Phylogeny, classification and biogeography of *Halfordia* (Rutaceae) in Australia and New Caledonia

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Abstract *Halfordia* F.Muell is a genus of rainforest trees or shrubs native to New Guinea, New Britain, New Caledonia, Vanuatu and eastern Australia. There is debate about the number of species that should be recognised in the genus. Four species have been named, but authors have commonly recognised only two species, and some recent treatments accept just one widespread species with a broad ecological range. We sequenced two nrDNA markers (ITS and ETS) and two cpDNA markers (*rbc*L and *trn*L–*trn*F) from samples across the range of *Halfordia* in Australia and New Caledonia. Three allopatric nrDNA groups were resolved: one from southeast Queensland and northern New South Wales (Group A); one from the Wet Tropics region of North Queensland (Group B); and one from the Cape York region of North Queensland, Torres Strait and New Caledonia (Group C). These groups were also partly differentiated by more slowly evolving cpDNA markers; the exception was one widespread haplotype in Australia (presumed ancestral). The nrDNA groups support

recognition of three previously described species: *H. leichhardtii* (Group A), *H. scleroxyla* (Group B) and *H. kendack* (Group C). Divergences among eastern Australia populations are best explained by vicariance and correlate with geographic breaks documented for other taxa (ranging in estimated ages from the mid-late Miocene to the Pleistocene). The broad distribution of Group C, from Cape York to New Caledonia, with less genetic divergence, arguably reflects recent range expansion into New Caledonia involving bird dispersal of fleshy fruits.

Introduction

Halfordia F.Muell is a genus of rainforest trees or shrubs native to New Guinea, New Britain, New Caledonia, Vanuatu and eastern Australia (Fig. 1). It is classified in Rutaceae subfamily Rutoideae (Groppo et al. 2012), or in subfamily Amyridoideae in the more finely divided classification of Morton and Telmer (2014). In molecular phylogenies (Groppo et al. 2008, 2012; Bayly et al. 2013a) it is placed as sister to a large clade of c. 15 genera (e.g. *Philotheca* Rudge, *Correa* Andrews, *Phebalium* Vent.) that are mostly endemic to Australia. When compared with these relatives, *Halfordia* is morphologically distinct and notable for its comparatively large leaves, fleshy, indehiscent fruits (Fig. 1 inset), and mostly rainforest habitat (in contrast to universally smaller leaves, dry dehiscent fruit, and occurrence mostly in sclerophyll forests, woodlands and shrublands).

There is a history of debate and confusion about the number of species that should be recognised in the genus. Four species names have been legitimately published: *Halfordia kendack* (Montrouz.) Guillaumin, based on a type from New Caledonia; *H. papuana* Lauterb. based on syntypes from several localities in New Guinea (no lectotype designated); *H. leichhardtii* based on a type from Moreton Bay, southeast Queensland; *H. scleroxyla* F.Muell based on a type from Rockingham Bay, north Queensland. Although some regional floras have suggested, without details, the recognition of four distinct species in the genus (e.g. Stanley and Ross 1983; Richards 2002), most accounts have recognised a smaller number.

Many treatments of the genus have recognised two species: *H. kendack* and *H. scleroxyla* (e.g. Bailey 1899; Francis 1951; Hyland and Whiffin 1993; Hyland et al. 1999, 2003; Centre for Australian National Biodiversity Research 2010). It has commonly been suggested that both species occur in north Queensland (Australia), and that *H. kendack* also extends to southern Queensland, New South Wales, New Guinea, New Britain, New Caledonia and Vanuatu. The distinction between the two species has most commonly been on fruit colour (variously described as black or purple in *H. kendack*, versus red in *H. scleroxyla*), but fruit size was also used by Hyland and Whiffin (1993) and Cooper and Cooper (2004).

In contrast, the recent *Flora of Australia* treatment (Hartley 2013), as well as earlier works (Hartley 2001; Forster 2002; Conn and Damas 2005), and the current Australian Plant Census (CHAH 2015), recognise just a single species, *H. kendack*, in Australia and throughout the range of the genus. This is in line with the comments of Francis (1956) that two distinct species were "scarcely separable", and with Forster et al. (2004) who considered fruit colour to be unreliable in distinguishing *H. kendack* and *H. scleroxyla* (in the sense of Hyland and Whiffin (1993)), and presented fruit measurements showing substantial overlap between them.

If just one species is recognised in *Halfordia*, it has both a very widespread distribution and a habitat breadth unmatched by other tree species in the Australian flora (Forster et al. 2004), with habitats ranging from coastal vine thickets at sea level to exposed montane rainforests (moss/fern microphyll thickets) at 1560 m altitude. On the basis of this habitat diversity, Forster et al. (2004) suggested that four distinct groups of allopatric populations in Australia could be defined as follows: lowland populations of north Queensland; montane populations of north Queensland; lowland populations of southeast Queensland and northern New South Wales; montane populations of southeast Queensland and northern New South Wales. Forster et al. (2004) conducted a study of leaf essential oils across all these population groups. That study showed some differentiation of lowland populations of north Queensland from those of other areas, but other variation did not correlate with locality, habitat or morphological variation. The authors concluded that this lent support to the notion that *Halfordia* includes just one variable species in Australia.

Apart from taxonomic questions, the widespread and disjunct distribution of *Halfordia* is biogeographically interesting. Within Australia there is a disjunction of ~ 800 km between populations in southern and northern Queensland, and there are clear disjunctions between populations of Australia and other land areas. As part of a broad study of the Australasian Rutoideae using the chloroplast markers *rbc*L and *atp*B, Bayly et al. (2013) showed limited sequence divergence between one sample of *Halfordia* from New Caledonia and two Australian samples; both Australian samples had identical sequences. The conclusion from that study, based on sequence divergence and corresponding age estimates, was that dispersal, rather than vicariance, potentially related to the fleshy bird-dispersed fruits, accounted for the

presence of *Halfordia* in both Australia and New Caledonia. However, that study provided little insight into the direction of dispersal between the two areas or into the biogeographic history of *Halfordia* within Australia.

The goal of the current study was to assess patterns of geographic variation in *Halfordia* in Australia and New Caledonia, using variable chloroplast and nuclear ribosomal DNA makers, and a broad sample of distinct populations. Our aim was to use these genetic data to: 1) test competing taxonomic hypotheses about the number of taxa that might be recognised in these areas; and 2) infer the biogeographic history of *Halfordia* in eastern Australia and New Caledonia.

