Relaxed Molecular Clock Provides Evidence for Long-Distance Dispersal of *Nothofagus* (Southern Beech)

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Nothofagus (southern beech), with an 80-million-year-old fossil record, has become iconic as a plant genus whose ancient Gondwanan relationships reach back into the Cretaceous era. Closely associated with Wegener’s theory of “Kontinentaldrift”, *Nothofagus* has been regarded as the “key genus in plant biogeography”. This paradigm has the New Zealand species as passengers on a Moa’s Ark that rafted away from other landmasses following the breakup of Gondwana. An alternative explanation for the current transoceanic distribution of species seems almost inconceivable given that *Nothofagus* seeds are generally thought to be poorly suited for dispersal across large distances or oceans. Here we test the Moa’s Ark hypothesis using relaxed molecular clock methods in the analysis of a 7.2-kb fragment of the chloroplast genome. Our analyses provide the first unequivocal molecular clock evidence that, whilst some *Nothofagus* transoceanic distributions are consistent with vicariance, trans-Tasman Sea distributions can only be explained by long-distance dispersal. Thus, our analyses support the interpretation of an absence of *Lophozonia* and *Fuscospora* pollen types in the New Zealand Cretaceous fossil record as evidence for Tertiary dispersals of *Nothofagus* to New Zealand. Our findings contradict those from recent cladistic analyses of biogeographic data that have concluded transoceanic *Nothofagus* distributions can only be explained by vicariance events and subsequent extinction. They indicate that the biogeographic history of *Nothofagus* is more complex than envisaged under opposing polarised views expressed in the ongoing controversy over the relevance of dispersal and vicariance for explaining plant biodiversity. They provide motivation and justification for developing more complex hypotheses that seek to explain the origins of Southern Hemisphere biota.

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**Introduction**

An important principle of evolutionary inference is that explanations for the past require an understanding of mechanisms and processes applicable in the present [1]. It is perhaps this sticking point more than any other that has polarised views over the relative importance of vicariance and dispersal for explaining extant plant biodiversity. In 1915, Alfred Wegener put forward a testable hypothesis and mechanism that could explain the transoceanic distribution of animal and plant species. In the 21st century, with many DNA studies now implicating the importance of long-distance dispersal for explaining plant biodiversity [2,3,4,5], it is disconcerting that there is currently a very poor understanding of the mechanisms of transoceanic dispersal (but see [6,7,8,9,10]). Indeed, the inference that the seeds of extant *Nothofagus* species are not suited for dispersal across large distances has played a major role in motivating the hypothesis that transoceanic distributions of *Nothofagus* (Figure 1) can only be explained by vicariance [11,12,13,14,15]. This hypothesis posits that following the Cretaceous breakup of Gondwana, *Nothofagus* rafted and evolved in situ upon different Southern Hemisphere lands. Whilst very attractive, this hypothesis fits somewhat uncomfortably with the findings from analyses of morphological and molecular data. In particular, whilst earlier molecular data have been insufficient for rigorous molecular clock analyses, their interpretation has favoured hypotheses of transoceanic dispersal [16,17,18].

Based on the sequence of Gondwana breakup, a hypothesis of vicariance most parsimoniously predicts that Australian *Nothofagus* species should be most closely related to South American species. This follows since South America and Australia were connected via Antarctica until approximately 35 million years (Myr) ago (Figure 1). In contrast, New Zealand is thought to have separated from Australia 80 Myr ago [19,20]. Thus to explain the close relationship between Australian and New Zealand species by vicariance, it is necessary to argue that extinction of Australian and/or closely related South American species has occurred [19]. Whilst this explanation is ad hoc, the fossil record does provide evidence...
for numerous Nothofagus extinctions in Australia, South America, and New Zealand [21,22,23].

However, the fossil record has also been interpreted as indicating multiple events of transoceanic dispersal of Nothofagus from Australia to New Zealand. Whilst the extinct “ancestral” Nothofagus pollen type occurred in New Zealand prior to the breakup of Gondwana, Fuscospora pollen first appeared in New Zealand during the Palaeocene (65 Myr ago) and Lophozonia pollen first appeared during the late Eocene (50 Myr ago; [24]). Sixty-five Myr ago the Tasman Sea had already reached its present-day size [19,20]. Hence it is possible that extant New Zealand Nothofagus subgenera did not have the opportunity to reach New Zealand via overland migration. Hill [25] has also described the species Nothofagus cethanica, which first appeared in Oligocene macrofossils from Tasmania. This species shares unique features with extant N. fusca and N. truncata from New Zealand and may share a sister relationship with these species explained by trans-Tasman Sea dispersal [26].

