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ORIGINAL ARTICLE

Molecular and fossil evidence disentangle the biogeographical history of *Podocarpus*, a key genus in plant geography

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ABSTRACT

Aim The genus *Podocarpus* (Podocarpaceae) provides an opportunity to contrast biogeographical hypotheses within and among continents, and to analyse divergence between disjunct tropical and temperate forests of South America. We developed a calibrated phylogeny of *Podocarpus* to reconstruct the ancestral areas and potential expansion routes within Podocarpaceae.

Location *Podocarpus* consists of two extant subgenera: *Foliolatus* from Asia and Oceania, and *Podocarpus* located in Gondwanan continents and north to the Caribbean. The paper focuses mainly on the area occupied by the latter subgenus.

Methods We combined previously published and novel DNA sequences with fossil records. New species sequenced are members of *Podocarpus* subgenus *Podocarpus* from South and Central America. We assembled DNA sequences of the chloroplast (*mat*K and *rbc*L) and nuclear (ITS1 and ITS2) to analyse phylogenetic relationships within *Podocarpus* subgenus *Podocarpus* by Bayesian methods, which were calibrated using macrofossils that could be confidently identified as modern genera. Ancestral areas were inferred using the dispersal–extinction–cladogenesis model.

Results The phylogenetic reconstruction inferred a minimum age for the origin of *Podocarpus* s.l. in the late Cretaceous–early Palaeogene (63 Ma) and strongly supported monophyly of the genus *Podocarpus* and of subgenera *Podocarpus* and *Foliolatus*. Subgenus *Podocarpus* consists of two monophyletic, latitudinally structured clades. One clade consists of temperate American species while the other includes species from tropical-subtropical Africa and South America.

Main conclusions The history of the subgenera within *Podocarpus* is older than previously reported: they can be traced back to late Cretaceous–early Palaeocene biogeographical connections between Australasia and South America through Antarctica. Latitudinally disjunct lineages within South America most probably diverged from widespread ancestors as a result of a persistent arid barrier that was established prior to the late Palaeogene. The calibrated age for the Tropical–Subtropical clade suggests an Atlantic–subtropical biogeographical corridor between South America and Africa long after the breakup of Gondwana and the stabilization of the circum-Antarctic current.

Keywords

Bayesian inference, Caribbean, Gondwana, historical biogeography, molecular dating, phylogeography, Podocarpaceae, *Podocarpus*, South America, vicariance

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INTRODUCTION

Taxa within families of southern origin, such as Podocarpaceae (podocarps) and Nothofagaceae (southern beech), are considered key sources of information in plant geography (Couper, 1960; van Steenis, 1972). Couper (1960: p. 492) stated: 'A study of the past distribution of Podocarpaceae and *Nothofagus* should then provide fundamental data for phytogeographical and palaeogeographical theories' and '. . .specific relationship (in the strict sense) is not inferred between fossil and recent plants, but simply that in our present state of knowledge the fossil forms are the most likely ancestral forms of the recent species'.

The biogeographical relevance of Podocarpaceae relies on the fact that it is the second largest conifer family (Farjon, 2010). In contrast to taxa within Nothofagaceae that are restricted to temperate areas, widespread genera within Podocarpaceae also reach subtropical and tropical areas. This is the case for *Podocarpus*, the most species-rich (*c.* 100 species) and widely distributed genus, which is found in Southeast Asia and almost all continents of the Southern Hemisphere that originated from Gondwana. Phylogenetic and phylogeographical studies of such a wide-ranging genus may prove valuable in addressing as yet largely unresolved biogeographical questions of long-distance dispersal and –vicariance within and among the southern continents.

The family Podocarpaceae was very diverse and broadly distributed during the Mesozoic and Cenozoic. However, few fossil records can be assigned to modern genera. The earliest accepted podocarp macrofossils are from the Triassic of Gondwana (Townrow, 1967; Morley, 2011), yet recent molecular dating studies and fossil data suggest a Jurassic origin of Podocarpaceae (Biffin *et al.*, 2011; Leslie *et al.*, 2012; Rothwell *et al.*, 2012; Escapa *et al.*, 2013). In addition, the divergence of the two *Podocarpus* subgenera from their common ancestor has been suggested to have occurred in the Palaeogene (Biffin *et al.*, 2011; Leslie *et al.*, 2012). A fair correspondence was shown between pollen types and major genera within Podocarpaceae (Morley, 2011), whereas those pieces of evidence were restricted to Old World Neogene podocarps and thus did not include all genera. Also, the lack of diagnostic features of *Podocarpus* pollen precludes identification of distinct species or subgenera (Pocknall, 1981; Hooghiemstra *et al.*, 2006; Morley, 2011). Furthermore, in the absence of specific synapomorphic characters, reproductive structures or whole-plant reconstructions, the phylogenetic placement of fossil conifers stay unresolved (e.g. Hill & Brodribb, 1999; Wilf *et al.*, 2009; Escapa *et al.*, 2010, 2013; Wilf, 2012; Escapa & Catalano, 2013).

Besides preservation biases and the lack of organic connection that fossils usually have, another problem that arises in fossil calibration relates to the precise chronology of the data source (Wilf & Escapa, 2015). Most historical fossil records are poorly time constrained, and the range of dates can be so wide that a defined age remains uncertain. Such records are therefore not useful for phylogenetic node calibration (Sauquet *et al.*, 2012). Therefore, hypotheses about the historical biogeography of *Podocarpus* based only on fossils continue to be extremely uncertain.