Materials and methods

DNA Isolation, amplification and sequencing

Plant material was obtained from field collections, cultivated plants of known provenance and one recently collected herbarium specimen (Table 1, Fig. 1). Leaf material was collected from 36 samples of *Halfordia* and five samples representing outgroup genera (*Neoschmidia* T.G.Hartley, *Eriostemon* Sm., *Drummondita* Harv., *Geleznowia* Turcz. and *Philotheca*) chosen on the basis of the higher-level phylogeny of Bayly et al. (2013). Material from field collections was dried in silica gel. Leaf tissue was disrupted using a mortar and pestle and DNA subsequently isolated using a DNeasy® Plant Mini Kit (Qiagen) following the manufacturer's instructions and using a final elution volume of 100 µL. We amplified and sequenced two nrDNA markers (ITS and ETS) and two cpDNA markers (*rbc*L and *trn*L–*trn*F; the latter including both the *trn*L intron and the *trn*L-*trn*F intergenic spacer). PCR amplifications and sequencing used the methods previously described for ITS and ETS (Bayly et al. 2015), *rbc*L (Bayly et al. 2013a) and *trn*L-*trn*F (Barrett et al. 2015; Bayly et al. 2015). All sequences were newly generated for this study, with the exception of three *rbc*L sequences from the study of Bayly (2013a).

Sequence editing and alignment

Contiguous sequences were assembled with Sequencher *v.* 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA) and manually aligned with Se-Al Sequence Alignment Editor *v.* 2.0 (Rambaut 2002). Individual sequences are available from GenBank (Table 1), and alignments are available in TreeBase (http://treebase.org/; accession no. xxx).

Phylogenetic analyses

Sequences were analysed using maximum parsimony (MP) with PAUP* 4.0 beta 10 Swofford (2002) and Bayesian inference (BI) using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). Separate analyses were performed for data from the combined nrDNA markers, the *trn*L-*trn*F cpDNA marker, and the combined cpDNA markers. Incongruence between nrDNA and cpDNA data was assessed using the Incongruence Length Difference Test (ILD; Farris et al. 1995a, b), implemented in PAUP* using 1000 heuristic search replicates (with MAXTREES for each replicate set at 2000). The *trn*L-*trn*F dataset was analysed individually, despite being congruent with the other cpDNA marker, *rbc*L, to assess the impact that the combination of markers had on the root position in the cpDNA dataset. To assess the effects of outgroup choice on the rooting of trees, MP and BI analyses were repeated including: A) only one of the five outgroup genera at a time; B) all five outgroup genera combined.

MP analyses involved a heuristic tree search with a CLOSEST addition sequence TBR branch swapping, and all characters equally weighted. A "simple indel coding" strategy (Simmons and Ochoterena 2000) was used, whereby all indels, whether single- or multi-base were represented as a single character in analyses. In the nrDNA analysis the MAXTREES was set at 10,000 (because large numbers of identical sequences resulted in many possible resolutions of polytomous nodes), and support for nodes was assessed by 1000 bootstrap replicates (with "full heuristic" searching and MAXTREES for each replicate set at 2000). The results of the cpDNA analyses were used to produce a haplotype networks in Haploviewer (Salzburger et al. 2011).

BI analyses used the GTR+G model of sequence evolution for ITS and ETS data partitions and the GTR+I model for *trn*L-*trn*F and *rbc*L partitions (with parameters unlinked between partitions), which were the preferred models using the Akaike Information Criterion as implemented in MrModeltest v. 2. (Nylander 2004). The "restriction" model was used for binary indel characters, which were coded using the same system as in MP analyses. Separate nrDNA and cpDNA analyses each included two runs of four chains run for five million generations, sampling trees every 1000 generations. A majority rule consensus was computed, with the first 25% of trees discarded as burn-in. That the two runs in each analysis had converged on a stationary distribution, and that the burn-in period was adequate, was judged by comparing the distribution of likelihood values in Tracer v.1.5 (Rambaut and Drummond 2009) and the standard deviation of split frequencies (which were < 0.01 at the end of the runs).

Results

Sequences of all DNA markers showed variation among *Halfordia* samples (Table 2), with nrDNA markers (ITS and ETS) being more variable than cpDNA markers (*trn*L––*trn*F and *rbc*L). An incongruence length difference (ILD) test revealed significant incongruence between the phylogenetic signal from nrDNA and cpDNA datasets ($p = 0.001$), and separate analyses of each dataset are shown here.

MP and BI analyses of combined nrDNA markers produced trees with similar topologies. Analyses including all five outgroups (combined) resolved three sequence groups in *Halfordia* that were geographically clustered (Figs 1, 2; root positions 1-4). These were: Group A from southeast Queensland and northern New South Wales; Group B from the Wet Tropics region of north Queensland; Group C from Cape York Peninsula, Torres Strait and New Caledonia. All of these groups had moderate to strong character support, BI posterior probabilities (PP 0.93-1.0), and MP bootstrap support (BS 71% –97%) when using the full complement of outgroups. Within group A there was some support (PP 0.78, BS 68%) for one divergent sample, PIF30512, being sister to other members of the group; that sample is one of several from upland rainforest (alt. ~740 m) of southeast Queensland (the others being PIF 34744, PIF

34745 and PIF 36184). Within Group C there was also weak support (a single substitution in ITS, and PP 0.87, BS 53%) for New Caledonian samples forming a monophyletic group.

Separate analyses of nrDNA data using different outgroup selections generally produced similar tree topologies to that shown in Fig. 2, but varied in the exact placement of the root. In most analyses the three geographic groups described above were each resolved as monophyletic (Figs 1, 2; root positions 1–4), but group A (root positions 6, 9) or group B (root positions 5, 7, 8) were paraphyletic in some analyses using only a single outgroup genus.

The results of the phylogenetic analyses are presented also as haplotype networks: Figure 3a for combined cpDNA markers and Figure 3b for *trn*L–*trn*F data only. Haplotypes of individual samples in analyses of combined data are summarised in Table 1. Outgroups were separated from the ingroup by relatively long branches and placement of the network root for the dataset of combined cpDNA markers varied between MP and BI analyses, and with choice of outgroup (Fig. 3a). In contrast, MP and BI analyses based only on *trn*L–*trn*F data were consistently rooted in a single position (Fig. 3b) regardless of the outgroup used.

The cpDNA networks agree with the nrDNA tree in showing New Caledonian haplotypes as a distinct lineage related to those of Cape York Peninsula. They also show a differentiated haplotype lineage from the Queensland Wet Tropics and another from southeast Queensland and northern New South Wales. However, the cpDNA groups do not exactly match the three nrDNA groups (Figs 2, 3). This incongruence is largely because the most common cpDNA haplotype (e.g. haplotype 1 in the analysis of combined markers) is widespread and is shared among samples from throughout the distribution of *Halfordia* in Australia, including Torres Strait (Table 1).

