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ORIGINAL ARTICLE

Biogeography and phylogeny of the New Zealand cicada genera (Hemiptera: Cicadidae) based on nuclear and mitochondrial DNA data

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ABSTRACT

Aim Determine the geographical and temporal origins of New Zealand cicadas.

Location New Zealand, eastern Australia and New Caledonia.

Methods DNA sequences from 14 species of cicadas from New Zealand, Australia, and New Caledonia were examined. A total of 4628 bp were analysed from whole genome extraction of four mitochondrial genes (cytochrome oxidase subunits I and II, and ribosomal 12S and 16S subunits) and one nuclear gene (elongation factor-1 alpha). These DNA sequences were aligned and analysed using standard phylogenetic methods based primarily on the maximum likelihood optimality criterion. Dates of divergences between clades were determined using several molecular clock methods.

Results New Zealand cicadas form two well-defined clades. One clade groups with Australian taxa, the other with New Caledonian taxa. The molecular clock analyses indicate that New Zealand genera diverged from the Australian and New Caledonian genera within the last 11.6 Myr.

Main conclusions New Zealand was likely colonized by two or more invasions. One NZ lineage has its closest relatives in Australia and the other in New Caledonia. These invasions occurred well after New Zealand became isolated from other land masses, therefore cicadas must have crossed large bodies of water to reach New Zealand.

Keywords:
Cicada, New Zealand, phylogeny, molecular clock, dispersal.

INTRODUCTION

While New Zealand contains many ancient 'Gondwanan' elements, the presumed ancient age and origin of many New Zealand taxa has been challenged in recent years (Raven, 1973; Dettman & Jarzen, 1990; Pole, 1994; Christidis *et al.*, 1996; Winkworth *et al.*, 2002). Several studies have looked at the origins of plants and vertebrates (Cooper *et al.*, 1992; Waters & Burridge, 1999; Chambers *et al.*, 2001) but few have focused on invertebrate taxa (e.g. Gleeson *et al.*, 1998). We examined the origins, both geographical and temporal, of a well-studied New Zealand insect group, the Cicadidae (order Hemiptera) (five genera, 36 described species + eight or more undescribed species).

New Zealand cicadas all belong to the tribe Cicadettini, a cosmopolitan tribe in the southern hemisphere that is found in Australia, New Caledonia, New Zealand, and Africa. They are divided into five genera: *Kikihia* (14 species) Dugdale, *Maoricicada* (14 species) Dugdale, *Rhodopsalta* (two species) Dugdale, *Notopsalta* (two species) Dugdale, and *Amphipsalta* (three species) Fleming. Nearly all New Zealand cicada species are endemic to the North and South Islands and to small surrounding islands. The only exception is a species of *Kikihia*, *K. convicta* (Distant), that is endemic to Norfolk Island (Australia), located between New Zealand and the Australian mainland. Such high levels of endemicity suggest that New Zealand cicadas have been isolated for some time. New Zealand separated from Gondwanaland 82 Ma and has remained isolated ever since (Cooper & Millener, 1993). Therefore, present day New Zealand cicadas must be the descendants of either Gondwanan species (i.e. older than 82 Myr) or of species that were able to cross hundreds of

kilometres of ocean to settle New Zealand; a remarkable feat for an insect not generally known for its dispersal abilities (Williams & Simon, 1995; de Boer & Duffels, 1996). The geographical origins of New Zealand cicadas have generally been traced to Australia or New Caledonia. Myers (1929), based on morphological evidence, hypothesized that all New Zealand cicadas were derived from a New Caledonian ancestor that invaded both New Zealand and Australia. However, Dugdale (1972) believed that New Zealand cicadas were the result of several, perhaps as many as five (one for each genus) separate invasions from Australia. Fleming (1975) believed that these invasions occurred only after angiosperms had extensively radiated throughout New Zealand 65 Ma. To distinguish between these hypotheses we determined the relationships between New Zealand cicadas and potential source populations and estimated the date of arrival of cicadas into New Zealand.

