Plant Syst. Evol. 249: 37–53 (2004)
DOI 10.1007/s00606-004-0209-0

Plant Systematics and Evolution
Printed in Austria

Phylogenetic implications of trans-specific chloroplast DNA sequence polymorphism in New Zealand Gnaphalieae (Asteraceae)

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Received December 2, 2003; accepted June 16, 2004
Published online: October 18, 2004

Abstract. The New Zealand endemic species of Gnaphalieae (Asteraceae) present a bewildering array of morphological forms recalcitrant to phylogenetic analysis using molecular or traditional characters. The chloroplast *psb*A-*trn*H intergenic spacer is highly variable in this group, with substitutions distinguishing 18 cpDNA haplotypes distributed among samples of *Raoulia* species and those of related genera. A subset of haplotypes was combined with sequences of the more slowly evolving *trn*L intron and *trn*L-*trn*F intergenic spacer region and subjected to median network and parsimony analyses. Although a number of chloroplast lineages were resolved, these do not correspond with taxonomic units, nor with groups supported by analysis of morphological characters, nor with groups indicated by nuclear rDNA ITS sequences. These data, considered in the context of morphological character state distribution in the group, suggest a complex set of interrelationships among extant species and genera as currently circumscribed. General implications for phylogeny reconstruction are discussed.

Key words: *Raoulia*, Asteraceae, phylogenetic incongruence, internal transcribed spacer, hybridisation, introgression, lineage sorting.

Phylogenetic studies using several chloroplast loci have become common since the advent of automated DNA sequencing. One reason plant molecular systematists rely heavily on the chloroplast genome is because it can generally be modelled as a single-copy, essentially non-recombining locus (Clegg and Zurawski 1992). In theory it has the additional advantage, as a result of uniparental inheritance, of reduced coalescence times. However, these characteristics (non-recombining and uniparental inheritance) also place serious limitations on the use of cpDNA for phylogeny reconstruction (e.g. Neigel and Avise 1986, Avise 1994, Ennos et al. 1999, Avise 2000). These limitations relate to the implications of intraspecific and intra-population sequence polymorphism in cpDNA under different demographic scenarios of speciation and to the possibility of chloroplast introgression between species. One problem has been termed lineage sorting and recognises that gene lineages in extant species may coalesce on individuals prior to the divergence of the species concerned and therefore may not reflect organismal or taxic phylogeny (Avise 1994). The length of time

that multiple gene lineages will be maintained in populations is in part dependent on demographic parameters such as effective population size and population substructure (Neigel and Avise 1986).

A related problem is the introgression of genes between divergent species. Chloroplast DNA introgression has been widely reported both in studies of contemporary hybridisation (reviewed in Rieseberg and Brunsfeld 1992) and as an inference based on incongruence between nuclear and chloroplast DNA phylogenies (e.g. Soltis et al. 1991, Jansen et al. 1992, Comes and Abbott 2001). This latter class of studies implies that fixation of an introgressed chloroplast in the recipient species, at least in part of its range, may be common in at least some angiosperm groups. Chloroplast introgression has also been suggested on the basis of discrepancies between cpDNA haplotype distribution and morphological species boundaries (e.g. Steane et al. 1998).

By far the most widely used nuclear DNA sequences employed in plant systematics are those of the nuclear ribosomal genes and spacers (ITS1, ITS2, and ETS: Small et al. 2004). Unlike cpDNA these nrDNA regions are biparentally inherited and show recombination (at least in some taxa) as a coevolving gene family in high copy number (Hamby and Zimmer 1992, Baldwin et al. 1995, Álvarez and Wendel 2003). These facts are often put aside as in general ITS and ETS trees have considerable congruence with prior hypotheses about phylogenetic relationships based on morphology, biogeography or other data, and ITS and ETS sequences are generally treated in the same ways as those of non-recombining single-copy genes in phylogenetic analyses (reviewed in Álvarez and Wendel 2003).

Where hybrid speciation or introgression is a factor in the evolution of a group, the behaviour of ITS and ETS sequences is likely to depart significantly from single-locus models. Studies examining the evolution of ITS sequences following crossing between parents with divergent sequences report that sequence capture of one parental sequence type by an introgressed population is possible (Comes and Abbott 1999) as is the generation of novel recombinant types displaying a combination of parental types (e.g. Campbell et al. 1997). By contrast, discussion of the ETS in this context is largely absent from the literature. It seems reasonable to expect that introgression may contribute to a high level of homoplasy in ITS and ETS sequence data sets as well as possible incongruence with other data sets and prior hypotheses about phylogeny.

One example of an angiosperm group where ITS sequences show these properties is the Asteraceae genus complex consisting of *Raoulia* Hook.f., *Leucogenes* Beauverd, *Rachelia* J.M.Ward et Breitw., *Anaphalioides* (Benth.) Kirp. and New Zealand species currently ascribed to *Ewartia* Beauverd and *Helichrysum* Mill. corr. Pers. All of the approximately 52 species in this assemblage are endemic to New Zealand except for two species of *Anaphalioides,* which are endemic to New Guinea.

Numerous anecdotal reports of hybrids among these genera led to the hypothesis that they shared a recent common ancestry (Ward 1981, 1997) rather than resulting from several separate entries into New Zealand from different sources, as was commonly assumed due to their extreme morphological diversity and similarities to genera elsewhere (e.g. Anderberg 1991). Subsequently, the hybrid parentage of some of these morphologically intermediate plants (McKenzie 2001, Smissen et al. 2003, McKenzie et al. 2004) has been verified. Analysis of ITS sequences by Breitwieser et al. (1999) recovered this generic complex as a strongly supported clade (hereafter referred to as the "New Zealand clade") but with very little internal resolution. This lack of resolution mirrors the findings of non-molecular studies, where different character sets support different taxonomic groupings due to the reticulate nature of the character state distributions (see for example Breitwieser and Ward 1993, 2003; Ward 1993; Wilton 1997).

The extent of morphological diversity within the New Zealand clade does not appear

to be the result of stabilised allopolyploidy. Polyploidy occurs in *Anaphalioides* (2 of 7 species: Glenny 1997), *Leucogenes* (2 of 4 species: Molloy 1995) and *Raoulia* (6 of c. 28 species: Dawson et al. 1993), but polyploids are almost always similar to extant diploid species.

Here we report the results of a survey of chloroplast *psb*A-*trn*H intergenic spacer, *trn*L-*trn*F intergenic spacer and *trn*L intron sequences from *Raoulia* and allied genera, focusing on the alpine cushion species linked to *Leucogenes* by their ITS sequences. We also report reanalysis of ITS sequences including important additional sampling.