A morphological description of reproductive and vegetative organs of extant and fossil Podocarpaceae was given by Florin (1940a). The most recent complete revision of *Podocarpus* is found in a long series of papers by Buchholz and Gray, the most pertinent to our work being Buchholz & Gray (1948) and Gray (1953, 1955, 1956, 1958). Later, de Laubenfels (1985) revised the genus, listing 95 species divided between two subgenera *Foliolatus* and *Podocarpus* that were based on characters of the female cone, foliar anatomy and cuticle; 18 sections were also recognized (nine per subgenus). A new revision of *Podocarpus* is in progress (Mill, 2014, 2015a,b) but meantime de Laubenfels (1985) and Farjon (2010) are the standard references. Earlier phylogenetic analyses of the Podocarpaceae (Chaw *et al.*, 1995; Kelch, 1997, 1998; Conran *et al.*, 2000) did not include all genera until Sinclair *et al.* (2002). In addition, more recent phylogeographical studies have shown that chloroplast regions, previously used in phylogenetic studies of Podocarpaceae, are polymorphic within species, such as *trn*L–*trn*F for *Podocarpus matudae* (Ornelas *et al.*, 2010) and *P. parlatorei* (Quiroga *et al.*, 2012). Omission of such intraspecific variation may bias phylogenetic reconstructions if not considered. While subgenera *Podocarpus* and *Foliolatus* have been confirmed as monophyletic by molecular analyses (Conran *et al.*, 2000; Knopf *et al.*, 2012; Leslie *et al.*, 2012), most of the sections recognized by de Laubenfels (1985) have not been supported by any phylogenetic reconstructions (Stark, 2004; Knopf *et al.*, 2012; Leslie *et al.*, 2012). Therefore, internal relationships of clades within subgenera remain to be explained.

A detailed analysis within subgenus *Podocarpus* may help to elucidate relevant biogeographical questions such as transcontinental disjunctions between temperate South America and Australia–New Zealand, as well as tropical Africa and South America-Central America. Phylogenetic reconstructions may elucidate alternative hypotheses that *Podocarpus* s.l. originated in the Palaeogene (Biffin *et al.*, 2011; Leslie *et al.*, 2012) and diversified to reach its present wide distribution as a consequence of long-distance dispersal, or that it consists of ancient (i.e. Cretaceous) widespread lineages that evolved within Gondwanan continents by vicariance (Warren & Hawkins, 2006). While the latter hypothesis, of a Gondwanan relict distribution, was used by Verboom *et al.* (2014) to explain the presence of Australasian lineages in the Greater Cape flora of South Africa, they said that it was much more difficult to explain the South American connections with that flora because of uncertainties over identifying sister lineages as well as the paucity of divergence time estimates. In addition, no biogeographical inferences have yet been made concerning the diversity of *Podocarpus* in the Neotropics, where the genus is the principal conifer element of montane forests. It has been hypothesized that Andean uplift stimulated the entrance of Austral–Antarctic elements (such as *Podocarpus*) into the temperate forests of the

Neotropics, and that new Neotropical taxa, adapted to the montane conditions caused by the Andean upheaval, were in turn derived from these (Van der Hammen & Hooghiemstra, 2000). We here present a fossil-calibrated molecular dating analysis, including new DNA sequences and macrofossil data of South American *Podocarpus* species that were not included in previous studies, in order to: (1) elucidate phylogenetic relationships among South American *Podocarpus* species using nuclear internal transcribed spacers 1 and 2 (ITS1 and ITS2) and conserved chloroplast DNA regions (*rbc*L and *mat*K); (2) estimate the divergence times of clades within subgenus *Podocarpus*, in order to detect whether naturally disjunct Neotropical species are the product of recent diversification from ancestors of austral origin or are relicts from widespread ancient lineages; and (3) reconstruct ancestral areas of *Podocarpus* lineages.

MATERIALS AND METHODS

Podocarpus species and their geographical distribution

Podocarpus is one of the largest extant conifer genera (Mill, 2014). We assembled a data set comprising the molecular sequences of species representing a near-complete sample of the world-wide distribution of the genus. To avoid possible synonymy, species names follow the Missouri Botanical Garden database (http://www.tropicos.org) and The Plant List (http://www.theplantlist.org). We used the Global Biodiversity Information Facility (http://www.gbif.org) as the most complete list of *Podocarpus* species and distribution range of *Podocarpus* (see Appendix S1 in Supporting Information).

Molecular data

Molecular data consisted of 108 sequences downloaded from GenBank and novel sequences of 15 American species (see Appendix S1 for accession numbers). Two of these 15 species (*P. glomeratus* and *P. ingensis*) have never been previously sequenced for any gene, while we provide sequences for additional genes for five species (*P. oleifolius, P. parlatorei, P. purdieanus, P. sellowii* and *P. trinitensis*) that were sequenced for other genes by Knopf *et al.* (2012) and/or Leslie *et al.* (2012). The remaining seven species (*P. angustifolius, P. coriaceus, P. hispaniolensis, P. lambertii, P. matudae, P. nubigenus*, *P. salignus*) were also studied by Biffin *et al.* (2011) and/or Leslie *et al.* (2012) but our sequences are newly generated. The polymerase chain reaction conditions for these fifteen species are described in Stark (2004) and Quiroga *et al.* (2012). In an effort to avoid missing taxa we requested samples from various herbaria; however, the resulting DNA products were too degraded for further analyses. The complete data set contained 71% of the total number of *Podocarpus* species. For the ingroup, we assembled four data sets comprising DNA sequences of *Podocarpus* species for each of two chloroplast [54 and 73 operational taxonomic units (OTUs) for *mat*K and *rbc*L respectively] and two nuclear (36 and 42 OTUs for ITS1 and ITS2 respectively) regions separately. These data sets were also combined as follows: for each type of marker separately consisting of 78 and 53 OTUs for chloroplast and nuclear regions, respectively, and all markers combined (49 OTUs) (see Table S2 in Appendix S2). The amount of missing data for the four-marker combined data set was: *mat*K = 9%, *rbc*L = 9%, ITS1 = 40% and ITS2 = 19%. The outgroup consisted of at least one species of each of the other 18 genera of Podocarpaceae, plus *Araucaria araucana* (Araucariaceae). We used Bayesian inference to estimate phylogenetic relationships for each marker and for the combined data set using MR BAYES 3.1.2 (Ronquist & Hulsenbeck, 2003). The parameters used to run the analyses are described in Appendix S2. We found that the variation in the numbers of missing data among markers did not affect branch length, and the topology of the phylogenies was independent of the numbers of taxa or DNA regions used (see Appendix S3).