This study focused on possible source populations in Australia and New Caledonia. While cicadas are found throughout the South Pacific we limited ourselves to these two countries for a number of reasons. First, populations in these countries had previously been suggested as possible ancestors (Myers, 1929; Dugdale, 1972; Fleming, 1975). Second, these are the two closest large land masses to New Zealand and they share a recent geological history when all three were connected by the supercontinent of Gondwanaland. Third, the cicadas of New Zealand, Australia, and New Caledonia are morphologically distinct from those of New Guinea and other islands north-west of New Zealand (Duffels, 1986). A distinct Australia – New Caledonia – New Zealand biogeographical region is supported by evidence in other taxa (Duffels, 1986). Fourth, all New Zealand cicadas belong to the tribe Cicadettini and current taxonomy does not recognize species of this tribe in South America.

By using selected species from each country we established a well-supported phylogeny containing all five New Zealand genera as well as selected Australian and New Caledonian genera. Furthermore, by using molecular sequence data we also examined the timing of arrival of cicadas into New Zealand.

MATERIALS AND METHODS

Taxon sampling

Cicadas collected in the field were either preserved in 95% ethanol, frozen on dry ice, or both; followed by storage at −70 °C. Voucher specimens of each species were deposited in the entomology collection of the Department of Ecology and Evolutionary Biology, University of Connecticut. All but two genera (see below) were represented by two species and all species by two individuals (Table 1). The choice of species to represent each genus was designed to include an early branching (basal) species and any other species in the genus, thus minimizing the occurrence of long branches on the resulting phylogenetic tree. Information on the phylogenetic relationships within each genus was obtained from the literature (Dugdale, 1972) and molecular systematic studies of *Maoricicada* (Buckley *et al.*, 2001a,b) and *Kikihia* (P. Arensburger, pers. comm.).

All five New Zealand genera were sampled. The single New Zealand species of the genus *Notopsalta*, *N. sericea* (Walker, 1850), was sampled to represent the earliest branch of the lineage leading both to *Notopsalta* and *Amphipsalta*. This was suggested by molecular phylogenetic analyses (T.R. Buckley *et al.*, unpub. data), which show all three *Amphipsalta* species and the one New Zealand *Notopsalta* species to form a monophyletic polytomy with similar terminal branch lengths.

Australia contains over 100 described species within the tribe Cicadettini with many remaining to be described (Moulds, 1990, M. Moulds unpub.). From the large number of cicadettine species in Australia we chose a subset for this study. Species in this subset were identified by Dugdale (1972) and M.M. as likely relatives to New Zealand cicadas based on morphological features. In a pilot sequencing study, fragments (between 385 and 684 bp) of the cytochrome oxidase II (COII) gene were sequenced for eleven Australian Cicadettini (*Cicadetta arenaria* (Distant), *C. celis* Moulds, *C. denisoni* (Distant), *C. torrida* (Erichson), *C. puer* (Walker, 1850), *C. tristrigata* (Goding & Froggatt, 1904), *C. labeculata* (Distant), *Urabunana marshalli* Distant, *Birrima varians* (Germar), *Pauropsalta aktities* Ewart, and *P. annulata* Goding & Froggatt, 1904). As done by other systematists (e.g. Kim *et al.*, 1999), species with unusually long branches were eliminated in order to minimize problems of long-branch attraction–uniting taxa at the tips of long branches because of parallel or convergent changes rather than homology–one of the most serious problems in molecular phylogenetic analysis (Felsenstein, 1978; Hendy & Penny, 1989; Swofford *et al.*, 2001). Even if other screened Cicadettini had been more closely related to New Zealand cicadas in terms of branching pattern, the large number of autapomorphies and shared convergent bases would have made phylogenetic analysis difficult. Based on these sequencing results, two species (*C. celis* and *C. puer*) with short branches (i.e. genetically less distant from the New Zealand cicadas) were chosen to represent the Australian Cicadettini.