Material and methods

The plants. Chloroplast *psb*A-*trn*H sequences were sampled from a total of 14 individuals from seven populations of *Raoulia eximia*, ten individuals from six populations of *Helichrysum lanceolatum,* three individuals from different populations of *R. grandiflora*, four individuals from four populations of *R. buchananii,* two individuals from different populations of *Leucogenes grandiceps,* two individuals from different populations of *R. mammillaris,* two individuals from the same population of *Anaphalioides bellidioides*, two individuals from the same population of *R. rubra,* and single specimens of *A. mariae, A. trinervis, Ewartia sinclairii, Helichrysum filicaule, Leucogenes leontopodium, R. beauverdii, R. bryoides, R. goyenii, R. hectorii, R. hookeri, Raoulia* sp. 'L', *R.* sp. 'M', *R. tenuicaulis* and *R. youngii*. Collection and voucher details are shown in Table 1. We also generated *trn*L-*trn*F intergenic spacer and *trn*L intron sequences from a subset of these samples including five individuals from four populations of *Raoulia eximia,* two individuals from different populations of *Helichrysum lanceolatum,* two individuals from different populations of *R. grandiflora,* and single individuals of *Anaphalioides bellidioides, A. trinervis, Leucogenes grandiceps, L. leontopodium, R.* sp. 'L', *R. hookeri,* and *R. rubra*. Representative sequences have been deposited in GenBank as detailed in Table 1.

To the ITS sequences already available (detailed in Breitwieser et al. 1999, Smissen et al. 2003) we added additional sequences for single specimens of *R. hectorii*, *R. hookeri*, *R. mammillaris*, R. sp. 'L', *R. youngii*, and three specimens of *R. grandiflora*. We also increased our sampling of ITS sequences from *R. eximia* and *R. buchananii* to include additional populations. Sequence alignments are available from the corresponding author.

DNA extraction, PCR and sequencing. Genomic DNA was extracted by the CTAB method as described in Smissen et al. (2003) or by Dneasy Plant Mini Kit (Qiagen) following the manufacturer's instructions with the addition of a phenol chloroform extraction following elution of DNA where necessary. ITS sequences were amplified by PCR and sequenced according to Smissen et al. (2003). Amplification of *psb*A-*trn*H used PCR cycling conditions with an initial denaturation at 97 °C for 120 s followed by 35 cycles of 94 °C for 30 s, 48 °C for 60 s, and 72 °C for 2 min. Primer sequences were taken from the web page http://www.paeon.de/h1/sang/dis4.html (*psb*Af 5' -GTTATGCATGAACGTAATGCTC-3′, *trn*Hr 5′-CGCGCATGGTGGATTCACAATC-3′). Sequencing used the same primers as PCR. Cycle sequencing was carried out using ABI BigDye 3.1 terminators and products were separated on an ABI 3730 Genetic Analyser. Sequences of *trn*L-*trn*F and *trn*L intron were generated using the primers of Taberlet et al. (1991) and PCR conditions as for the *psb*A-*trn*H detailed above. ITS sequences were generated as described in Smissen et al. (2003).

Data analysis. All New Zealand Gnaphalieae cpDNA sequences analysed in this study were readily aligned by eye. Likewise, the ITS sequences of members of the New Zealand endemic clade were readily aligned by eye. Ingroup ITS sequences were aligned with outgroup ITS sequences using ClustalX (Thompson et al. 1997) and subsequent visual inspection. Median networks were constructed using splits generated via Hadamard conjugation by Spectronet (Huber et al. 2002). The option of Hadamard conjugation was employed as Spectronet is unable to generate splits directly from data columns with more than two states and thus information is lost. An alternative strategy available in Spectronet, reducing sequence data to purine/pyrimidine (r/y) information at these sites, is not justified for chloroplast sequences, as these generally do not show a transition/transversion bias. Shortest parsimony trees for ITS sequences were found using PAUP4.0b10 heuristic search with TBR branch swapping, steepest descent and multrees in effect, and a simple addition sequence. Searches for shortest trees for ITS

Table 1. Geographic sources, voucher numbers and GenBank accession numbers for samples included in this study, except those for ITS sequences previously published in Breitwieser et al. (1999) and Smissen et al. (2003)

Species	Location	Vouchers	*psb*A-*trn*H	*trn*L intron	*trn*L-*trn*F	ITS
Anaphalioides bellidioides (G.Forst.) Glenny	South Island, Canterbury, Port Hills	CANU38726	AY611223	AY606891	AY606902	
		CANU38727				
Anaphalioides mariae (G.Muell.) Glenny	Papua New Guinea, Mt Wilhelm	CHR492179	AY611228			
Anaphalioides trinervis (G.Forst.) Anderb.	North Island, North Auckland, Huia Dam	CHR569864	AY611234	AY606890	AY606901	
Ewartia sinclairii (Hook.f.) Cheeseman	South Island, Marlborough, Hodder River	CANU37227	AY611218			
Helichrysum filicaule Hook.f.	South Island, Canterbury, Port Hills	CHR569874	AY611219			
Helichrysum lanceolatum (Buchanan) Kirk	South Island, Canterbury, Port Hills	CHR565241	AY611220	AY606889	AY606900	
	North Island, South Auckland, Karangahake Gorge	CHR569867	AY611222			
		CHR569871				
	North Island, North Auckland, Piha	CHR569868				
		CHR569869	AY611221			
		CHR569870				
	North Island, North Auckland, Huia	CHR569872, CHR569873				
	North Island, Gisborne, Hicks Bay	CHR569866	AY611233			
	North Island, Wellington, Pohangina Totara Reserve	CHR569865				
Leucogenes grandiceps (Hook.f.) Beauverd	South Island, Nelson, No Mans Creek	CHR514141	AY611224	AY606885	AY606896	
	South Island, Canterbury, Waimakariri Valley, Black Range	CANU38729				
Leucogenes leontopodium (Hook.f.) Beauverd	North Island, Wellington, Mt Holdsworth	CHR569876[a]				
	ex nursery stock	CHR510025	AY611227			
Raoulia beauverdii Cockayne	South Island, Canterbury, Mackenzie Basin, near Tekapo	CANU38724	AY611225			
Raoulia bryoides Hook.f.	South Island, Nelson, Richmond Range	CHR502568	AY611215			

Table 1 (continued)