Discussion

Rooting of gene trees and incongruence between nuclear and chloroplast markers

Rooting of both nrDNA and cpDNA analyses is uncertain, which creates challenges in making taxonomic and biogeographic conclusions. Uncertainty about rooting most likely relates to substantial divergence between *Halfordia* and outgroup genera, which attach to the ingroup by relatively long branches (i.e., 8–29 times longer than any ingroup branches). Long branches can be placed in erroneous phylogenetic positions, and long branches connecting ingroup/outgroup taxa can commonly create difficulties in phylogeny reconstruction (Bergsten 2005).

From nrDNA data, evidence is in favour of the three geographic groups in *Halfordia* (as identified on Figs 1 and 2) each being monophyletic, but with uncertain relationships between them. This is supported by both MP (root position 1) and BI analyses (root position 2) including the full complement of outgroups, and at least some analyses involving each of the other outgroup taxa (Table 3; Fig. 2). It is only in some analyses using individual outgroup taxa (root positions 5–9) that monophyly of either nrDNA sequence group A or group B was not resolved. In those analyses, branches connecting to the ingroup are substantially longer than in analyses using multiple outgroups.

CpDNA analyses show evidence of incongruence with nrDNA data, but interpreting the cause of that incongruence depends on how cpDNA analyses are rooted. Several lines of evidence suggest that the root position indicated in analyses of *trn*L–*trn*F data (Fig. 3b) is more plausible than any of those suggested by analyses of the combined cpDNA dataset (Fig. 3a).

Root positions shown in analyses of combined cpDNA markers (Fig. 3a) lead to convoluted biogeographic interpretations that are not supported by nrDNA variation. If root positions shown in Fig. 3a were taken at face value, it would be inferred that ancestral haplotypes are represented by "missing" (undetected) states in the centre of the network and that the most common, haplotype (1) is a derived condition (peripheral on the network) that, in contrast to all other haplotypes, has managed to obtain a widespread geographic distribution (from New South Wales to

Torres Strait; Table 1). That interpretation would infer relatively recent seed-mediated gene flow (chloroplast introgression), involving just one derived chloroplast haplotype, between populations that are now substantially geographically disjunct (e.g. some by ~800 km in mainland Australia). If there were such widespread seed-mediated gene flow between areas, it would seem likely that: a) some evidence of gene flow would be also seen in nrDNA sequences, and b) that more than one cpDNA haplotype could be shared across areas. Such a scenario is not readily supported by the data.

The change in root positions when *rbc*L data are added to *trn*L–*trn*F data should also be viewed cautiously because central branches of the haplotype network (Fig. 3a), around which the root positions are clustered in the combined dataset, are associated with non-synonymous changes in *rbc*L, i.e., that affect amino acid composition of the translated protein. Recent studies have highlighted that changes in *rbc*L, which codes for a key photosynthetic enzyme, Rubisco, can be incongruent with overall phylogenetic signal from other cpDNA markers (Bayly et al. 2013a) and that some sites, including those involved in this network (Fig. 3a), show evidence of positive selection in other groups of flowering plants (Kapralov and Filatov 2007; Bock et al. 2014). This raises the prospect that shared states in *rbc*L between ingroup and outgroup, which affect placement of the root in combined cpDNA analysis, could reflect parallel evolution (influence of selection), rather than similarity through descent.

The simplest interpretation of cpDNA variation in *Halfordia* is that haplotype 1 is an ancestral haplotype (established prior to the differentiation of geographic areas) that was historically widespread in eastern Australia and has been retained in some individuals across all areas and in each of nrDNA groups A-C. This explanation is consistent with the root position in separate analyses of *trn*L–*trn*F data (Fig. 3b), and also with a slower relative mutation rate of the cpDNA, which, based on the proportion of variable sites within *Halfordia*, was 4.0–7.3 times lower than that observed in nrDNA markers (Table 2). Thus, incongruence between nrDNA and cpDNA phylogenies could simply reflect greater geographic differentiation of nrDNA markers coupled with slower divergence of cpDNA markers, including retention of ancestral cpDNA sequences in a large proportion of individuals.

Our taxonomic and biogeographic interpretations, discussed in the following sections, are predicated on the justifiable assumptions that: a) geographic nrDNA groups A, B and C are each monophyletic and; b) that cpDNA haplotype 1 is likely an ancestral haplotype in the genus.

Implications for taxonomy

Patterns of genetic variation in *Halfordia*, in particular the geographic groupings resolved by analyses of nrDNA (Figs 1, 2), are not consistent with the taxonomic boundaries between *H. kendack* and *H. scleroxyla* proposed by Hyland et al. (2003) and other similar treatments (e.g. Hyland et al. 1999; Hyland and Whiffin 1993; Cooper and Cooper 2004; Centre for Australian National Biodiversity Research 2010). The genetic data, together with previous discussions on the lack of reliable characters to support such circumscriptions (Forster et al. 2004), are reasonable grounds to reject such a classification.

Recognising a single species in the genus, *H. kendack*, as proposed by Hartley (2001, 2013), is one taxonomic alternative, but we suggest such species limits would be unnecessarily broad. Given the differentiation between the three nrDNA groups (Fig. 2) they could be considered to represent three distinct species, to which the names *H. leichhardtii* (nrDNA Group A), *H. scleroxyla* (nrDNA Group B) and *H. kendack* (nrDNA Group C) would apply.

A classification recognising three species would be consistent with patterns of variation in nrDNA. However, further sampling for molecular analysis is preferable before robust species boundaries can be identified. First, sampling from New Guinea and New Britain would help to identify the relationships of plants from those areas; our assumption, based on proximity of distributions is that they could be most closely related to plants of Cape York Peninsula and Torres Strait, but without further study there is the possibility that more than one taxon might be present there. Second, the exact geographic boundary between the two nrDNA groups in North Queensland could be clarified by more comprehensive sampling. Third, identifying

morphological features correlated with genetic groups would both corroborate their distinctiveness and provide a basis for morphological identification of specimens.

Some limited data from Forster et al. (2004), based on samples at BRI, supplemented here by additional data from specimens at MEL (Table 4), suggest plants of Cape York, New Caledonia and Vanuatu generally have smaller (shorter) fruit than those of other areas, and that plants of some regions might differ in petal or filament lengths. In New Guinea, fruit size seems to vary (Table 4; Conn and Damas 2005) with some specimens having fruit in the range of those seen on Cape York, but others being more in the range seen in other populations. Far more comprehensive comparisons are required, including assessment of the extent to which fruit development might depend on plant exposure (e.g. canopy tops as opposed to within the canopy or on a sheltered forest margin). Some difficulties in gathering morphological data, also noted by Forster et al. (2004), are that: most herbarium collections, including many made for this study, are sterile; there is heterophylly between juvenile and adult foliage, especially in exposed canopy positions, and this is not readily appreciated from herbarium material; fertile collections often have fruit or flowers, but not both. Consequently, *Halfordia* is not well represented in herbaria by specimens that are suitable for detailed comparisons of flowers, fruits and leaves, and the absolute numbers of flowering specimens, in particular, are quite low (e.g. 6 out of 80 collections at MEL in total).