The relationships of the New Caledonian cicadas are less well understood than those of cicadas found in either New Zealand or Australia. Cicadas collected during a single sampling trip in 1998 were identified by M. Boulard (Muséum d'Histoire Naturelle, Paris) and M. M. Two New Caledonian cicadettine species were used in this study, *Pauropsalta johanae* Boulard and *Myersalna depicta* (Distant), based on morphological similarity with New Zealand cicadas. The majority of cicada species collected in New Caledonia belonged to the tribe Taphurini and were not used in our study.

In addition to the above species, two outgroup species were included. These were two Australian species of the tribe Parnisini, *Diemeniana frenchi* (Distant) and *D. tillyardi* Hardy. Dugdale (1972) hypothesized that based on morphology, members of this genus may be closely related to New Zealand cicadas.

Table 1 Location and date of collection of cicadas in New Zealand, Australia, and New Caledonia. Two individuals were sequenced for each species

Species	Country	Collection date	Collection location	Notes
Maoricicada hamiltoni	New Zealand	4 January 1998	Featherston, Wairarapa	
Maoricicada hamiltoni	New Zealand	4 January 1998	Featherston, Wairarapa	
Maoricicada cassiope	New Zealand	26 January 1997	Mt Ruapehu, Tongariro National Park	
Maoricicada cassiope	New Zealand	26 January 1997	Mt Ruapehu, Tongariro National Park	
Rhodopsalta cruentata	New Zealand	29 January 1997	Smith's Ford, Nelson District	
Rhodopsalta cruentata	New Zealand	29 January 1997	Smith's Ford, Nelson District	
Rhodopsalta leptomera	New Zealand	23 February 1994	Waikanae Beach, Wellington District	Specimen provided by D. Lane
Rhodopsalta leptomera	New Zealand	23 February 1994	Waikanae Beach, Wellington District	Specimen provided by D. Lane
Kikihia cauta	New Zealand	23 February 1994	Rimutaka summit, Wellington District	Specimen provided by D. Lane
Kikihia cauta	New Zealand	23 February 1994	Rimutaka summit, Wellington District	Specimen provided by D. Lane
Kikihia scutellaris	New Zealand	13 February 1994	Queen Elizabeth II park, Wellington District	Specimen provided by M. Richards
Kikihia scutellaris	New Zealand	28 January 1997	Johnston's Hill, Wellington District	
Pauropsalta johanae	New Caledonia	1 February 1998	Mont Koghis, province Sud	Specimens courtesy of the Australian Museum
Pauropsalta johanae	New Caledonia	1 February 1998	Mont Koghis, province Sud,	Specimens courtesy of the Australian Museum
Myersalna depicta	New Caledonia	6 February 1998	Park de la rivière bleue, province Sud	Specimens courtesy of the Australian Museum
Myersalna depicta	New Caledonia	6 February 1998	Park de la rivière bleue, province Sud,	Specimens courtesy of the Australian Museum
Cicadetta celis	Australia	21 January 1997	Prospect Park, Sydney, New South Wales	Specimen provided by D. Emery
Cicadetta celis	Australia	24 January 1997	Prospect Park, Sydney, New South Wales	Specimen provided by D. Emery
Cicadetta puer	Australia	12 February 1997	Mount Kapatur National Park, New South Wales	Specimens courtesy of the Australian Museum
Cicadetta puer	Australia	12 February 1997	Mount Kapatur National Park, New South Wales	
Notopsalta sericea	New Zealand	4 February 1993	Warkworth, Auckland District	
Notopsalta sericea	New Zealand	4 February 1993	Warkworth, Auckland District	
Amphipsalta cingulata	New Zealand	28 January 1997	Day's Bay, Wellington District	
Amphipsalta cingulata	New Zealand	28 January 1997	Day's Bay, Wellington District	
Diemeniana frenchi	Australia	22 January 1998	Kosciusko National Park, New South Wales	Specimens courtesy of the Australian Museum
Diemeniana frenchi	Australia	18 January 1998	Kosciusko National Park, New South Wales	Specimens courtesy of the Australian Museum
Diemeniana tillyardi	Australia	26 January 1998	South West National Park, Tasmania	Specimens courtesy of the Australian Museum
Diemeniana tillyardi	Australia	26 January 1998	South West National Park, Tasmania	Specimens courtesy of the Australian Museum