Raoulia buchananii Kirk	South Island, Southland, Gertrude Saddle	CHR546190	AY611212			
	South Island, Southland, MacKinnon Pass	CHR546188				
	South Island, Otago, Minaret Burn	CHR565559	AY611213			AY606872
	South Island, Otago, Young River	CHR569758				
Raoulia eximia Hook.f.	South Island, Canterbury, Mt Potts	CHR546177				
		CHR546178	AY611207	AY606884	AY606895	
		CHR546180	AY611208			
		CHR546182		AY606886	AY606897	
		CHR569875				
	South Island, Nelson, Lyell Range	CHR546162				
		CHR546160				
		CHR546164				
	South Island, Westland, Paparoa Range	CHR546168				
	South Island, Nelson, Lookout Range	CHR546154				
	South Island, Nelson, St Arnaud Range	CHR546147				AY606873
		CHR546148	AY611209			
	South Island, Nelson, Mt Arthur	CHR546146	AY611210			
	South Island, Nelson, Lead Hills	CHR546151		AY606883	AY606894	
	South Island, Nelson, Glasgow Range	CHR509743				
Raoulia grandiflora Hook.f.	South Island, Marlborough, Altimarlock	CANU38728	AY611231	AY606888	AY606899	AY606879
	South Island, Canterbury, Lagoon Saddle	unvouchered	AY611232	AY606887	AY606898	AY606880
	North Island, Wellington, Mt Holdsworth	CHR569863[a]				AY606881
Raoulia goyenii Kirk	Stewart Island, Mt Anglem	CHR560933	AY611214			
Raoulia hectorii Hook.f.	South Island, Otago, Treble Cone	CANU38722	AY611230			AY606878
Raoulia hookeri Allan	cultivated, Landcare Research, Lincoln	CHR569861	AY611216			AY606875
Raoulia sp. 'L'	South Island, Otago, Mt Pisa	CANU38725	AY611226	AY606892	AY606903	AY606877
Raoulia sp. 'M'	South Island, Canterbury, Mackenzie Basin, Upper Cass River	CANU38721	AY611235			

Table 1 (continued)

Species	Location	Vouchers	*psb*A-*trn*H	*trn*L intron	*trn*L-*trn*F	ITS
Raoulia mammillaris Hook.f.	South Island, Canterbury, Foggy Peak South Island, Canterbury, Mt Hutt	CHR569860 CANU38629	AY611206			AY606874
Raoulia rubra Buchanan	North Island, Wellington, Mt Holdsworth	CHR546183 CHR546184	AY611211	AY606882	AY606893	
Raoulia tenuicaulis Hook.f.	cultivated, Landcare Research, Lincoln	unvouchered	AY611217			
Raoulia youngii (Hook.f.) Beauverd	South Island, Otago, Treble Cone	CANU38723	AY611229			AY606876

[a]These samples are included only in ITS analysis, no chloroplast DNA sequences are available for them.

sequences using TBR branch swapping and 1000 random addition replicates, with multrees but without steepest descent in effect (see http://paup.csit.fsu.edu/problems.html), were also conducted. DELTRAN character optimisation was used as PAUP4.0b10 reports incorrect branch lengths for outgroup rooted trees for our data with ACCTRAN optimisation (see http://paup.csit.fsu.edu/problems.html). Bootstrap values were calculated from 1000 bootstrap replicates resampling the original number of characters using full heuristic search, multrees and steepest descent in effect, collapsing branches if their minimum length was zero. Alignment gaps were treated as missing data for ITS sequence analysis. Heteroplasmy, where detected in ITS sequences, was treated as ambiguity for the purposes of analysis. For the *psb*A-*trn*H analysis alignment gaps were coded as presence/absence characters and included in analyses. Analysis of chloroplast DNA sequences for New Zealand endemic Gnaphalieae together with those of *Euchiton audax, Argyrotegium mackayi, Pterygopappus lawrencei, Ewartia catipes* and several *Craspedia* samples (Smissen, unpublished sequences) was also undertaken using similar methods to those described above.

Results

Eighteen haplotypes were identified from *psb*A–*trn*H sequences. Distribution of these among the species sampled is shown in Table 2 and relationships inferred among them by median network analysis are shown in Fig. 1. Nucleotide substitutions are apparent at a total of 19 sites and four alignment gaps attributable to insertion/deletion events (indels) unique to haplotypes were detected. Alignment gaps range in size from 1 to 36 base pairs in length. Two characters support conflicting splits between samples and this is reflected in the box linking haplotypes C1, C2, C3 and C4 in the median network. Maximum nucleotide divergence among the haplotypes (between A5 and C6) was 3.8%. The maximum haplotype divergence detected within a species was 3.1%. This was between the A5 and C5 haplotypes detected in *R. buchananii*.

The *trn*L intron and *trn*L-*trn*F intergenic spacer appear to have slower rates of

Table 2. Distribution of cpDNA haplotypes among plant samples

Haplotype	Species occurrence	Specimens
A1	*Raoulia rubra*	CHR 546183, CHR 546184
	Leucogenes leontopodium	CHR510025
A2	*Leucogenes grandiceps*	CHR514141, CANU38729
A3	*Anaphalioides bellidioides*	CANU38726, CANU38727
A4	*Anaphalioides mariae*	CHR492179
A5	*Anaphalioides trinervis*	CHR569864
	Raoulia buchananii	CHR 546188, CHR546190
A6	*Helichrysum lanceolatum*	CHR569865, CHR569866,
A7	*Helichrysum lanceolatum*	CHR565241
A8	*Helichrysum lanceolatum*	CHR569867, CHR569871
A9	*Helichrysum lanceolatum*	CHR569868, CHR569869, CHR569870, CHR569872, CHR569873
A10	*Ewartia sinclairii*	CANU37227
B1	*Raoulia grandiflora*	Lagoon saddle (unvouchered)
B2	*Raoulia hectorii*	CANU38722
	Raoulia eximia	CHR546180, CHR546182
C1	*Raoulia* sp. 'L'	CANU38725
C2	*Raoulia bryoides*	CHR502568
	Raoulia eximia	CHR546147
	Raoulia sp. 'M'	CANU38721
C3	*Raoulia tenuicaulis*	unvouchered[a]
	Raoulia hookeri	CHR569861
C4	*Raoulia beauverdii*	CANU38724
	Helichrysum filicaule	CHR569874
C5	*Raoulia buchananii*	CHR565559, CHR 569758
	Raoulia eximia	CHR546162, CHR546168, CHR546177, CHR546178, CHR546154, CHR546160, CHR546164, CHR569875
	Raoulia goyenii	CHR560933
	Raoulia grandiflora	CANU38728
	Raoulia mammillaris	CHR569860, CANU38629
	Raoulia youngii	CANU38723
C6	*Raoulia eximia*	CHR546146, CHR546151, CHR509743