A contribution to the debate over the relative importance of vicariance and dispersal can be made by estimating the divergence times of extant species. However, DNA sequences of insufficient length have prevented robust molecular clock analyses from being undertaken. For this reason, we report the sequencing of a 7.2-kb chloroplast genome fragment covering the gene regions (trnL–trnF and atpB–psaI; see Table 1 for accession numbers) for 11 species of three Nothofagus subgenera (Lophozonia, Fuscospora, and Nothofagus). Our aim has been to date divergence of extant species in the subgenera Lophozonia and Fuscospora. We have carried out relaxed molecular clock analyses using the methods of Sanderson [27,28] and Thorne et al. [29]. Our findings are that, whilst vicariance is likely to explain some transoceanic relationships amongst Nothofagus species, phylogenetic relationships between trans-Tasman species in both Lophozonia and Fuscospora can only be explained by mid- to late-Tertiary transoceanic dispersal.

Results

Figure 2 shows an optimal maximum-likelihood reconstruction of phylogenetic relationships for chloroplast DNA sequences (7.2-kb comprising the atpB–psaI region and the trnL–trnF region; 7,269 nucleotide sites) for Nothofagus (subgenera or pollen groups (a) Lophozonia, (b) Fuscospora, and (c) Nothofagus) and outgroup Castanea sativa (not shown). In a sensitivity analysis of 60 substitution models, the tree shown in Figure 2 was always recovered with very little difference in branch lengths regardless of the substitution model used. Of all substitution models evaluated, K81uf+G was identified as the best fitting one for the data based on hierarchical likelihood ratio tests and the Akaike Information Criterion. This substitution model and also the F84+Γ8 model were used for further analyses. The latter was included because the Bayesian relaxed molecular clock (BRMC) approach as implemented in the program MULTIDIVTIME (see Materials and Methods) only allows the use of the JC and the F84 models. Thus analysis with the F84+Γ8 model allowed a comparison of date estimates to be obtained using different relaxed molecular clock methods. All nodes of the optimal ML tree recovered in the sensitivity analysis received nonparametric bootstrap support greater than 97%, with the only exception being the grouping of N. cunninghamii with N. moorei, which received 72% support.

Divergence times for the nodes in this tree (Figure 2) were estimated using the penalized likelihood (PL) method [27] and BRMC method [29,30,31]. For these analyses, a period of 70–80 Myr was used to calibrate the divergence between the three fossil pollen groups representing subgenera Lophozonia, Nothofagus, and Fuscospora. These four pollen groups all first appeared in the fossil record approximately 75 Myr ago [32]. A second constraint of a minimum of 20 Myr for the divergence of N. cunninghamii and N. moorei was also used. This constraint was based on observations reported by Hill [26] that 20-Myr-old fossils intermediate between N. moorei and N.
produced unrealistic age estimates for all basal nodes. For vicariance. Constraining these two nodes in this way provided us with a lower bound for divergence times of trans-Tasman Sea reached its present position; thus this date constrained the divergence of Australian and New Zealand sister taxa to 65 Myr (the time before present when the Tasman Sea separated from Australia). Violet numbers show bootstrap values. The pollen grains represent the first appearance of the respective pollen type in the New Zealand fossil record. Plio, Pliocene; Oligo, Oligocene; Palaeo, Palaeocene; Ma, Maastrichian; Campan, Campanian. L1–L4, Lophozonia 1–4; F1–F2, Fuscospora 1–2; F/N1, Fuscospora/Nothofagus 1. DOI: 10.1371/journal.pbio.0030014.g002