Molecular techniques

For each species, DNA was extracted from two individuals, amplified, and sequenced on different days to insure that contaminants had not mistakenly been sequenced. Total genomic extractions were performed using the CTAB/DTAB (Gustincich *et al.*, 1991) and 'salting out' (Sunnucks & Hales, 1996; Buckley *et al.*, 2001a) protocols using thoracic and/or ovarian tissue. Portions of five genes were amplified using the polymerase chain reaction (PCR): (1) the entire COII gene (693 bp), (2) a section at the 3′ end of the cytochrome oxidase I gene (COI) (753 bp), (3) domain III of the 12S small subunit region (12S) (389 bp), (4) domains IV and V of the 16S mitochondrial large subunit gene (16S) (504 bp), and (5) a large section of the elongation factor-1 alpha gene (EF1α) (2289 bp). PCR primers and conditioners are listed in Table 2. This is the same data set used by Buckley *et al.* (2002), but it is described in more detail here and examined with a different emphasis. DNA products were purified for sequencing using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Purified PCR products were sequenced using Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Amersham Biosciences, Piscataway, NJ, USA). Cycle sequencing products were cleaned by ethanol precipitation or by Sephadex spin columns and analysed on an ABI Prism™ 377 DNA Sequencer (Amersham Biosciences). Sequences were aligned using amino-acid sequences for protein coding regions and

Table 2 Primer names, sequences, and conditions used in PCR reactions of genes sequenced

Primer for gene	Primer name	Primer sequence 5′–3′	Reference	Thermal cycling conditions
Cytochrome oxidase I	C1-J-2195	ttgatttttggtcatccagaagt	Simon *et al.* (1994)	94° for 45 s 56° for 45 s
	TL2-N-3014	tccaatgcactaatctgccatatta	Simon *et al.* (1994)	72° for 75 s
Cytochrome oxidase II	TL2-J-3034	aatatggcagattagtgca	Simon *et al.* (1994)	94° for 45 s 50° for 45 s
	A8-N-3914	tcatattattggtgatatttgagg	Simon *et al.* (1994)	72° for 75 s
Domain III	SR-J-14233	aagagcaacggggcgatgtgt	Simon *et al.* (1994)	94° for 45 s 55° for 45 s
12S ribosomal small subunit	SR-N-14588	aaactaggattagataccctattat	Simon *et al.* (1994)	72° for 75 s
16S ribosomal large subunit	LR-J-12887	ccggtctgaactcagatcacgt	Simon *et al.* (1994)	94° for 45 s 56° for 45 s
	LR-N-13398	cgcctgtttaacaaaaacat	Simon *et al.* (1994)	72° for 75 s
Elongation factor 1-alpha	EF1-F001-cicada	tctacaaatgtggtggtatc	Created for project	94° for 60 s 60° for 60 s
	EF1-R752-cicada	tcagtgttgtccattttgtt	Created for project	72° for 75 s
Elongation factor 1-alpha	EF1-F650-cicada	tgctgctggtactggtgaat	Created for project	94° for 60 s 60° for 60 s
	EF1-R114-cicada	ttgatagacttgggattttc	Created for project	72° for 75 s

secondary structure for ribosomal DNA (Hickson *et al.*, 1996; Buckley *et al.*, 2000). Coding regions of the elongation factor 1-alpha sequences were aligned using the amino acid sequence. Non-coding regions were aligned using the program CLUSTALW (Thompson *et al.*, 1994) and adjusted by eye. Sequences were deposited into Genebank, accession numbers: AF314151 through AF314162 (12S); AF426274 through AF426287 (16S); AF426438 through AF426448 (COI); AF313500, AF313507, AF313515, AF313517 through AF313525 (COII); AF313526 through AF313537, AY271925, AY271926, AY271928 through AY271930 (EF1α).