[a]The DNA extract used for this sample is the same as that used by Glenny and Wagstaff (1997) for which no voucher was made.

nucleotide substitution than the *psb*A-*trn*H intergenic spacer in this group (maximum divergence between the sampled haplotypes of 1.2% for each locus). Combining *psb*A-*trn*H sequences with those of the *trn*L intron and *trn*L-*trn*F intergenic spacer region for a subset of 12 haplotypes gives a total of 27 variable sites, 8 of which are potentially informative about phylogenetic relationships among haplotypes. A median network generated for these data (Fig. 2) shows slightly improved resolution of relationships over that for the *psb*A-*trn*H sequences alone. An unrooted strict consensus of shortest trees from a parsimony analysis for these data is also presented (Fig. 3). We explored possible outgroup rooting with Australian Gnaphalieae and New Zealand Gnaphalieae previously evidenced to be outside the New Zealand endemic clade (Ward 1993, Breitwieser et al. 1999, Smissen et al. 2003, Breitwieser and Ward 2003). Different outgroups grouped with different parts of the

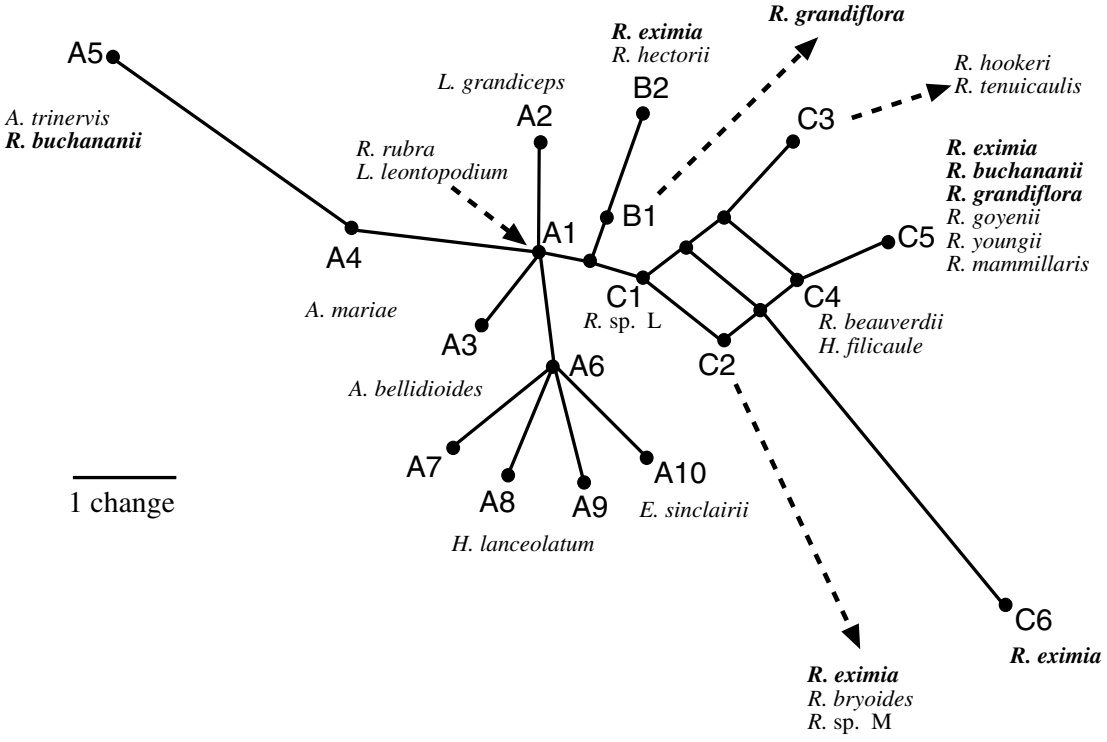

Fig. 1. Median network for *psb*A-*trn*H haplotypes of *Raoulia* and allied genera. "Boxes" represent conflicting signals in the data. Branch lengths are drawn proportional to the number of differences between sequences. The species from which each haplotype has been sampled are listed. Species shown in bold are those from which non-monophyletic groups of haplotypes have been sampled

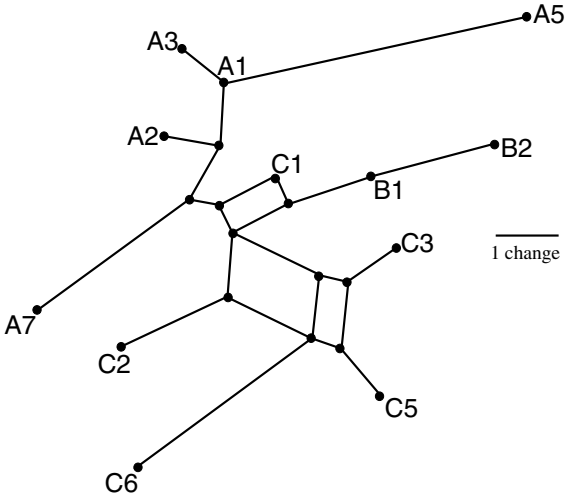

Fig. 2. Median network for combined *trn*L intron, *trn*L-*trn*F intergenic spacer and *psb*A-*trn*H intergenic spacer sequences of selected haplotypes. Labels refer to haplotypes shown in Fig. 1 and Table 2. Branch lengths are drawn proportional to the number of differences between sequences

ingroup (attributable to homoplasy) giving no clear picture as to where the root should be placed (not shown). Consequently we have

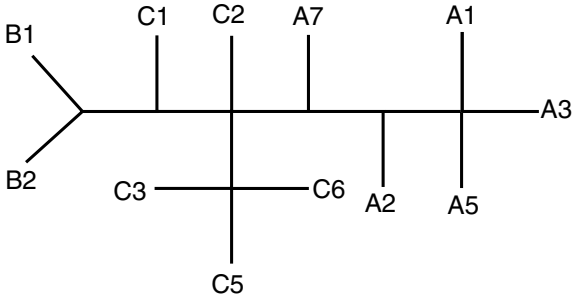

Fig. 3. Unrooted strict consensus of four most parsimonious trees for combined *trn*L intron, *trn*L-*trn*F intergenic spacer, and *psb*A-*trn*H intergenic spacer sequences. Labels refer to haplotypes shown in Fig. 1 and Table 2. The most parsimonious trees contributing to this consensus had a Consistency Index of 0.967, Retention Index of 0.970 and Rescaled Consistency Index of 0.937

limited our conclusions to those that can be drawn from un-rooted networks.