Of note when considering species-level taxonomy is the position of sample PIF 30512 in the nrDNA tree (Fig. 2). That sample is grouped with others from southeast Queensland, but as sister to them and on a long branch. It is a representative of montane rainforest from southern Queensland or northern New South Wales (alt. ~ 740 m). Forster et al. (2004) suggested the presence of allopatric montane and lowland populations in this area and that differences between them were worthy of investigation. Given the results obtained here, it would be worth sampling more extensively the montane populations of *Halfordia* from this area to identify whether any form a distinct nrDNA group and whether there are morphological differences correlated with genetic groups. From the present study, however, it is clear that three other samples from montane populations in this area (PIF 34744, PIF

34745 and PIF 36184) do not group with PIF 30512 and have nrDNA sequences identical to those from a range of lowland populations.

In assessing species taxonomy, the lack of cpDNA divergence between some geographic areas should not be seen as evidence against the recognition of distinct taxa. CpDNA variation is commonly incongruent with species limits based on morphology or nuclear DNA markers (e.g., Rieseberg and Soltis 1991; Meudt and Bayly 2008; Petit and Excoffier, 2009; Hollingsworth et al. 2011; Holmes et al. 2014; Nevill et al. 2014; Barrett et al. 2015). As inferred above, this is often attributed to low cpDNA mutation rates and incomplete sorting of cpDNA lineages (e.g. Meudt and Bayly 2008), but can also result from introgression of cpDNA between species (McKinnon et al. 2001; Pollock et al. 2013).

Biogeography

Eastern Australia

Within eastern Australia, the distribution of *Halfordia* and of the distinct genetic lineages correlates well with known biogeographic patterns. Rainforest vegetation, in which *Halfordia* occurs, has a discontinuous distribution in eastern Australia. These forests were historically more widespread and contracted substantially since the mid-late Miocene and with climatic fluctuations of the Quaternary (Hugall et al. 2002; Yeates et al. 2002; Byrne et al. 2011; Chapple et al. 2011; Burke et al. 2013). Today, between southern and northern Queensland, areas of rainforest are separated by a number of 'dry corridors' identified as common biogeographic barriers (geographic breaks) in a range of plant and animal groups. The large disjunction in *Halfordia* distribution between southern Queensland (Group A) and northern Queensland (Group B and C) fits with two of these barriers (Fig. 1), abutting the Dawson-McKenzie Gap (Schodde and Mason 1999) in the south and the Burdekin Gap (Keast 1957, Chapple et al. 2011) in the north, and spanning the St Lawrence (Burke et al. 2013) or Broad Sound Gap (Ford 1987) in between. In north Queensland, the two distinct nrDNA and cpDNA lineages in the Wet Tropics and Cape York are separated

by the Normanby Basin (Cracraft 1986; Ford 1986), also known as the Laura Gap (Moritz et al. 2005).

The fleshy fruit of *Halfordia* is consumed by a number of frugivorous birds that have wide geographic ranges in eastern Australia (Barker and Vestjens 1989; Floyd 1989; Cooper and Cooper 2004; Forshaw and Cooper 2015), including the Topknot Pigeon (*Lopholaimus antarcticus*), Wompoo Fruit-dove (*Ptilinopus magnificus*) and White-headed Pigeon (*Columba leucomela*). It is, thus, possible that the disjunct distribution of *Halfordia* in eastern Australia could reflect a history of bird-mediated dispersal via endozoochory, at least in the past when there were more continuous 'stepping stones' of rainforest patches along the east coast of Australia. However, if that is the case, the presence of geographically distinct nrDNA lineages (Figs 1, 2) suggests a history of strong geographic isolation of disjunct lineages and provides no evidence of ongoing gene flow between them.

A vicariant history, associated with rainforest contraction since the mid-late Miocene, presents the simplest explanation for the distribution of the inferred ancestral chloroplast haplotype 1 across disjunct areas of eastern Australia, and the pattern of geographic differentiation between other haplotypes. Also consistent with a vicariance explanation is a molecular age estimate (Bayly et al. 2013) that dates the age of *Halfordia*, based on the divergence between haplotype 1+2 and haplotype 9, as (0.9–) 6.6 (–14.9) million years. This estimate, although potentially problematic (Bayly et al. 2013), and having a large margin of uncertainty, overlaps the periods of substantial rainforest fragmentation inferred since the mid-Miocene. This is consistent with estimated ages for vicariant divergences in other rainforest taxa, including plants (e.g. Burke et al. 2013) and animals (e.g. Moritz et al. 2000; O'Connor and Moritz 2003; Moussalli et al. 2005; Chapple et al. 2011), although estimated divergences in an ancient group of spiders are considerably older (Rix and Harvey 2012).

In north Queensland, the exact geographic boundary between the Cape York and Wet tropics genetic lineages is not clearly defined. Based on current sampling the nrDNA clades are separated by the Normanby Basin, but there is a gap of c. 300 km between our northernmost sample from the Wet Tropics clade and our southernmost sample from the Cape York clade. Further sampling of intervening populations is required to identify the boundary between these groups and more clearly define the biogeographic patterns. Of note, however, is that the Wet Tropics clade is clearly

distributed across the Black Mountain Corridor (Fig. 1). This relatively low altitude, dry corridor separates more mesic, upland areas that are postulated to have acted as refugia for rainforest taxa in more arid periods (Moritz et al. 2000; Hugall et al. 2002). A range of animal groups show strong genetic differentiation across this corridor (e.g. Joseph et al. 1995; Schneider et al. 1998; Moritz et al. 2000), sometimes with evidence of vicariance followed by subsequent range expansion associated with climatic fluctuations (Moritz et al. 2009). Genetic differentiation associated with the Black Mountain Corridor was found in some, but not all, species of *Elaeocarpus* trees studied by Rossetto et al. (2009) who concluded that plant functional traits and environmental factors associated with altitude also affect plant distribution patterns in this region. Based on markers used in this study, there is no evidence of genetic structuring of *Halfordia* associated with the Black Mountain Corridor. This could be related to the biology of *Halfordia* and its history in this area, but it could also reflect the fact that the DNA sequencing markers used here are not as variable as the microsatellite markers or mtDNA sequences commonly used in other studies (e.g. Rossetto et al. 2009, Moritz et al. 2000).