Fossil calibration and molecular dating

The phylogeny of *Podocarpus* was calibrated based on well-known, precisely dated macrofossils that could be confidently assigned to modern genera (Table 1, Fig. 1). Macrofossils were used according to their earliest appearance in the fossil record; this does not mean that the age of the fossil corresponds to a point of divergence but rather indicates the confirmed presence of a taxon at a given point in geological time.

We revised recent literature on fossil podocarps (Hill & Brodribb, 1999; Wilf, 2012) and used several new fossil age data, which come from isotope geochronological dating or that can be well correlated with a specific geological time period (Table 1, Fig. 1). Fossil calibrations at generic nodes (Table 1) are described in Appendix S2.

Few fossil records can be certainly assigned to *Podocarpus*, the great majority of them restricted to the Palaeogene (66–23 Ma). To date, the oldest reliable fossil corresponds to *P. andiniformis* from the Río Pichiléufu flora of Patagonia (USNM40384: Fig. 1e–g; Berry, 1938; Florin, 1940a). As noted in Table 1, recent radiomagnetic dates give a well-constrained age of 47.74 ± 0.18 Ma for the Río Pichiléufu fossiliferous strata (Wilf *et al.*, 2005; Wilf, 2012). *Podocarpus andiniformis* was later found in the Laguna del Hunco flora (Wilf *et al.*, 2005; Wilf, 2012), which is slightly older with a well-constrained age of 52.22 ± 0.29 Ma (early Eocene; Wilf, 2012). This pushes back the oldest reliably dated fossil evidence for *Podocarpus* by 5 Myr.

Species divergence times were estimated using the combined data set by two different Bayesian approaches as implemented in BEAST2 1.8.0 (Bouckaert *et al.*, 2014). The settings to run BEAST2 and the calibration nodes used are described in Appendix S2.

Table 1 Fossil calibration points used for divergence time estimation in Ma. Node numbers correspond to those in Fig. 2.

Node	Fossil species	Epoch	Minimum age (Ma)	Localization
Root Araucariales*	*Araucaria* spp.	Late Triassic	214 ± 10*	Antarctica[1,2] Australia[3]
1. Podocarpaceae*	*Rissikia media*	Triassic?-Jurassic	160 ± 10*	Argentina, India, Australia, Antarctica[3,4,5]
2. *Acmopyle*	*Acmopyle florinii* *Acmopyle engelhardtii*	Late Palaeocene	57.5 ± 1.5	Australia[6] Argentina[7,8,9]
3. *Dacrycarpus*	*Dacrycarpus* sp.	Early Palaeocene	64.48 ± 0.59	Salamanca Fm., Argentina[10,11]
4. *Dacrydium*	*Dacrydium* sp.	Middle Eocene	44 ± 4.0	Australia[12]
6. *Retrophyllum*	*Retrophyllum* sp.	Early Eocene	52.22 ± 0.22	Laguna del Hunco, Argentina[8]
7. *Nageia*	*Nageia hainanensis*	Eocene	34–55	China[13]
8. *Podocarpus*	*Podocarpus andiniformis*	Early Eocene	52.22 ± 0.22	Laguna del Hunco, Argentina[8,14,15]

*The Araucariales node was set with a maximum age estimation based on transitional conifers. For Podocarpaceae node we used minimal age estimation based on recent literature. We also ran analyses without using these values, obtaining similar results that do not substantially modify other node values.

[1]Escapa *et al.* (2010); [2]Escapa & Catalano (2013); [3]Rothwell *et al.* (2012); [4]Townrow (1967); [5]Escapa *et al.* (2013); [6]Hill & Carpenter (1991); [7]Florin (1940b); [8]Wilf (2012); [9]Fig. 1d; [10]Iglesias (2007); [11]Fig. 1a–c; [12]Carpenter & Pole (1995); [13]Jin *et al.* (2010); [14]Florin (1940a); [15]Fig. 1e–g.