Of the eight species for which more than one individual has been sampled (*Anaphalioides bellidioides, Leucogenes grandiceps*, *Raoulia eximia, R. mammillaris, R. rubra, R. buchananii, R. grandiflora, Helichrysum lanceolatum*), three possess non-monophyletic haplotype combinations. Two haplotypes were sampled from *R. buchananii* and these are separated by nine substitutions in Fig. 1 (haplotype A5 and C5 respectively). Four haplotypes were observed in *R. eximia*: C6 is so far unique to this species, C5 is shared with five other *Raoulia* species, B2 is shared with *R. hectorii,* and C2 is shared with *R. bryoides* and *R.* sp. 'M'. Two individuals of *R. grandiflora* had the C5 haplotype shared by six *Raoulia* species while the third had the unique B1 haplotype related to the B2 haplotype shared by *R. eximia* and *R. hectorii*. Of the remaining three species, only two individuals of *R. rubra*, from the same population, have been sequenced. *Raoulia rubra* is endemic to a small area in the North Island (Smissen et al. 2003) and probably has little cpDNA diversity. Only a small part of the geographic range of *L. grandiceps* is represented by the two individuals sampled, so little can be read from the fact that they display haplotypes identical at the *psb*A-*trn*H locus. Some diversity has been sampled from the widespread *Helichrysum lanceolatum* with four closely related haplotypes so far identified (A6-9). Relationships between these *H. lanceolatum* haplotypes and the related A10 haplotype (sampled from *Ewartia sinclairii*) are unresolved (Fig. 1).

Heuristic search on ITS sequences returns 22958 trees of 291 steps (Consistency Index 0.659, Retention Index 0.832 and Rescaled Consistency Index 0.548 excluding uninformative characters). These 22958 trees condense to a single topology if branches with a minimum length of zero (i.e. no unambiguous character support) are collapsed (Fig. 4). Searches with random addition sequence did not report any trees shorter than 291 steps nor any of 291 steps not agreeing with Fig. 4. Although PAUP4.0b10 reported finding more shortest trees in the random addition search (23374) than in the simple addition sequence search, the program appears to be saving too many trees (see http://paup.csit.fsu.edu/problems.html) as filtering the saved trees for "best score" retains only 22958 trees. The single condensed tree for the most part confirms the previously reported pattern of relationships (Smissen et al. 2003). A significant new result compared with previously published analyses (Breitwieser et al. 1999, Smissen et al. 2003) is the exclusion of *R. grandiflora* from a well-supported group containing the remaining sampled species of *Raoulia* and allied genera (93% bootstrap). Although Fig. 4 depicts *R. grandiflora* as the sister clade to a clade comprising the New Zealand endemic clade and sampled species of *Argyrotegium*, *Euchiton*, Australian species of *Ewartia,* and *Pterygopappus lawrencei*, bootstrap support for this clade is essentially absent (52%). The ITS data can therefore be viewed as consistent with *R. grandiflora* being sister group to the New Zealand endemic clade. There is little resolution within the New Zealand endemic clade, but three sub-groupings are noteworthy. Firstly, sequences from the species of *Anaphalioides* group weakly together (bootstrap 57%). Secondly, the ITS sequences of South Island alpine cushion species *R. eximia, R. buchananii,* and *R. goyenii* group with the sequence of *Leucogenes leontopodium* (bootstrap 64%). Thirdly, the sequences from a group of morphologically similar lowland mat-forming *Raoulia* species (*R. hookeri, R. monroi*, and *R. tenuicaulis*) form a well-supported clade (bootstrap 91%).

Discussion

cpDNA haplotype distribution. Median network and parsimony analysis of combined *psb*A-*trn*H, *trn*L intron, and *trn*L-*trn*F sequences produced very similar results, differing only in the capacity of median networks to display conflicting signal, and allow conflicting phylogenetic signal to be distinguished