New Caledonia

Halfordia samples from New Caledonia cluster together in a terminal group, related to samples from Cape York, in both the nrDNA tree and cpDNA haplotype networks, under any of the rooting alternatives suggested in this study. The simplest explanation for this pattern, for the low level of molecular divergence (1–2 bp in nrDNA and cpDNA markers), and for the monophyly of New Caledonian samples, is relatively recent colonisation of New Caledonia by a single *Halfordia* lineage, as previously inferred (Bayly et al. 2013). It seems likely that bird-mediated seed dispersal (endozoochory) has played a role in this colonisation. Although it is speculative to identify potential vectors, a number of extant frugivorous birds have distributions ranging from New Guinea or northern Australia to New Caledonia (Gibbs et al. 2001). The data here show a close relationship between populations on Cape York and New Caledonia, but genetic relationships of plants from New Guinea, New Britain and Vanuatu are unknown. Colonisation of New Caledonia via New Guinea, New Britain and islands of the Greater Melanesian Arc into the South-West Pacific, possibly facilitated by periods of low sea-level, reducing distances for bird dispersal over water, is conceivable (see Brown et al. 2012).

Vicariance, as an alternative explanation for the distribution of *Halfordia* in New Caledonia, is unlikely. Its plausibility depends on the timing of final separation between the eastern margin of Australia and the continental block including New Caledonia (called Zealandia; Mortimer 2004), and in particular, the submergence of microcontinental blocks of northeastern Zealandia. Inferred submergence times for these regions are Late Eocene for the Kenn Plateau (Exon et al. 2006; Ladiges and Cantrill 2007), Late Oligocene for the Lord Howe Rise (De Beuque et al. 1998; Sutherland et al. 2010), and Late Eocene for the Norfolk Ridge (Bache et al. 2012), i.e., all pre-dating the estimated age of *Halfordia* by a considerable period of time. Nonetheless, the geology of the area surrounding New Caledonia is complex (Ladiges and Cantrill 2007), and the source area for potential colonisers is not clear.

Compliance with ethical standards
Conflict of Interest: The authors declare that they have no conflict of interest

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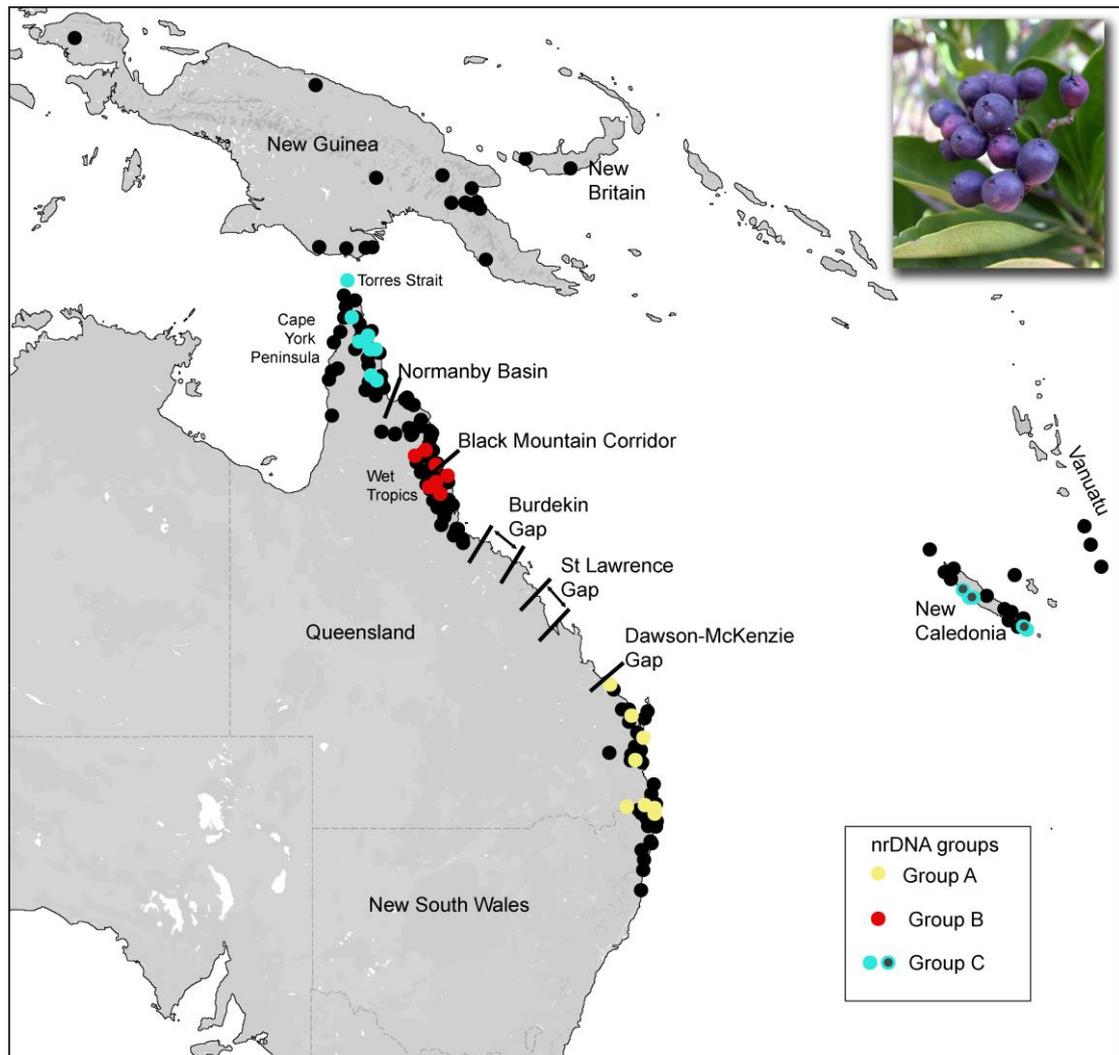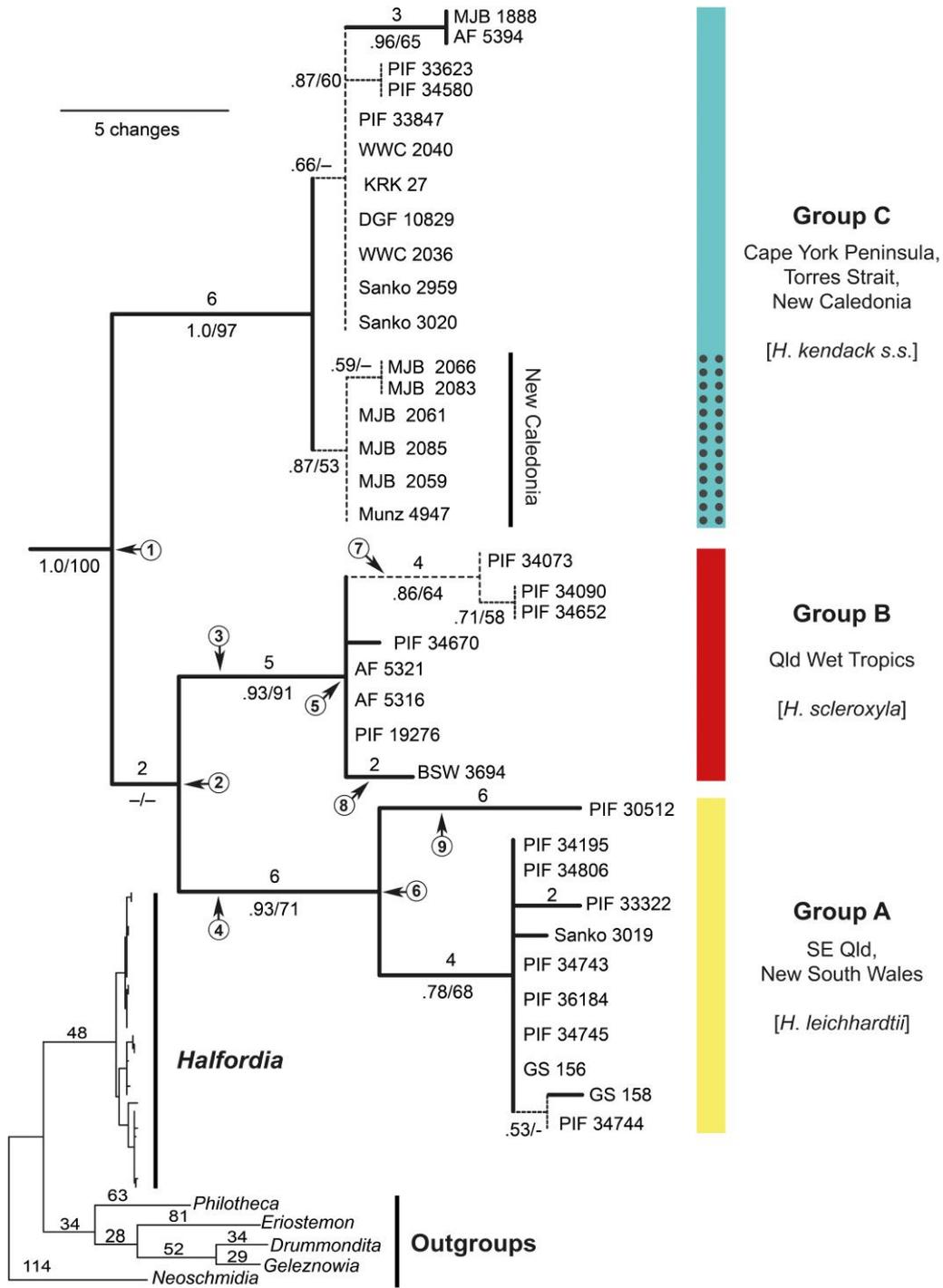
Figure Legends