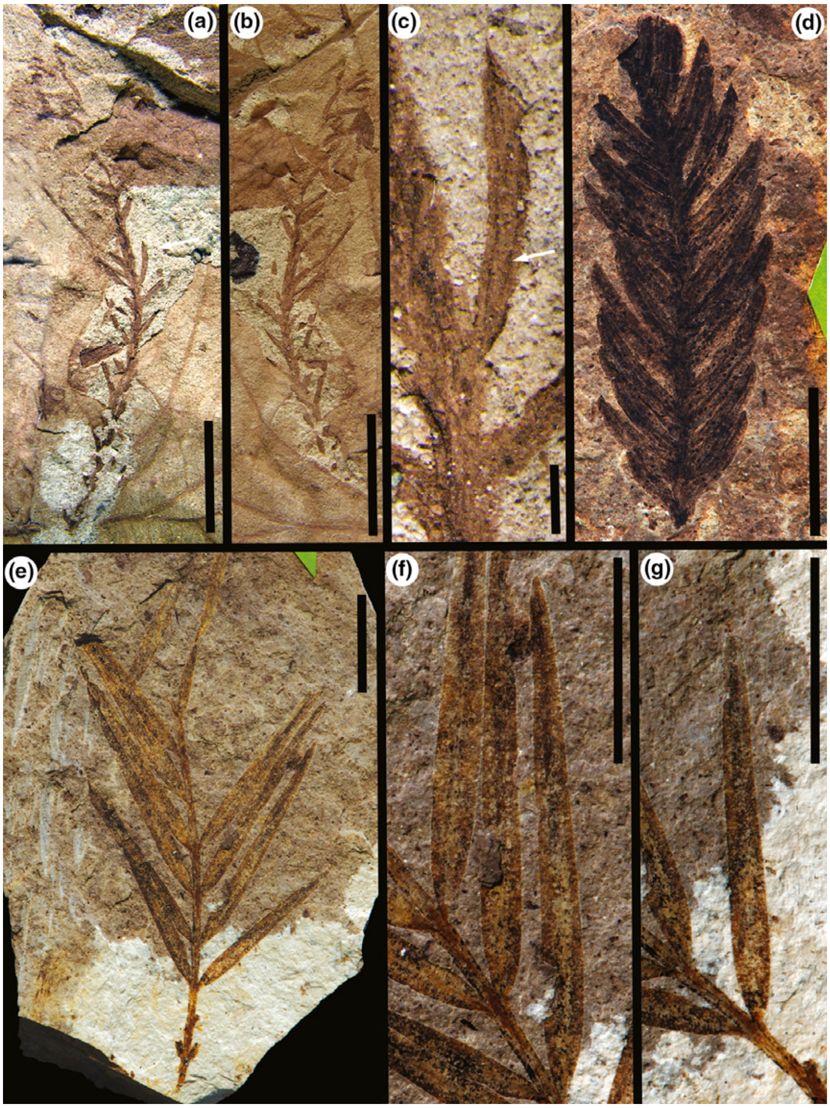

Figure 1 Images of precisely dated fossil materials (Table 1) used for phylogenetic calibrations and confidently identified as modern genera: (a) *Dacrycarpus* sp. from Salamanca Fm. (early Danian) from Iglesias (2007). White arrow indicates leaf magnified in Fig. 1c. (b) Counterpart of material in Fig. 1a. (c) Closer view of a leaf from Fig. 1a, note typical *Dacrycarpus* leaf characters: fine-needled bilaterally flattened leaves; acuminate apex with a long fine mucro; and typical linear pair of stomata bands (white arrow) deployed at equal distance from the mid-vein (Wilf, 2012). Scale = 1 mm. (d) *Acmopyle engelhardtii* (Berry) Florin, type specimen (USNM 40385b); note distinctive distichous bilaterally flattened leaves, larger and wider leaf shape (Wilf, 2012). (e) *Podocarpus andiniformis* Berry, type specimen (USNM 40384) from the Eocene Pichileufú flora, Patagonia. (f–g): closer view of leaves in (e), note the large adpressed leaf region, wider base shape, straight apex shape, and strong middle vein that characterize the leaves in the genus (Florin, 1940a). All scales represent 1 cm (except in c).

Ancestral area reconstruction

Six broad geographical areas were delimited based on the current distribution range of *Podocarpus*. These areas were: Austral (southern South America, Antarctica, Australia); Oceania (Malaysia, Micronesia, New Zealand, New Caledonia, Papuasia); Asia (China, Indonesia, Japan, Malaysia, Philippines, Taiwan); Tropical America (Central America and Caribbean); Subtropical America (South America Neotropical); and Africa (see Appendix S2). To reconstruct ancestral areas of *Podocarpus* we used the dispersal–extinction–cladogenesis (DEC) model implemented in LAGRANGE 20130526 (Ree & Smith, 2008), modifying the adjacency matrix, the number of time slices, and the dispersal probabilities according to geological epoch since the late Cretaceous and the corresponding climatic and geological settings (see Appendix S2).

RESULTS

Phylogenetic analysis

Nuclear and chloroplast DNA yielded two monophyletic clades with maximum branch support that matched the distribution of extant *Podocarpus* subgenera. The *Foliolatus* clade included only species from Asia, Oceania and Australasia while the *Podocarpus* subgenus clade contained species from South and Central America, Africa, New Zealand, New Caledonia and eastern Australia (Fig. 2). The results of molecular dating suggest that both subgenera may have diverged in the early Eocene or even before (82–52 Ma; Table 2).

Within subgenus *Podocarpus*, two latitudinally structured, well-supported major clades can be distinguished, Austral and Tropical-Subtropical (Fig. 2), that may have diverged in the Palaeogene (42 Ma; Table 2). The Austral clade consists of species from temperate latitudes of southern South America, New Zealand, New Caledonia and Australia. The Tropical-Subtropical clade is composed of species from tropical and subtropical America and Africa. The Tropical-Subtropical clade in turn includes three well-supported subclades: the subtropical South American subclade, which is sister to the subclade of subtropical African species, and a third monophyletic group that includes all species with tropical distributions in Central America, Caribbean and South America (north of Amazonia, as well as the tropical and subtropical Andes) (Fig. 2).

Biogeography and divergence time

The minimum divergence time of *Dacrycarpus* and *Dacrydium* (Table 2, Fig. 2) was 91 and 54 Ma (late Cretaceous) respectively. The estimated minimum divergence times obtained for the sister group of *Podocarpus* (*Retrophyllum*, *Nageia* and *Afrocarpus*) were also in the late Cretaceous (85 Ma) (Table 2). The estimated minimum age for *Podocarpus* s.l. was 63 Ma (Table 2), and 52.22 ± 0.29 Ma based on the evidence of Patagonian fossils (Tables 1 & 2). Thus, *Podocarpus* may have already been differentiated from the rest of Podocarpaceae by the latest Cretaceous (Fig. 2). Subgenera within *Podocarpus* may have diverged prior to the late Palaeogene, although the range of minimum age estimations for the *Foliolatus* node indicate that this is much younger (23 Ma) than subgenus *Podocarpus* (42 Ma) (Table 2). Based on these results and modern distribution, the ancestral area of subgenus *Foliolatus* was restricted to East Gondwana, while subgenus *Podocarpus* was widely distributed in West Gondwana, including the tropical climatic belt and Antarctica (DEC model $P = 0.385$; Fig. 2). Two nearly synchronic diversifications of subgenus *Podocarpus* can be recognized: (1) the C1 Austral clade (25 Ma) within the cool climatic belt in Gondwana, including temperate species distributed in

Table 2 Bayesian relaxed molecular clock age estimates in million years (Ma) for different species of *Podocarpus* phylogeny, using two Bayesian approaches in BEAST: birth/death and Yule. 95% highest posterior density (HPD) values are given in parentheses, *fossil calibrated.