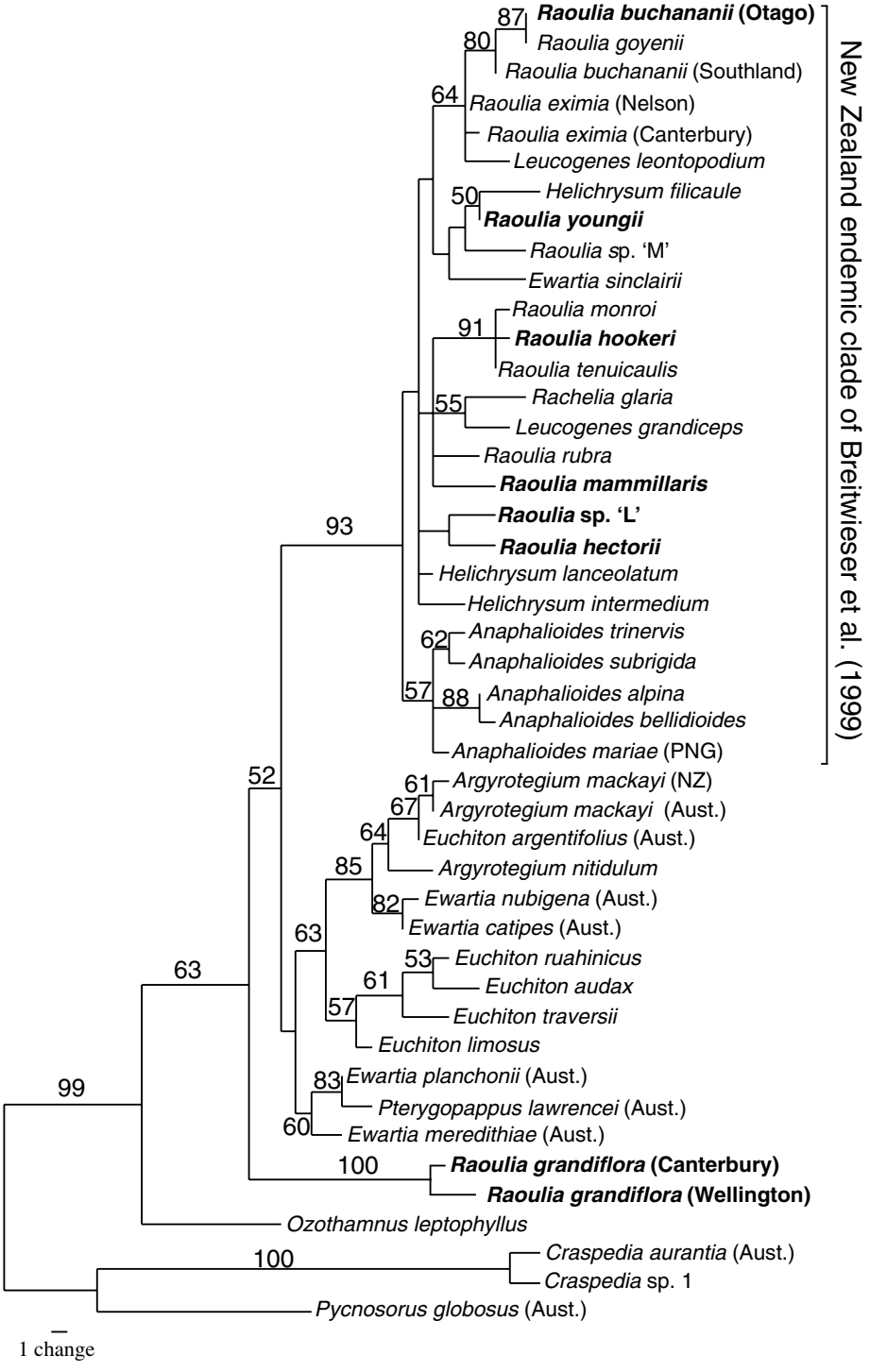

Fig. 4. Most parsimonious tree for ITS sequences. Numbers above branches are bootstrap percentages from 1000 replicates (where >50). Note that the "New Zealand endemic clade" includes the Papua New Guinea endemic *Anaphalioides mariae*. Country of origin is New Zealand unless otherwise noted (Aust = Australia, PNG = Papua New Guinea). Sequences of *Craspedia* and *Pycnosorus* have been defined as outgroups for this graph. Branch lengths are drawn proportional to the inferred number of substitutions. Species in bold are samples new to this study, others are detailed in Smissen et al. (2003) or Breitwieser et al. (1999)

easily from lack of phylogenetic signal. We present the parsimony network (Fig. 3) to demonstrate the loss of information in parsimony networks relative to the median network, and to demonstrate the general agreement over groups recovered. Combining *psb*A-*trn*H sequences with those of the *trn*L-*trn*F intergenic spacer and *trn*L intron provides improved resolution (compare Fig. 1 and Fig. 2) but adds little additional conflict, suggesting that data from these different regions of the chloroplast genome are congruent and, therefore, that the apparent pattern of chloroplast haplotype sharing observed across species is not the product of misleading homoplastic changes in the *trn*H-*psb*A intergenic spacer.

Even with the limited sampling reported here, it is clear that significant cpDNA polymorphism in New Zealand Gnaphalieae is common, and that attempts to recover a species phylogeny from cpDNA sequences are likely to be highly misleading. It should be noted that we took no steps to increase the probability of sampling introgressed individuals. In fact, in the case of *R. eximia*, we specifically tried to avoid hybrid populations. Likewise, we had no reason to expect *R. buchananii* specimens displaying the A5 haplotype shared with *Anaphalioides* to be unusual in any way.

However, some haplotype sharing observed in our study is consistent with expectations of relationships based on morphology and, in some cases, ITS sequences. The three alpine cushion species linked by similar morphology and ITS sequences, *R. eximia*, *R. goyenii* and *R. buchananii*, all share haplotype C5, although other haplotypes were also sampled from both *R. eximia* and *R. buchananii*. A fourth alpine cushion species, *R. rubra*, is morphologically very similar to the other three but has a distinct ITS sequence type and has cpDNA haplotype A1. Two lowland mat-forming *Raoulia* species, *R. hookeri* and *R. tenuicaulis*, have identical *trn*H-*psb*A sequences and have ITS sequences differing by a single unambiguous substitution. A very similar ITS sequence (differing from the *R. tenuicaulis* sequence only by the presence of an ambiguous site) is also shared by the morphologically similar species *R. monroi*, for which no cpDNA sequence is available. The *Anaphalioides* species sampled all share group A haplotypes, although these were also found in some other species.

Although numerous hybrid combinations are known among the species within the New Zealand clade that were sampled in this study, as well as other species for which sequence data are unavailable, it is not possible to explain all the haplotype sharing observed among morphologically dissimilar species on the basis of contemporary hybridisation. However, *R. bryoides* and *R. eximia* are sympatric in the St Arnaud Range where *R. eximia* samples display the C2 haplotype shared with *R. bryoides*. Occasional putative hybrids have been collected elsewhere where the two species grow together (Allan 1961, Ward 1997). Our samples of *Raoulia rubra* and *Leucogenes leontopodium* share the A1 haplotype. These species were both collected from Mt Holdsworth (Wellington, North Island) where they occur in sympatry, and putative hybrids displaying intermediate morphology and additive ITS sequences have been collected (Allan 1961, Smissen et al. 2003). The three South Island alpine cushion species *R. buchananii, R. eximia*, and *R. goyenii* possess an entirely different set of cpDNA haplotypes from *R. rubra* and *Leucogenes*, but share ITS sequence characters with *L. leontopodium* that are not shared by *R. rubra* or *L. grandiceps*. No hybrids between any of these three South Island alpine cushion species and *L. leontopodium* are known (but there is little or no overlap in their ranges). Further complicating matters, *L. grandiceps* is widespread in the South Island and does hybridise with at least *R. eximia* (McKenzie 2001), and, on Stewart Island, *R. goyenii* (Allan 1961, I.B. pers. observation). It is likely that the alpine cushion group of *Raoulia* species and the species of *Leucogenes* are linked by past and possibly ongoing reticulation, but in the absence of knowledge of ancestral ranges and the direction of hypothetical

sequence introgression, specific hypotheses accounting for the sequences we have observed would be highly speculative.

Haplotype A5 was sampled from *Anaphalioides trinervis* and *Raoulia buchananii*. No hybrids between this pair of species are known but hybrids of *A. trinervis* and *A. bellidioides* are common. *Anaphalioides bellidioides* is reported to hybridise with a range of *Raoulia, Helichrysum* and *Leucogenes* species (Glenny 1997). Also, experimental crosses between *A. trinervis* and other New Zealand Gnaphalieae, including species of *Raoulia* and *Leucogenes*, set seed (McKenzie 2001). Likewise, hybrids between *R. eximia* and *R. hectorii* (which share the B2 haplotype) are unknown, but the two are sympatric at Mt Potts, and the combination is not unlikely.