Fig. 1 Distribution of *Halfordia* based on herbarium records. Coloured circles indicate the provenance of samples included in the current study, colour coded to match the nrDNA clades identified in Fig 2. Inset in top right shows fruit and leaves of *Halfordia* (Boulinda, New Caledonia, same population as *MJ Bayly* 2083)

Fig. 2 Gene tree based on phylogenetic analysis of nrDNA (ITS + ETS) sequences and including all five outgroup genera. This is one of the equally most parsimonious trees produced by MP analysis (length = 532 steps, consistency index = 0.83, retention index = 0.84), drawn with branch lengths proportional to inferred sequence change. The tree is drawn in two parts: the upper part shows only the ingroup (*Halfordia*) portion of the tree; the lower inset shows overall tree shape with all outgroups included. Dashed lines indicate branches that were not present on the strict consensus tree. Support values shown are BI posterior probabilities/MP bootstrap support. MP branch length values are shown for branches with length > 1 step. Sample details are provided in Table 1. Numbered arrows indicate root positions obtained by separate analyses using different combinations of outgroups (details in Table 3).

Fig. 3 Haplotype networks for *Halfordia* based on (a) combined cpDNA sequences (*rbc*L, *trn*L–*trn*F) or (b) *trn*L–*trn*F sequences only. Filled circles represent distinct haplotypes (numbered), and colour-coding of circles indicates the proportion of individuals with each haplotype that are placed in the nrDNA clades identified on Fig 2. Note that haplotype 1 is geographically widespread. Inferred mutational steps between observed haplotypes in *Halfordia* are represented by unfilled circles. Each branch unit represents a single DNA sequence difference, which are colour coded by cpDNA marker and whether changes in *rbc*L affect amino acid composition of the translated protein. All non-synonymous changes in *rbc*L (black branches) are in sites inferred to be under positive selection in other groups of flowering plants by Kapralov

and Filatov (2007); in order, from the bottom of the figure upwards, these branches represent changes at codons 320, 240 and 225, relative to the spinach chloroplast genome, as used by Kapralov and Filatov (2007). For (a), only the ingroup portion of the network is shown and numbered arrows indicate root positions obtained by separate analyses using different combinations of outgroups (see Table 3). All analyses of *trn*L–*trn*F data, regardless of analysis method or outgroup selection, showed the same root position as indicated in (b).

Group C
Cape York Peninsula,
Torres Strait,
New Caledonia

[*H. kendack s.s.*]

Group B
Qld Wet Tropics

[*H. scleroxyla*]

Group A
SE Qld,
New South Wales

[*H. leichhardtii*]

25
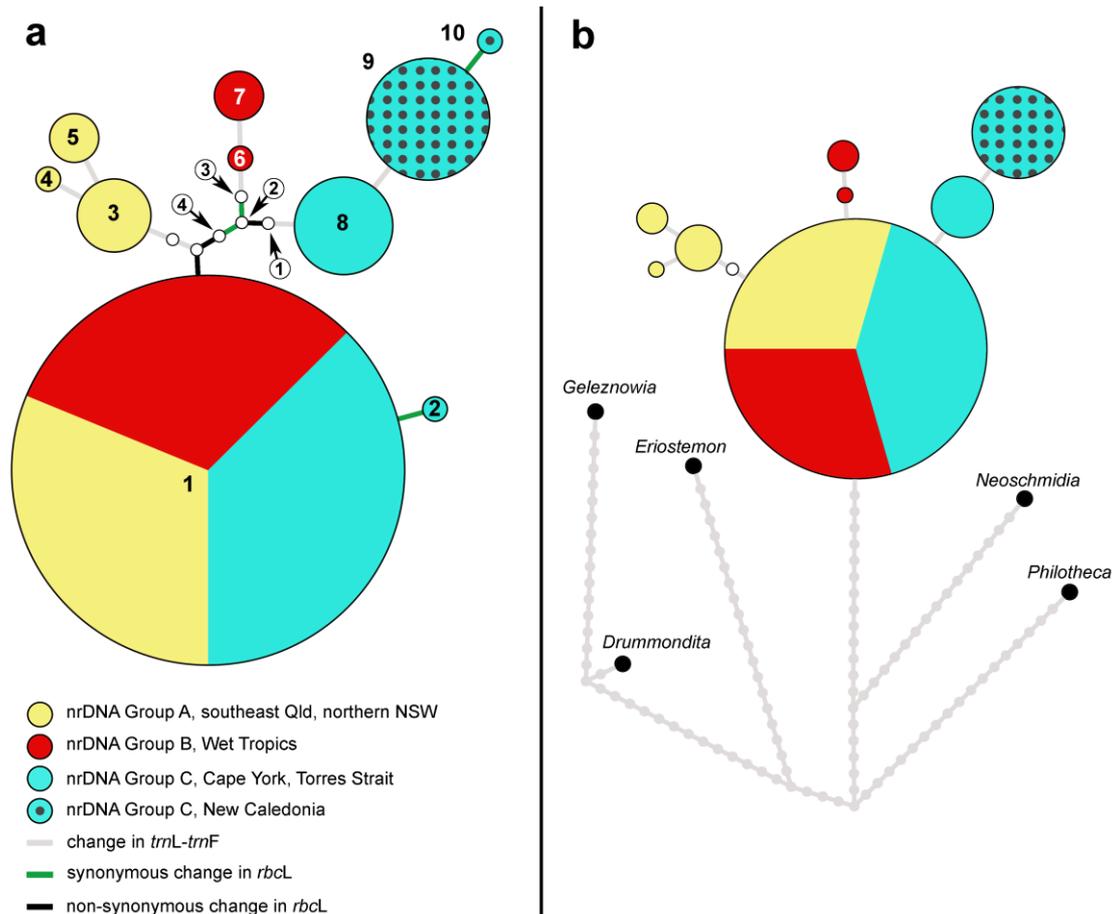
Table 1 Details of sequences (GenBank numbers) and samples used in this study