Node	Clade	Birth/death	95% HPD (Ma)	Yule	95% HPD (Ma)
1.	Podocarpaceae*	230.25	(199.15, 258.15)	226.89	(196.70, 257.21)
2.	*Acmopyle**	141.84	(114.80, 168.18)	141.68	(114.13, 170.37)
3.	*Dacrycarpus**	90.73	(69.29, 113.41)	91.62	(67.63, 115.07)
4.	*Dacrydium**	52.63	(44.00, 66.93)	54.49	(44.00, 71.67)
5.	Sister group to *Podocarpus*	82.62	(67.99, 98.48)	87.46	(69.40, 108.06)
6.	*Retrophyllum**	64.94	(53.00, 77.88)	68.34	(53.40, 84.98)
7.	*Nageia**	48.78	(44.5, 56.77)	50.58	(44.50, 61.92)
8.	*Podocarpus**	60.01	(52.22, 71.80)	65.21	(52.22, 81.83)
9.	*P.* subgenus *Foliolatus*	20.31	(13.18, 28.48)	26.24	(15.78, 38.58)
10.	*P.* subgenus *Podocarpus*	38.78	(27.97, 51.05)	46.12	(32.54, 62.46)
11.	Austral clade	22.27	(13.96, 31.46)	27.82	(16.89, 39.75)
12.	Tropical–Subtropical clade	30.51	(21.27, 41.14)	37.00	(24.71, 50.28)
13.	SA–African subclades	28.97	(18.59, 40.21)	35.11	(21.00, 49.05)
14.	SA Subtropical subclade	17.88	(11.91, 24.26)	22.57	(14.49, 32.07)
15.	African subclade	8.42	(3.78, 13.84)	10.93	(4.53, 19.07)
16.	C-SA Tropical subclade	5.02	(1.60, 9.28)	6.58	(1.76, 12.92)

