2010) are taken into consideration, the relative ages obtained can be transformed into absolute ages. Placement of fossils in the correct position in the phylogenetic tree is crucial for accurate interpretation (Forest, 2009). Some previous studies have been published on the subject of the age of asterids (e.g. Millán-Martínez, 2010; Bell et al., 2010; Bremer et al., 2004) to which Ericales belong, and fossil *Diospyros* are known from some localities (mainly in India and North America), but none has been found in New Caledonia. *Austrodiospyros cryptostoma* (Basinger and Christophel, 1985), a fossil from Australia has many morphological similarities to *D. australis* of clade II (Duangjai et al., 2009). It is thus far the only fossil belonging to a clade that includes *Diospyros* species from New Caledonia. We treat *A. cryptostoma* as member of clade II in this study.

Genome sizes vary nearly 2400-fold across angiosperms (Pellicer et al., 2010). Most variation in DNA amount is caused by different amounts of non-coding, repetitive DNA, such as pseudogenes, retrotransposons, transposons and satellite repeats (Leitch, 2007; Bennett and Leitch, 2005; Parisod et al., 2009; Petrov, 2001). Genome sizes and chromosome numbers of *Diospyros* are within the range of those of other members of Ericales (Bennett and

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