Collecting number/taxon	Provenance; Location of voucher specimen(s)	cpDNA hHaplotype*	ITS	ETS	*trn*L-*trn*F	*rbc*L
Halfordia: **New Caledonia**						
MJB 2059	Grand Kaori; MEL 2383631, BRI, NOU	10	KU861233	KU861274XXXX	KU861315XXXX	KU861353XXXX
MJB 2061	Plaine des Lacs; MEL 2383630, BRI, NOU	9	KU861234	KU861275XXXX	KU861316XXXX	KU861354XXXX
MJB 2066	Koniambo; MEL 2383625, BRI, NOU	9	KU861235	KU861276XXXX	KU861317XXXX	JN987112
MJB 2083	Boulinda; MEL 2383626, BRI, NOU	9	KU861236	KU861277XXXX	KU861318XXXX	KU861355XXXX

MJB 2085	Pindai; MEL 2383627, BRI, NOU	9	KU861237	KU861278XXXX	KU861319XXXX	KU861356XXXX
Munz 4947	Pic du Grand Kaori; NOU 030757	9	KU861238	KU861279XXXX	KU861320XXXX	KU861357XXXX

***Halfordia*: Torres Strait and Cape York, north Queensland, Australia**

DGF 10829	Moa Island, Torres Strait; BRI	1	KU861228	KU861269XXXX	KU861310XXXX	KU861348XXXX
AF 5394	McIlwraith Range NP, CSIRO EP/32 off Leo Creek Rd E of Coen; CNS 129738	8	KU861226	KU861267XXXX	KU861308XXXX	KU861346XXXX
KRK 27	Jardine River Catchment, Twin Eliot Falls walking track; BRI AQ0745473	1	KU861231	KU861272XXXX	KU861313XXXX	KU861351XXXX
MJB 1888	CSIRO arboretum, Atherton (tag. no. 473), ex Timber Reserve 14 (82,658; JC 145) McIlwraith Range; MELU 105895	8	KU861232	KU861273XXXX	KU861314XXXX	KU861352XXXX
PIF 33623	19 km along Middle Peak track to Shelburne Bay, Richardson Range; BRI AQ0743481	1	KU861242	KU861283XXXX	KU861324XXXX	KU861360XXXX
PIF 33847	5 km W of Indian Bay, 11 km S of Thorpe Point; BRI AQ0743298	1	KU861243	KU861284XXXX	KU861325XXXX	KU861361XXXX
PIF 34580	Bolt Head, Olive River Environmental Reserve; BRI AQ0745524	1	KU861247	KU861288XXXX	KU861329XXXX	KU861365XXXX
Sanko 2959	Cult. Tolga, ex Cape York, Qld; MELU	1	KU861255	KU861296XXXX	KU861337XXXX	JN987111
Sanko 3020	Cult. Tolga, ex Leo Ck, McIlwraith Ra, arb 8282; MELU 105888	8	KU861257	KU861298XXXX	KU861339XXXX	KU861374XXXX
WWC 2036	N of Massy Ck crossing, Silver Plains; BRI AQ0745519	1	KU861258	KU861299XXXX	KU861340XXXX	KU861375XXXX

WWC 2040	Chili Beach Rd, Iron Range NP QLD; BRI AQ074551	8	KU 861 259	KU8613 00XXX XX	KU8613 41XXX XX	KU8613 76XXX XX

Halfordia: Wet Tropics, north Queensland, Australia

AF 5316	Daintree NP, Hilda Creek area, c.700m WNW of Thornton Peak; CNS 129662	7	KU 861 224	KU8612 65XXX XX	KU8613 06XXX XX	KU8613 44XXX XX
AF 5321	Daintree NP, Hilda Creek area near camping area, c.800 m WNW of Thornton Peak; CNS 129667	7	KU 861 225	KU8612 66XXX XX	KU8613 07XXX XX	KU8613 45XXX XX
BSW 3694	Black Mt N of Kuranda; CANB 597970	1	KU 861 227	KU8612 68XXX XX	KU8613 09XXX XX	KU8613 47XXX XX
PIF 19276	Bell Peak North summit, Malbon Thompson Range; BRI AQ0587790, MEL 0253671	1	KU 861 239	KU8612 80XXX XX	KU8613 21XXX XX	KU8613 58XXX XX
PIF 34073	Topaz, Westcott road; BRI AQ0743506	1	KU 861 244	KU8612 85XXX XX	KU8613 26XXX XX	KU8613 62XXX XX
PIF 34090	S.F.194 Longlands Gap, Herberton Range Forest Reserve; BRI AQ0743446	1	KU 861 245	KU8612 86XXX XX	KU8613 27XXX XX	KU8613 63XXX XX
PIF 34652	Sutties Gap Rd, Walter Hill Range, Wooroonooran NP, QLD; BRI AQ0745460	1	KU 861 248	KU8612 89XXX XX	KU8613 30XXX XX	KU8613 66XXX XX
PIF 34670	Mt Windsor NP, 5km past forestry barracks, QLD; BRI AQ0745458	6	KU 861 249	KU8612 90XXX XX	KU8613 31XXX XX	KU8613 67XXX XX