The distribution of cpDNA polymorphism among species of New Zealand Gnaphalieae reported here is difficult to explain other than through introgression among species after species divergence. However, the level of polymorphism found and the seemingly random association of haplotypes with species precludes specific simple hypotheses of introgression events. Given that haplotypes coexisting in species are widely separated in the network (Fig. 1), an alternative explanation based on maintenance of ancestral polymorphism would imply that divergence of species occurred after the evolution of most of the haplotype diversity observed (i.e. that the hypothetical ancestor possessed haplotypes indistinguishable from extant haplotypes at the *psb*A–*trn*H locus). A rapidly expanding population can be expected to maintain greater haplotype diversity than static or shrinking populations (Avise 2000), as lineage extinction is comparatively rare under such circumstances. Therefore, if speciation events producing most of the extant species of the New Zealand clade occurred in rapid succession, or even contemporaneously, and occurred after a period of major population growth in their common ancestor (perhaps following dispersal to New Zealand) this level of polymorphism might conceivably have been developed. However, such a hypothetical ancestral species would have needed both a high level of inter-population gene-flow and a high level of local differentiation to produce the extant pattern of morphological and cpDNA diversity seen today. Given these sorts of constraints, the differences between the two scenarios (ancestral polymorphism with local differentiation and inter-population gene-flow, and introgression among divergent species) start to become quantitative rather than qualitative and at least partly semantic. Importantly, both involve reticulation among diverging populations. Whether these are viewed as species, races or populations depends on the degree of divergence and definitions of the categories.

Other studies of cpDNA polymorphism in vascular plants have revealed strong associations of haplotypes with geographical areas, either as phylogeographic structure within a species or across species boundaries, perhaps as a result of co-occurring glacial refugia (Steane et al. 1998, Trewick et al. 2002, Petit et al. 2004). No such pattern associating haplotypes with geographical areas is evident from our data.

ITS sequence types. Few clades within the New Zealand endemic clade are well supported by the ITS sequence analysis. However, the grouping of *Anaphalioides* species accords with morphology and is not strongly contradicted by the cpDNA data. As discussed above, *R. monroi, R. tenuicaulis*, and *R. hookeri* are morphologically similar and their grouping in the ITS tree is therefore not unexpected. As discussed in Smissen et al. (2003) the grouping of South Island alpine cushion species *R. eximia, R. buchananii* and *R. goyenii* is consistent with morphology, but inclusion of *Leucogenes leontopodium* in this clade is not. The groupings of *R. hectorii* with *R.* sp. 'L' and the grouping of *Rachelia glaria* with *Leucogenes grandiceps* are weakly supported by ITS sequences and by their morphological similarity, whereas the pairing of *Helichrysum filicaule* with *Raoulia youngii* is weakly supported by ITS sequences and strongly contradicted by morphology.

Importantly, the well-supported branch separating the outgroup taxa and *Raoulia grandiflora* from the New Zealand endemic clade including the rest of *Raoulia* in the shortest ITS tree is not reflected in the cpDNA evidence. Also, phenetic and cladistic analyses of morphology, leaf anatomy and flavonoid profiles do not provide any support for this separation (Ward 1993; Breitwieser and Ward 1993, 2003). Overall morphology suggests a close relationship between *R. grandiflora* and *R. youngii*. Of the two cpDNA haplotypes observed in *R. grandiflora*, one is so far unique but nested well within the diversity of the rest of the group (grouped with a haplotype shared by *R. hectorii* and *R. eximia*); the other is shared with at least five other *Raoulia* species.

However, this well-supported branch in the ITS tree, and the ITS sequence data in general, should not be ignored. Firstly, these data seem to indicate a longer history of the New Zealand clade than previously evidenced (Breitwieser et al. 1999). Because ITS sequences are biparentally inherited and potentially recombining, their divergence presumably requires a low level or absence of interbreeding. The ITS separation between *R. grandiflora* and the New Zealand endemic clade strongly suggests that diversified lineages were present from an early stage in the history of the group in New Zealand (conflicting with explanations of cpDNA diversity relying exclusively on incomplete lineage sorting). Secondly, this result necessitates a re-evaluation of the conclusion of Breitwieser et al. (1999) that the New Zealand clade has evolved in New Zealand following a single dispersal event from Australia. ITS sequences provide no unambiguous evidence as to whether *R. grandiflora* is sister to the rest of *Raoulia* and allies, or originated within a distinct clade (as shown in Fig. 4). Thirdly, the ITS sequence analysis provides a temporal context for understanding the evolution of diversity in New Zealand Gnaphalieae. This analysis suggests an early divergence of *R. grandiflora* from the rest of the group, a later radiation generating most of the morphological and taxonomic diversity, and a second phase of species radiations evidenced for the South Island alpine cushion *Raoulia* species, lowland mat-forming *Raoulia* species, and *Anaphalioides* species. Reticulation may have played a role in any of these phases.

Evolutionary consequences of reticulation. Although we have presented evidence for cpDNA introgression between endemic New Zealand Gnaphalieae, there is as yet no firm evidence of nuclear gene introgression, and in particular no evidence of adaptively significant introgression. Thus, while reticulation seems to be a good explanation for the failure of DNA sequences to recover phylogenetic structure, the importance of reticulation in adaptive evolution is still a matter for speculation. However, Kim and Rieseberg (1999) have shown that nuclear gene introgression is not only possible, but has probably resulted in the formation of new taxa in *Helianthus* L. Also, given the lack of congruent hierarchical structure in morphological and chemical data sets previously analysed in the New Zealand Gnaphalieae (Breitwieser and Ward 1993, Ward 1993), it is tempting to speculate that these characters have also been subject to reticulate evolution. Future investigations of New Zealand Gnaphalieae should focus on nuclear gene introgression in selected hybridising species using multi-locus approaches such as AFLP or simple-sequence-repeat markers.

A cautionary note for phylogenetic studies. Regardless of the importance of reticulation in adaptive evolution, the high degree of intraspecific cpDNA polymorphism apparent from our limited sampling has important implications for the use of cpDNA sequences for phylogeny estimation in Asteraceae. The large within-species divergences (up to 3.1%) reflect, to a large part, the high substitution rate of the *psb*A-*trn*H intergenic spacer region of the chloroplast DNA molecule; approximately three-fold lower divergences were found in the *trn*L intron and *trn*L-*trn*F intergenic spacer. Rapid species radiations, such as those that appear to have occurred in New Zealand Gnaphalieae, may be a common

pattern in angiosperms (Bateman 1999). A DNA-sequence-based phylogenetic study of Australian Gnaphalieae (Bayer et al. 2002) also indicates a rapid radiation of genera. Moreover, patterns of transpecific chloroplast polymorphism have been reported for *Senecio* sect. *Senecio* (Senecioneae: Asteraceae) by Comes and Abbott (1999, 2001) on the basis of restriction fragment length polymorphism. The concurrent appearance of Asteraceae pollen in the fossil records of several continents (Graham 1996) in the late Oligocene to early Miocene is consistent with a rapid spread and diversification of Asteraceae on a worldwide scale in the ancient past. Given these facts, it seems reasonable to assume that at least some of the major extant lineages of Asteraceae are descended from rapid species radiations similar to those seen in the New Zealand flora and elsewhere in the world today.