Phylogenetic analyses

Most analyses were conducted using PAUP*4.0b2a (Swofford, 1998). Aligned nucleotide sequence data were partitioned into three data sets: (1) combined ribosomal 12S and 16S nucleotide sequences (12S + 16S data set, 893 bp), (2) combined COI and COII sequences (COI + COII data set, 1446 bp), and (3) elongation factor 1-alpha sequences (EF1α data set, 2289 bp, excluding 1072 bp found in two large insertions). Combining the 12S and 16S sequences and combining COI and COII sequences was justified because a parametric bootstrap analysis (Efron, 1982; Bull *et al.*, 1993a; Huelsenbeck *et al.*, 1995) showed no significant character incongruence between individual gene sequences (all $P > 0.05$).

The following analyses were performed for each data set individually (12S + 16S, 893 bp; COI + COII, 1446 bp; EF1α 2289 bp), on a data set including all mitochondrial genes (12S + 16S + COI + COII; 2339 bp), and on a data set including all sequences combined (12S + 16S + COI + COII + EF1α; 4628 bp). Because differences in base frequencies between taxa can mislead some phylogenetic methods (Lockhart *et al.*, 1994), these were examined on all sites and on parsimony informative sites only using heterogeneity chi-square tests as implemented in PAUP*. Phylogenetic analyses were conducted using maximum likelihood (Felsenstein, 1981) and maximum parsimony (Fitch, 1971) optimality criteria. An initial maximum parsimony tree search with tree-bisection-reconnection followed by branch swapping under equal weights was performed. Using a tree obtained from this search the fit of the data to this tree under a range of substitution models was calculated, following the method of Frati *et al.* (1997). Substitution models tested were those of Jukes & Cantor (1969) (JC), Kimura (1980) (K2P), Hasegawa *et al.* (1985) (HKY85), and general-time reversible (e.g. Yang, 1994) (GTR). Among-site rate variation was accommodated in three different ways: (1) assuming a proportion of sites were invariable (*I*) (e.g. Hasegawa *et al.*, 1985), (2) assuming all sites free to vary with rates among sites following a discrete approximation to the gamma distribution (*G*) (Yang, 1994), and (3) assuming that a proportion of sites were invariable with the remainder free to vary following a gamma distribution (*G* + *I*) (Gu *et al.*, 1995). The ln-likelihood scores under the above range of substitution models were evaluated using likelihood ratio tests (Goldman, 1993; Frati *et al.*, 1997, Huelsenbeck & Crandall, 1997). The model with the fewest parameters that did not differ significantly from the lowest ln

likelihood score across all models was selected for further phylogenetic analyses.

Estimating dates of divergence

Two dating methods were used, (1) a traditional global molecular clock but with taxa removed that violated the clock assumption and (2) the local clock method of Yoder & Yang (2000) that allowed different lineages to evolve at different rates.

Traditional global clock

The 12S + 16S + COI + COII gene sequences were tested for uniformity of evolutionary rate using a likelihood ratio test suggested by Felsenstein (1993). In this test, the likelihood scores of a tree constrained to evolve in a clock-like fashion and an unconstrained tree are compared. If the two scores are not significantly different the sequences are assumed to have evolved at a uniform rate. Data sets (12S and 16S) and taxa (*P. johanae* and *M. depicta*) did not evolve at a constant rate among lineages and were removed from the global clock analysis. Sequence divergences between taxa were estimated using maximum likelihood with the best-fitting GTR + G + I model. Two clock calibrations were used to obtain dates of divergence. First, the calibration of Brower (1994) of insect mitochondria (0.023 pairwise sequence divergence between taxa every million years) was used. Second, to obtain the rate of evolution, the average corrected genetic distance of the genera *Maoricicada*, *Kikihia*, and *Rhodopsalta* to each other was divided by the estimated age of these three genera based on independent geological calibrations for the genera *Maoricicada* and *Kikihia* of 9.3 Myr (C. Simon *et al.*, pers. comm.). This yielded a calibration of 0.016 sub/site/my for the COI + COII data set, and 0.020 sub/site/my for the COII data set.