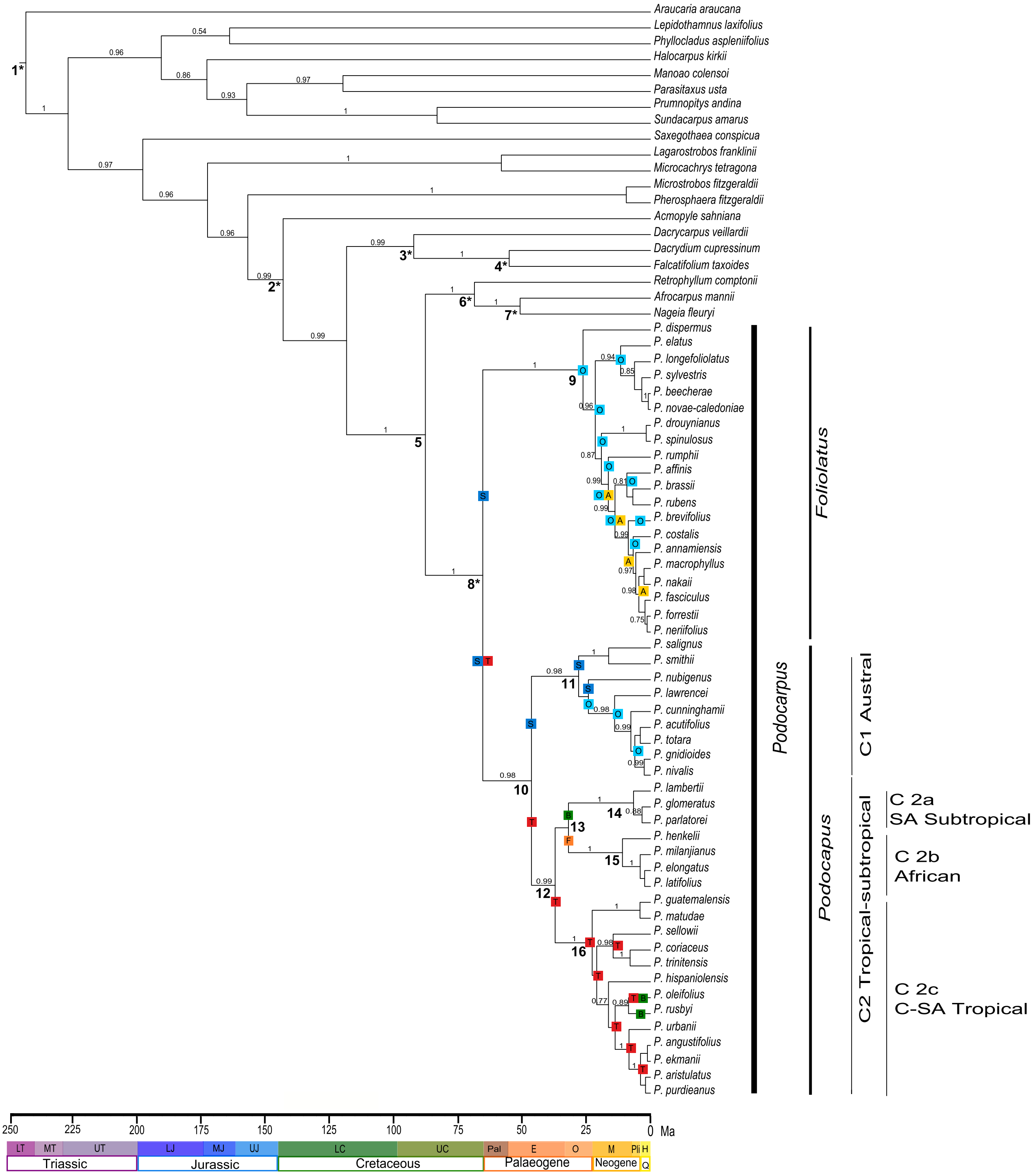

Figure 2 Chronogram indicating the evolutionary relationships among Podocarpaceae genera, and *Podocarpus* species by means of *mat*K, *rbc*L and ITS1–2 consensus tree calculated with Yule estimation. The numbers above the branches indicate the highest posteriori probability values (95% highest posterior density) for Bayesian inference analysis. Bold numbers correspond to the nodes in Table 2. Asterisks in nodes indicate fossil calibration points. Ancestral areas inferred in LAGRANGE as indicated with squares as S, Austral SA; O, Oceania; A, Asia; T, Tropical; B, Subtropical SA; F, Africa. The geochronological scale is based on ICS (2014).

southern land masses: southern South America, Australia, New Zealand, New Caledonia and (2) the C2 Tropical–Subtropical clade (34 Ma), comprising species distributed in tropical and subtropical latitudes of America and Africa (Fig. 2). Clade C2 contained three subclades (Fig. 2): (1) subclade C2a (SA Subtropical: subtropical South America), which was sister to (2) subclade C2b, the African subclade (species from subtropical Africa) and (3) subclade C2c (C-

SA Tropical: species with tropical distributions in northern Amazonia, Antilles, Caribbean Islands and Central America, as well as species from tropical and subtropical Andes). Species in subclade C2c occupy the tropical and warm climatic belt in Central and South America (Fig. 3).

DISCUSSION

We aimed to evaluate biogeographical hypotheses regarding the intercontinental geographical distribution of the conifer genus *Podocarpus* (Podocarpaceae) in the Southern Hemisphere mainly using a molecular, fossil-calibrated phylogenetic hypothesis, and to reconstruct the ancestral areas and dispersal routes among continents using the DEC model. We found that the minimum age of *Podocarpus* ranged between 82 Ma and 52 Ma, 20 Myr older than previously reported (Biffin *et al.*, 2011; Leslie *et al.*, 2012). We obtained a strongly supported monophyly for *Podocarpus* divided into two main clades, *Podocarpus* and *Foliolatus*, which were widely distributed within Gondwana. The *Podocarpus* clade was further divided into two subclades (C1 Austral, C2 Tropical–Subtropical). Subclade C2 Tropical-Subtropical could itself be subdivided into three subclades (C2a SA Subtropical, C2b African, C2c C-SA Tropical), whose compositions and relationships to one another are defined in the Results section. Our fossil-calibrated relationships and ancestral area reconstruction suggest an Atlantic subtropical biogeographical corridor and also that latitudinally disjunct lineages within South America probably diverged from widespread ancestors resulting from the persistent arid barrier.

Fossil inference

Our results suggest that the origin of *Podocarpus* could be traced back to the late Cretaceous. The improvement in both the updated fossil ages and well-recognized fossil records provides older minimum ages for several nodes in the Podocarpaceae phylogeny. Because fossil records estimate minimum ages, they may push back the ages for nodes when adding older fossil calibrations. For instance, when using the Patagonian *P. andiniformis* macrofossil to calibrate modern South American species (i.e. the *Podocarpus* subgenus node), the *Podocarpus* node was extended 37–34 Myr back (data not shown, although performed as separate analysis).

In Patagonia, the conifer fossil record is abundant through the Jurassic and Cretaceous periods (Archangelsky & Romero, 1974; Iglesias *et al.*, 2011; Wilf *et al.*, 2013). Currently, in temperate Austral and Neotropical forests of South America, conifers are reduced to three families: Cupressaceae (three monotypic genera); Araucariaceae (one genus and two