Halfordia: Southeast southeast Queensland/northern New South Wales, Australia

GS 156	Toogoom/Craignish, Harvey Bay; BRI AQ0745282	1	KU 861 229	KU8612 70XXX XX	KU8613 11XXX XX	KU8613 49XXX XX
GS 158	Rees Lane, Reesville, Maleny; BRI AQ0745281	4	KU 861 230	KU8612 71XXX XX	KU8613 12XXX XX	KU8613 50XXX XX

			KU	KU8612	KU8613	KU8613
PIF 30512	The Head, Lower slopes of Wilson's Peak; BRI AQ0648623	3	KU 861 240	KU8612 81XXX XX	KU8613 22XXX XX	KU8613 59XXX XX
PIF 33322	Eurimbula, Qld; BRI AQ0739798	2	KU 861 241	KU8612 82XXX XX	KU8613 23XXX XX	JN9871 13
PIF 34195	Freshwater road, Cooloola section, Great Sandy NP; BRI AQ0743516	3	KU 861 246	KU8612 87XXX XX	KU8613 28XXX XX	KU8613 64XXX XX
PIF 34743	Cult. Cooroy, ex Brunswick Heads, adjacent to nature reserve in area under housing development, NSW; BRI	5	KU 861 250	KU8612 91XXX XX	KU8613 32XXX XX	KU8613 68XXX XX
PIF 34744	Cult. Cooroy ex Toonumbar NP, Sheep Station Ck walking Track (formerly Wiangaree SF), NSW; BRI	3	KU 861 251	KU8612 92XXX XX	KU8613 33XXX XX	KU8613 69XXX XX
PIF 34745	Cult. Cooroy ex Nightcap NP, walking Tk near Mt Nardi television transmitter towers, NSW; BRI	1	KU 861 252	KU8612 93XXX XX	KU8613 34XXX XX	KU8613 70XXX XX
PIF 34806	Jones Rd, Wooyung, near Billinudgel, NSW; MEL 2325314, BRI	5	KU 861 253	KU8612 94XXX XX	KU8613 35XXX XX	KU8613 71XXX XX
PIF 36184	Lamington NP; BRI AQ0813869	1	KU 861 254	KU8612 95XXX XX	KU8613 36XXX XX	KU8613 72XXX XX
Sanko 3019	Cult. Tolga, ex Mt Tamborine South Qld (arb. 276); MELU 105887	1	KU 861 256	KU8612 97XXX XX	KU8613 38XXX XX	KU8613 73XXX XX
Outgroups						
Drummondita calida	Bulleringa NP, Qld, PIF 22556; BRI AQ0605109	–	KU 861 221	KU8612 62	KU8613 03	JN9870 97
Eriostemon australasius	Cult. Rosanna, Vic., MJB 1869; MELU 105864	–	KU 861 222	KU8612 63	KU8613 04	JN9870 99

Geleznowia verrucosa	Tathra NP, WA, MJB 1910; MEL 2383587	–	KU 861 223	KU8612 64	KU8613 05	JN9871 10
Neoschmidia pallida	Cult. Royal Botanic Gardens Sydney, ex Mt Dore, New Caledonia, P.H. Weston 3303; NSW	–	KU 861 260	KU8613 01	KU8613 42	JN9871 26
Philotheca spicata	Alexander Morrison NP, Western Australia, MJB 1907; MEL 2383588	–	KU 861 261	KU8613 02	KU8613 43	JN9871 32

* cpDNA haplotype numbers are based on the analysis of combined cpDNA markers (Fig. 3a). Abbreviations: AF, Andrew Ford; BRI, Queensland Herbarium, Brisbane; BSW, Bruce S Wannan; CNS, Australian Tropical Herbarium, Cairns; Cult., cultivated; DGF, David Fell; GS, Greg Smyrell; KRK, Kris Kupsch; MEL, National Herbarium of Victoria, Melbourne; MELU, University of Melbourne Herbarium, Melbourne; MJB; Michael Bayly; Munz, Jérôme Munzinger; NOU, IRD herbarium in Nouméa; NP, National Park; NSW, New South Wales, Australia; NT, Northern Territory, Australia; PIF, Paul Forster; Sanko, Garry Sankowsky; Qld, Queensland, Australia; WWC, W Cooper.

Table 2 Comparison of sequence variation within *Halfordia* (ingroup only) in DNA regions used for phylogenetic analyses

	ITS	ETS	*trn*L– *trn*F	*rbc*L
Total no. of variable characters (no. of indel characters in brackets)	28 (4)	15 (1)	8 (30)	7 (0)
Proportion of variable characters	4.4%	3.2%	0.8%	0.06%
Parsimony informative characters (no. of indel characters in brackets)	20 (1)	8 (1)	7 (3)	5 (0)

Table 3 Details of root positions in phylogenetic analyses

Root position	Outgroup (analysis method)
nrDNA analyses	
1	All (MP), *Neoschmidia** (MP)
2	All (BI)
3	*Eriostemon* (MP), *Neoschmidia** (MP),
4	*Drummondita* (MP), *Eriostemon** (MP),
	Geleznowia (MP), *Philotheca* (BI, MP)
5	*Eriostemon* (BI), *Neoschmidia* (BI),
6	*Geleznowia* (BI)
7	*Eriostemon** (MP)
8	*Eriostemon** (MP)
9	*Drummondita* (BI)
cpDNA analyses	
(combined dataset)	
1	All (BI), *Eriostemon* (BI, MP),
	Philotheca (MP, BI), *Geleznowia* (BI,
	MP)
2	All (MP), *Drummondita** (MP)
3	*Neoschmidia* (BI, MP),
4	*Drummondita** (BI, MP)

Root position numbers match those shown on Fig. 2 for nrDNA and Fig. 3a for cpDNA analyses. Abbreviations: All, *Neoschmidia*, *Eriostemon*, *Drummondita*, *Geleznowia* and *Philotheca*; BI, Bayesian inference, MP, maximum parsimony. * indicates rooting only recovered in some equally most parsimonious trees.

Table 4 Flower and fruit measurements for *Halfordia*

Geographic area	Petal length (mm)	Filament length (mm)	Fruit length × width (mm)
Cape York Peninsula	3–3.8	1.8–3.1	6–8(–9.5) × 4–8
New Guinea	–	–	6.5–7 × 6 or 10–15 × 10–13

New Caledonia	4–4.5	c. 3	6–8(–9) × 5–6.5
Vanuatu	4–4.5	–	c. 9 × 6.5
Qld Wet tropics	c. 5.5	4–5	(9)10–15 × 7–15
Southeast Qld/northern NSW	4–5.8	2.8–5.5	7–13 × (5–)6–13

Data come from Forster et al. (2004; based on specimens at BRI) supplemented by measurements of additional specimens at MEL. Abbreviations: NSW, New South Wales; Qld, Queensland.

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