Local clock

To account for the difference in evolutionary rate of the New Caledonian taxa, a 'local clock calibration' was used following the method of Yoder & Yang (2000). Four rates were specified for this method: (1) one rate for the terminal branch leading to *P. johanae*, (2) one rate for the terminal branch leading to *M. depicta*, (3) one rate for the branch uniting the two New Caledonian taxa, (4) one rate for all the remaining branches. The clock was calibrated by setting the divergence date of *Kikihia*, *Rhodopsalta*, and *Maoricicada* to 9.3 Ma (C. Simon *et al.*, pers. comm.).

RESULTS

Pattern of sequence variation

Alignment of the 12S and 16S genes was consistent with secondary structure and the conserved motifs described in Hickson *et al.* (1996) and Buckley *et al.* (2000). An aligned RNA data matrix contained a total of 893 bp (389 bp from 12S, 504 bp from 16S). A few regions of ambiguous alignment were excluded from the analysis (99 bp). PAUP* chi-square tests of homogeneity of base frequencies across taxa revealed no significant differences whether using all sites ($P = 1.0$), or parsimony informative sites only ($P = 0.93$). The two rRNA genes were combined into a single data set with the simplest most likely model of evolution a GTR + G + I ($G = 0.607$, $I = 0.490$) model. There were 128 parsimony informative sites in this data partition.

The COI and COII data matrix included the entire COII gene (693 bp) and 753 bp of the COI gene. The number of parsimony informative sites were, respectively, 143 and 149 bp. Alignment of these genes using amino acid sequences did not include any insertions or deletions and did not include any ambiguously aligned sites. A test of homogeneity of base frequencies across taxa did not indicate any significant differences when all sites were included ($P = 1.0$). However, a significant difference was detected when only parsimony informative sites were included ($P < 0.001$). Further analysis of this data set showed that this test on parsimony informative sites only was non-significant when only COII sequences were used ($P = 0.21$), but was significant when only COI sequences were used ($P = 0.03$). To test for the effect of unequal base composition on phylogenetic reconstruction when data sets were combined, an analysis of the data using a model of evolution with log-determinant corrected distances with a proportion of invariant sites removed (LogDet + I) was performed on the COI + COII data set. This model has been shown to be robust to changes in base pair composition among taxa (Lockhart *et al.*, 1994; Steel, 1994). The simplest model of evolution with the highest likelihood score was a GTR + G + I ($G = 0.51, I = 0.94$). The LogDet + I analysis produced the same topology as the other phylogenetic methods (shown in Fig. 1).

Independent analyses of the 12S + 16S data set and COI + COII data set using both maximum likelihood and maximum parsimony produced topologies consistent with the topology in Fig. 1. This same topology was well supported when all the mitochondrial genes (12S + 16S + COI + COII) were combined into a single analysis. The simplest model of evolution with the lowest ln-likelihood score was a GTR + G + I ($G = 0.49$, $I = 0.72$). The total number of parsimony informative sites in the mtDNA data was 391 bp.

A total of 2289 bp were sequenced from the elongation factor 1-alpha gene, of which 148 bp (4.40%) were parsimony informative. This included six exons (2303 bp) and five introns (1058 bp). Coding regions were highly conserved and easily aligned (5.9% of amino acids were variable). Intron regions were aligned using the program CLUSTALW (Thompson *et al.*, 1994). Two taxa had large insertions in different introns: *D. frenchi* had a 463 bp insertion, and *P. johanae* had an insertion *c.* 700 bp in size (this insertion was not completely sequenced; the size was approximated from PCR fragment size). Exons and introns were pooled into a single data set. The simplest model of evolution with the highest likelihood score was a GTR + G ($\alpha = 0.49$) model.