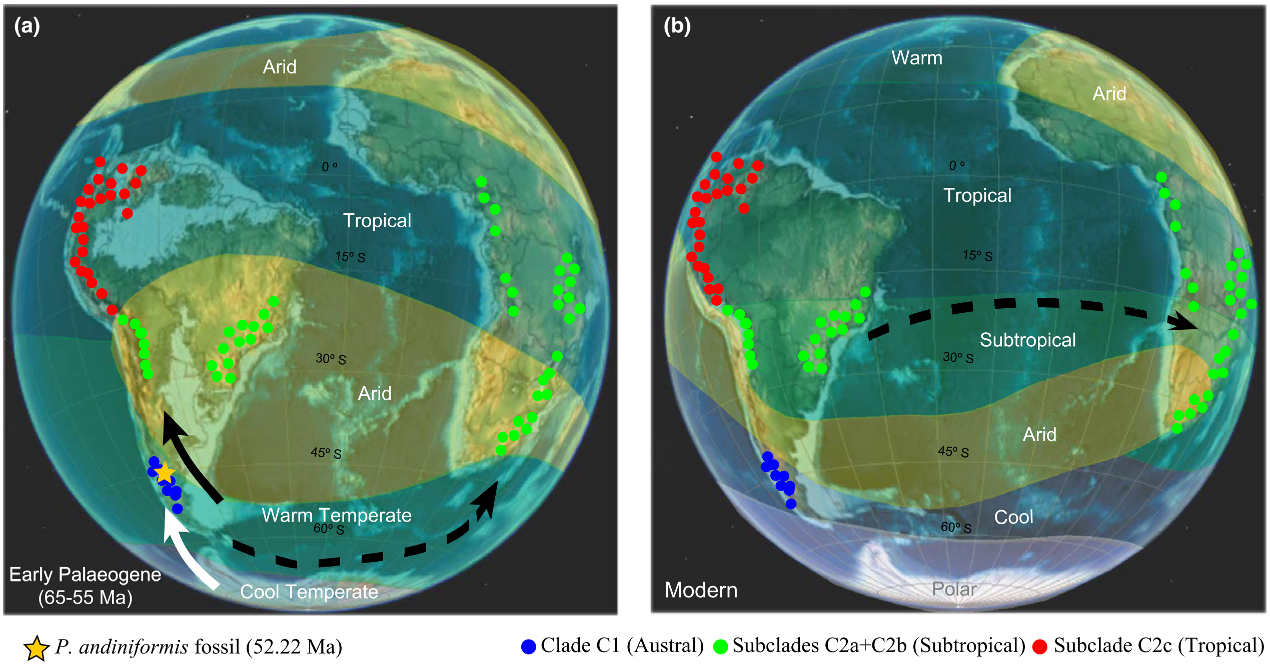

Figure 3 Modern distribution of *Podocarpus* subgenus *Podocarpus* in South America and Africa superimposed to (a) Early Palaeogene (65–55 Ma) palaeoclimatic and palaeogeographical reconstruction based on Scotese (2010). Note the broad subtropical arid climatic belt at the region where the SA and African Subtropical subclades (C2a and C2b respectively) are distributed today. The solid black arrow indicates probable direction of movement for both the warm temperate climatic belt and the SA Subtropical subclade C2a. The white arrow indicates probable direction of movement for both the cool temperate climatic belt and the Austral clade C1. The black dashed arrow indicates probable direction of dispersion for the African subclade C2b within the warm temperate climatic belt; (b) Modern geography and climate. Note the possible subtropical connection between the SA and African Subtropical subclades (C2a and C2b respectively) that may have occurred after climatic belt migration during the Oligocene (black dashed arrow).

species); and Podocarpaceae (five genera). However, during the Mesozoic and early Palaeogene, a significant number of modern conifer taxa occupied Patagonia that now are extinct in South America, including *Acmopyle*, *Agathis*, *Dacrycarpus* and *Papuacedrus* (Wilf *et al.*, 2009, 2014; Wilf, 2012). Our estimated minimum age for divergence indicates that the ancestor of *Podocarpus* may have diverged from its sister group (*Retrophyllum*, *Nageia* and *Afrocarpus*) earlier than the latest Cretaceous. Re-calculated ages based on fossil-calibrated Bayesian molecular dating gives a minimum age of 85 Ma for the sister group of *Podocarpus* (Table 2). Afterwards, in the Palaeogene, *Podocarpus* (63 Ma) had already diverged into the two geographically structured subgenera: *Foliolatus* (23 Ma) and *Podocarpus* (42 Ma). Therefore, the ancestral lineage from which both subgenera are presumed to have originated may have survived the Cretaceous–Palaeogene global extinction event.

Ecological tolerance

Although the widespread subgenus *Podocarpus* has been reported as having originated in the Palaeogene–Neogene transition (Biffin *et al.*, 2011), our results show that the ancestors of the Austral and Tropical–Subtropical clades may have differentiated earlier, during the warm Palaeogene in western Gondwana. According to the current biogeography and temperature tolerances, *Podocarpus* species present a highly conservative niche. Today, the Austral clade mainly occurs south of latitude 20° S in South America, Australia and New Caledonia. The only exception is *P. smithii* (Australia), which is distributed in montane rain forest between 15° S and 17° S. Following Biffin *et al.* (2012), the species of the Austral clade may occupy environments with high rainfall and average minimum temperatures below 0 °C of the coldest month. The ancestors of the Tropical–Subtropical clade probably remained confined to warmer climates in tropical South America, while cold-tolerant lineages of the Austral clade adapted to novel cooling trends that developed after the Eocene–Oligocene boundary (Zachos *et al.*, 2001; Bowen & Zachos, 2010). This biogeographical pattern is similar to that suggested for cold-hardy lineages of the Nothofagaceae that also evolved once cold climates began in southern South America (Premoli *et al.*, 2012). The establishment of cool trends at high latitudes was synchronic with the development of the Antarctic Circumpolar Current when Antarctica separated from South America and Australia and with the onset of glaciation Antarctica during the late Palaeogene (Zachos *et al.*, 2001; Iglesias *et al.*, 2011; Lawver *et al.*, 2011). These events probably deepened clade differentiation within subgenus *Podocarpus*, although the overall diversification of extant taxa within the Austral clade has been poor.

Phylogeny and biogeography of subgenus *Podocarpus*

The reciprocal monophyly of the Austral clade containing species of South America and Oceania, and the Tropical-Subtropical clade including South American and African taxa, suggests that these latitudinally divergent lineages probably originated from a widespread common ancestor during the late Cretaceous. The ancestral area reconstruction, molecular and fossil analyses support the hypothesis of a Gondwanan wide-range distribution of ancestral clades during or previous to the Eocene (Fig. 2). Further data from Oceania are needed to test the alternative hypothesis that subgenus *Podocarpus* developed in South America and then migrated to Oceania through Antarctica, a connection that seems to have persisted up to the middle Eocene (Iglesias *et al.*, 2011; Wilf *et al.*, 2013). Our results challenge the hypothesis of Van der Hammen & Hooghiemstra (2001) who proposed that tropical and subtropical South American *Podocarpus* were derived as a result of northward migration by cold-tolerant ancestors of Austral-Antarctic origin (i.e. the Austral clade). The early divergence of the Austral and Tropical–Subtropical lineages reflect their independent evolution on either side of the arid belt (Fig. 3) as suggested by the ancestral area analysis. Therefore, the evidence presented here supports a vicariance hypothesis within South America that closely matches tectonic and climatic events such as the latest Cretaceous–earliest Palaeocene marine ingressions and the long-lasting arid (dry and hot) belt at mid-latitudes of South America (Iglesias *et al.*, 2011; Woodburne *et al.*, 2014; Fig. 3). These probably acted as a persistent, effective barrier in South America that reinforced the south–north isolation of the Austral and Tropical-Subtropical clades (see below). Nonetheless, the fact that South American temperate *Podocarpus* taxa within the Austral clade are closely linked to those from Australia, New Zealand and New Caledonia strengthen the southern South America–Oceania connection, as suggested for widespread ancestral *Nothofagus* lineages (Hill, 1991, 2001).