It is clearly important that before relationships among extant lineages are inferred from chloroplast phylogenies, the possibility that lineage sorting of ancestral cpDNA polymorphism or chloroplast introgression has influenced chloroplast lineage distribution be evaluated. Neigel and Avise (1986) conclude from modelling experiments that "phylogenetic distributions of mtDNA can lack concordance with species boundaries when species are recently separated". Resolving this lack of concordance requires sufficient generational time for matrilineal coalescence in each species, and sufficient reproductive isolation to prevent exchange of maternally inherited DNA between the new species. If further speciations occur before both of these conditions are met then maternally inherited DNA trees may lack concordance with species phylogeny, unaffected by the passage of further time (see hypothetical example, Fig. 5).

In the hypothetical example depicted in Fig. 5 it is assumed that nucleotide substitutions occur such that the true chloroplast phylogeny can be estimated (Fig. 5b). The true species tree for these hypothetical species is (AB)(CD). Because the divergence of species C and D occurred relatively soon after the divergence of their parent species Y from X, a subset of the chloroplast lineages present in Z was retained and subsequently sorted so that the cpDNA supports the tree ((AB)C)D. The branch supporting the A + B + C clade is relatively short, reflecting the shallow divergence of chloroplast lineages at the time of speciation. Conversely, the divergence of species A and B occurred after lineage sorting had left species X with only

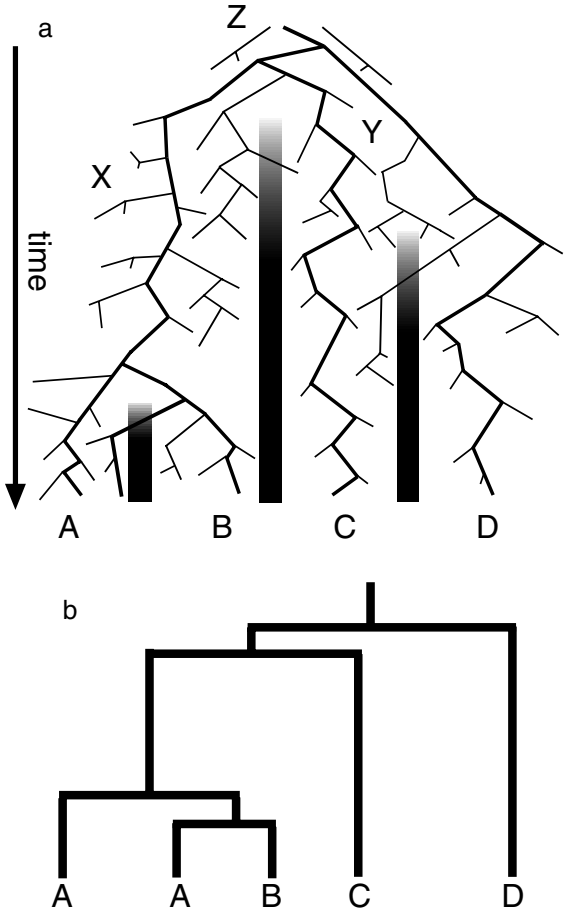

Fig. 5. Fate of chloroplast lineages during a hypothetical species radiation illustrating potential consequences of introgression and lineage sorting. Adapted from Avise (2000, p. 65). b. Expected chloroplast DNA tree for taxa A, B, C, and D given sufficient sampling of nucleotide sites. Relationships between chloroplast lineages are correctly estimated, but these do not correspond with the order of divergence of the species

one of the chloroplast lineages inherited from Z, and after reproductive isolation between species X and Y was strong enough to prevent chloroplast introgression. As a result the branch supporting the A + B clade is relatively long. Species A appears twice in the cpDNA tree as a result of polymorphism resulting from reticulation early in the divergence of species A and B. Given sufficient further time, in the absence of further chloroplast introgression, lineage sorting will occur and each of A and B will lose all but one of the chloroplast lineages shown.

As in the hypothetical example in Fig. 5, lineages descending from ancestors that evolved during rapid radiations, whose relationships are likely to be obscured by lineage sorting or introgression effects, will be grouped by relatively short branches in phylogenetic trees derived from cpDNA. Except in exceptional cases where inferences about ancestral demographics can be made, we suggest cpDNA clades grouped by branch lengths shorter than distances between haplotypes co-occurring in species should not be used to infer species phylogeny.

Importantly, these caveats apply not only to interspecific comparisons (Wendel and Doyle 1998), but also to any lineages descending from ancient rapid radiations (Avise and Wollenberg 1997). In the past, short internal branches in cpDNA trees that are likely to be misleading if used to infer species phylogeny will have tended to attract little statistical support as measured by bootstrapping, and little decay analysis (Bremer 1994) support because of their short length. Thus a conservative view of the tree will have placed little confidence in these branches. However, as multigene studies analysing larger numbers of nucleotides are undertaken, shorter branches (as measured by substitutions per site rather than absolute numbers of changes) in chloroplast phylogenetic trees will be estimated with statistical confidence (as measured by tests including bootstrapping).

Where short branches in cpDNA trees are independently supported (for example by morphological or nuclear DNA data) then it may be reasonable to place confidence in them. However, where independent support is lacking, short internal branches in phylogenetic trees estimated with the aid of very long cpDNA sequences (especially multiple chloroplast genes) should not be considered reliable estimators of species phylogeny, regardless of statistical significance, unless they exceed the level of contemporary intraspecific cpDNA variation. It should also be noted that parsimony optimisations may tend to overestimate the lengths of internal branches in trees (relative to external branches) in a process similar to the comparatively well understood phenomenon of long-branch attraction (Felsenstein 1978). Maximum likelihood optimisations are probably more appropriate for this comparison (Smissen unpublished).

Here we have shown that levels of intraspecific cpDNA sequence polymorphism can be sufficient that attempts to estimate phylogenetic relationships among well-differentiated species are completely confounded.

The authors thank Robert McKenzie for plant material and discussion, and David Glenny, Peter Lockhart and Phil Novis for field collections. Previous versions of the manuscript benefited from review by Christine Bezar, Peter B. Heenan, Steven J. Wagstaff and two anonymous reviewers.

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