Table 1. Origin of Nothofagus Samples and Sequence Accession Numbers

| Species | Source | Country | Herbarium Voucher | Accession Number |
|---------|--------|---------|------------------|-----------------|
| N. menziesii | Te Aroha | New Zealand | MPN 27272 | AY605494 |
| N. solandri | Waikaremoana | New Zealand | MPN 27273 | AY605497 |
| N. truncata | Taranaki | New Zealand | MPN 24995 | AY605498 |
| N. fusca | Ruahine Range | New Zealand | MPN 27275 | AY605491 |
| N. moorei | Barrington Tops National Park | Australia | MPN 27271 | AY605495 |
| N. cunninghamii | Cultivated Tasmania | Australia | MPN 25020 | AY605493 |
| N. gunnii | Cultivated Tasmania | Australia | MPN 27274 | AY605493 |
| N. alessandri | Cultivated Valdivia | Chile | MPN 27277 | AY605503 |
| N. obliqua | Cultivated Valdivia | Chile | MPN 27278 | AY605503 |
| N. glauca | Cultivated Valdivia | Chile | MPN 27279 | AY605512 |
| N. nitida | Cultivated Valdivia | Chile | MPN 28699 | AY745879 |
| C. sativa | Cultivated variety | Tuscany | – | AY548965 |

The inferred ages for the remaining nodes of the tree, closely resembling Nothofagus species were also present at that time. The inferred ages for the remaining nodes of the tree, obtained under the F84+$\Gamma_8$ model of substitution are given in Table 2 and graphically illustrated on Figure 2. The robustness of the estimates to calibration error was tested by constraining the divergence of Australian and New Zealand sister taxa to 65 Myr (the time before present when the Tasman Sea reached its present position; thus this date constrained the divergence of Australian and New Zealand sister taxa to 65 Myr (the time before present when the Tasman Sea reached its present position; thus this date provided us with a lower bound for divergence times of trans-Tasman Nothofagus disjunctions that might be explained by vicariance). Constraining these two nodes in this way produced unrealistic age estimates for all basal nodes. For constrained.

Table 2. Estimated Divergence Dates and Standard Deviations (in Brackets) of Different Nothofagus Clades

| Node Number/Constraint | BRMC Date Estimate | PL Date Estimate |
|------------------------|--------------------|-----------------|
| Root: 70–80 Myr F/N1: 65 Myr | F1, L3: 65 Myr | F1, L3: 65 Myr |
| L1: 20 Myr | 65 Myr |
| F/N1 | 20 |
| F2 | 20 |
| L2 | 65 |
| F1 | 65 |
| L3 | 65 |
| L4 | 65 |
| F | 65 |
| Node Number/Constraint | BRMC Date Estimate | PL Date Estimate |
|------------------------|--------------------|-----------------|
| Root: 70–80 Myr F/N1: 65 Myr | F1, L3: 65 Myr | F1, L3: 65 Myr |
| L1: 20 Myr | 65 Myr |
| F/N1 | 20 |
| F2 | 20 |
| L2 | 65 |
| F1 | 65 |
| L3 | 65 |
| L4 | 65 |
| F | 65 |

The numbers in bold are all the nodes that were estimated without constraints. Dates are based on different calibration dates and estimation approaches and are given in Myr before present.

* Node fixed

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example, using the BRMC method, which additionally required a prior expectation to be specified for the age of the root node (which we specified at 75 Myr—the time of appearance of all four extant pollen types), we estimated a more likely age for the root node at 191 Myr. For the PL approach, which does not require specification of a prior, we estimated the age of the root node at 634 Myr. Other basal nodes, the age of the root node is 634 Myr. Other basal nodes, including Ficus sporofora and Lophozonia lineages, were also much older than reasonably expected (see Table 2).

**Discussion**

Our findings from molecular clock analyses using five independent calibrations (for four nodes), suggest that the sister relationships of the Australasian (Australia and New Zealand) species within both Lophozonia and Ficus sporofora lineages are too young to be explained by continental drift (as indicated by the inferred ages of nodes F1 and L3). Transoceanic dispersal appears the most likely explanation for the trans-Tasman sister relationships indicated in Figures 1 and 2. In contrast, the age inferred for node F2, using both relaxed clock methods is compatible with a hypothesis of continental drift as an explanation for the sister relationship between South American and Australasian Ficus sporofora lineages. The age for node L4, which separates Australasian and South American Lophozonia, may also be consistent with vicariance. The BRMC method dates it at 34 Myr before present. However, the PL method estimates this node to be only 25 Myr old, an age too recent to be consistent with vicariance. Thus we regard our results for node L4 as equivocal. Nevertheless, southern beeches are likely to have been present in Antarctica 25 Myr ago [33], and thus long-distance dispersal across the young southern ocean between South America and Australia via Antarctica may be conceivable.