Climate affinity

Our data support a long-lasting persistence of lineages of subgenus *Podocarpus* within the South American Plate. The ancestors of the SA Subtropical lineage were probably located south of its current distribution under the warm temperate climatic belt (Fig. 3a, black arrow). Similarly, the ancestral lineage of the Austral clade was probably located under the Palaeogene cool temperate climatic belt (Fig. 3a, white arrow), which may have been further south (e.g. Antarctica). In addition, the Tropical-Subtropical clade, with a minimum estimated divergence age prior to the Neogene, and probably earlier than the late Eocene (Table 2), shows that the African and SA Subtropical subclades fall within the same palaeolatitudinal belt, which in turn were isolated from those with tropical distributions (Fig. 3). We propose two alternative hypotheses for the relationship between the African and South American subtropical subclades: (1) an early migration of *Podocarpus* from South America to Africa within the southern warm temperate climatic belt (Fig. 3a, black dashed arrow) during the uppermost Cretaceous–early Palaeogene (minimum age in the Eocene–Oligocene transition; Table 2); or (2) a later migration of *Podocarpus* from South America to Africa once the subtropical climatic belt was established during the Oligocene, when the humid belt shifted towards lower latitudes (Fig. 3b, black dashed arrow).

The subtropical subclades seem to have originated before the late Palaeogene, as evidenced by highly supported long branch lengths (Fig. 2). However, short branch lengths within the SA Subtropical and African subclades probably indicate more recent species radiations during the Neogene (Fig. 2), most likely at the middle Miocene climatic optimum (Zachos *et al.*, 2001). Although members of the Podocarpaceae were probably present in ecologically suitable areas of eastern Africa (i.e. humid; Fig. 3a) since the late Cretaceous and early Palaeogene, they migrated into subtropical regions when the climate cooled in the Pliocene (Morley, 2011). Subtropical South American species of the Tropical-Subtropical subclade constitute a well-supported subclade (C2a) that unites species from the Andes and the Atlantic coast. This subclade is sister to the African species (subclade C2b) and is clearly phylogenetically separated from South American species of northernmost latitudes (subclade C2c, C-SA Tropical). Dry seasonal climates of northern South America during the uppermost Cretaceous and Palaeocene (Dino *et al.*, 1999; Hoorn *et al.*, 2010) may have constituted an effective long-lasting barrier that isolated the SA Subtropical and C-SA Tropical subclades. Afterwards, during the Miocene, shallow marine ingressions over tropical lowlands in north-western Brazil, Colombia and Peru (e.g. Pebas System: Rossetti & Netto, 2006; Hovikoski *et al.*, 2010; Hughes *et al.*, 2013; Boonstra *et al.*, 2015) may have also kept these subclades isolated. Meanwhile the arid diagonal was expanding geographically in South America, broadening in subtropical areas (Uba *et al.*, 2005), thus maintaining the divergence between the Austral and Tropical-Subtropical clades.

Divergence times of lineages within subgenus *Foliolatus* occurred at the Palaeogene–Neogene boundary (Table 2), shortly after the separation of South America and Australia from Antarctica (Zachos *et al.*, 2001). Further molecular studies are required to better elucidate biogeographical and phylogenetic relationships within subgenus *Foliolatus*.

CONCLUSION

The phylogenetic reconstruction of *Podocarpus* presented here shows a strong geographical control. The use of new DNA sequences helps to resolve some inconsistencies that appeared in previous studies, particularly among South American and Caribbean species. Similarly, the inclusion of novel fossil data from Patagonia made an invaluable contribution to this work. If additional diagnostic subgeneric characters were considered in future analyses, this could lead to better resolution of the phylogeny. The estimated minimum age of *Podocarpus* s.l. gives origination times in the late Cretaceous–early Palaeogene (63 Ma), much older than that estimated previously. The new minimum age estimates also suggest that all lineages (subclades) within *Podocarpus* were already present by the Eocene. Thus, the biogeographical patterns of extant *Podocarpus* clades are the result of vicariance events related to palaeoclimatic changes and tectonic events that have affected the latitudinal distribution of such ancient lineages almost since the late Cretaceous–early Palaeogene. The recent diversification of African and northern South American species occurred as a result of dispersal events during the Neogene. Finally, our results suggest a biotic connection between South America and Africa, which may be related to palaeoclimatic and palaeo-biogeographical belts at subtropical latitudes. The evidence presented here, in addition to previous morphological, phylogenetic, and phylogeographical studies, highlight the need to revisit the taxonomy of *Podocarpus*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 List of species, distribution and accession numbers of *Podocarpus* and outgroup taxa.
Appendix S2 Extended Materials and Methods section.
Appendix S3 Phylogenetic trees for each independent DNA region.

BIOSKETCH

M. Paula Quiroga conducts research in phylogeny, phylogeography and population genetics of trees in South America. She has a special interest in interpreting the biogeographical history in disjunct genera and species on the continent.

Author contributions: M.P.Q. led the research, analyses and writing, P.M. analysed divergence time and DEC model. A.I. contributed with the fossil evidence of several Podocarpaceae genera. A.C.P. conceived the ideas. R.R.M. provided expertise on *Podocarpus* biogeography.

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