The robustness of our phylogenetic inferences has been investigated by varying the substitution model (60 symmetric models were used), estimating the variance of age estimates, and evaluating the influence of calibrations on divergence times. With the exception of the root node, the PL method consistently gave more recent age estimates than did the BRMC method. Both methods showed sensitivity to the divergence time estimates be truly convincing. DNA sequence analyses have also suggested that long-distance dispersal and continental drift are both important for explaining distributions of the conifer Agathis (Araucariaceae) in the South Pacific [35]. Although the molecular evidence for Agathis is not as strong as it is for Nothofagus, the findings from the molecular studies on these genera highlight the importance of considering more complex hypotheses of relationship in debates concerning the relative importance of dispersal and vicariance.

**Materials and Methods**

**Sequence data.** Chloroplast DNA sequences (7.2 kb comprising the atpB–psaD region and the trnL–trnF region) were determined for each of 11 accessions of Nothofagus (subgenera or pollen groups Lophozonia, Ficus sporofora, and Nothofagus) sampled in South America, Australia, and New Zealand (see Table 1). These genome regions were also determined for C. sativa (an outgroup taxon from Fagaceae) and aligned using progressive multiple-sequence alignment: ClustalX version 1.81 [36]. This resulted in an unambiguous alignment of 7,269 nucleotide sites. Data are missing for approximately 250 bp of the atpB gene and atpB–rbcL intergenic region of Nothofagus.

**Table 3. Variation of Estimated Divergence Times (in Myr) under 60 Symmetrical Models of DNA Substitution**

| Node Number/Constraint | L1 | L2 | L3 | L4 | F1 | F2 | FN1 | Root |
|------------------------|----|----|----|----|----|----|-----|------|
| Root                   | Min| Max| Min| Max| Min| Max| Min| Max  |
| F/N1: 70–80 Myr        | 20 | 20 | 21.7| 21.8| 26.6| 27.0| 41.1| 42.4 |
| L2: 20 Myr             | 0.7| 0.7| 0.7 | 20  | 20  | 21.7| 21.8| 26.6 |

Dates estimated using PL approach.
* Node constrained.
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Tree building. Phylogenetic analyses were conducted with PAUP* version 4.0b10 [37] under the ML criterion. A model sensitivity test was conducted, investigating a range of 60 symmetrical models of DNA substitution corresponding to the 56 implemented in MODELTEST version 3.06 [38] (http://darwin.uvigo.es/software/modeltest.html) plus F84, F84+I, F84+Γ, and F84+Γ+I. ML parameters of these models were estimated by PAUP* following the approach used in MODELTEST. These parameters were then used to conduct 60 individual ML heuristic searches in PAUP* with tree bisection-reconnection branch swapping and a neighbour-joining starting tree.

ML bootstrap proportions were obtained after 100 replications, using the same search strategy and ML parameters as for the analysis of the original dataset.

Molecular dating: The PL method. Divergence dates were estimated using the PL method of Sanderson [27] as implemented in the program r8s (http://ginger.ucdavis.edu/r8s/). The program ML and inos was used to generate 100 bootstrap resampled datasets of 7,269 sites in length. ML branch lengths of the optimal nonparametric bootstrapping of the original dataset as suggested by Sanderson [27], and the correcting smoothing parameter \( k \) defined accordingly. Divergence dates were estimated on the 60 ML phylograms recovered in the phylogenetic model sensitivity analysis. Ages for each node across the 60-ML trees were summarized using the “profile” command. Confidence limits on dating estimates were computed by using cross-validation as suggested by Sanderson [27]. Divergence dates were estimated on the 60 ML phylograms recovered in the phylogenetic model sensitivity analysis. Ages for each node across the 60-ML trees were summarized using the “profile” command. Confidence limits on dating estimates were computed by using cross-validation as suggested by Sanderson [27].

Supporting Information

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/) accession numbers for the sequences discussed in this paper are given in Table 1.

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Author contributions. MK, KS, DH, and PJL conceived and designed the experiments. MK and KS performed the experiments. DH and FS contributed reagents/materials/analysis tools. MK, DH, and PJL wrote the paper.

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