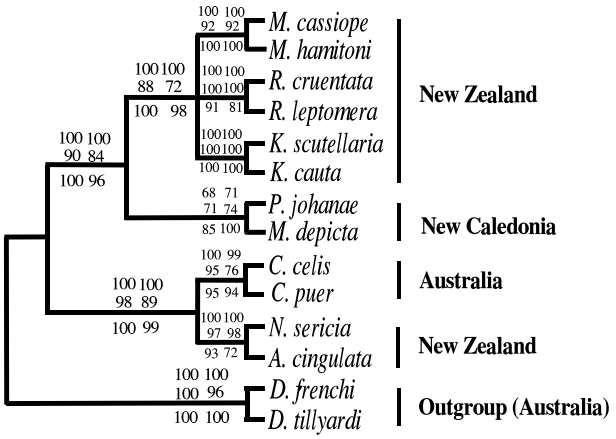

Figure 1 Consensus tree of three molecular data sets (described below) using both maximum parsimony and maximum likelihood optimatility criteria. Numbers next to each node represent bootstrap values; each column of bootstrap values corresponds to the following data sets, from top to bottom: mitochondrial (12S + 16S + COI + COII) and EF1α data sets combined, mitochondrial data set only, and EF1α data set only. Bootstraps in left columns are from maximum likelihood analyses, while bootstraps in right columns are from unweighted maximum parsimony analyses. To the right of the species names is the country where these cicadas are found.

The 12S, 16S, COI, COII, and EF1α data sets were combined into a single data set. The combinability of these data was examined by Buckley *et al.*, 2002 using a Bayesian approach. The simplest model of evolution with the highest likelihood score was a GTR model with among site rate variation accommodated using both a gamma distribution (alpha = 0.66) and invariant sites ($I = 0.41$).

Results of the phylogenetic analyses

Three of the above data sets (mtDNA alone, 12S + 16S + COI + COII; nuclear DNA alone, EF1α; and all data, 12S + 16S + COI + COII + EF1α) were used in maximum likelihood (using models of evolution described above) and evenly weighted maximum parsimony phylogenetic analyses. These analyses produced the consensus topology in Fig. 1. As expected with more data, the combined 12S, 16S, COI, COII, and EF1α data set yielded the highest bootstrap values. Maximum likelihood analyses had generally higher bootstrap values than the parsimony analyses. The only unresolved node was the trifurcation uniting the genera *Maoricicada*, *Kikihia*, and *Rhodopsalta*.

Estimates of dates of divergence

A test of uniformity of evolutionary rates on all branches of the tree was performed on the 12S + 16S + COI + COII data set. The COI + COII data set with the New Caledonian *P. johanae* excluded, and the COII data set with the *P. johanae* excluded passed the Felsenstein (1993) test of uniformity of evolutionary rates (i.e. the likelihood scores of a tree constrained to evolve in a clock-like fashion and an unconstrained tree were not significantly different), but barely ($P = 0.11$ for the COI + COII data set, $P = 0.12$ for the COII data set). However, when both New Caledonian cicadas (*P. johanae* and *M. depicta*) were excluded, the significance of the result decreased (i.e. the *P*-value increased) dramatically ($P = 0.43$ for the COI + COII data set, $P = 0.57$ for the COII data set). Exclusion of other taxa in the analysis did not yield such large changes in the significance of the test. The remaining data sets failed the Felsenstein (1993) test, even when New Caledonian and other taxa were excluded individually or in pairs. These results indicated that (1) neither the 12S, 16S, nor the 12S + 16S data sets evolved at a uniform rate among lineages, and (2) for the COI + COII and COII data sets, the New Caledonia taxa evolved at a different rate from the remaining taxa. Therefore, only the COI + COII and COII data sets with New Caledonian taxa excluded appeared to evolve at a uniform rate among lineages. Fig. 2b shows the ML (GTR + I + G) phylogram for the COI + COII data set. Divergence dates are shown on Fig. 2a.

DISCUSSION

Congruence of phylogenetic trees

New Zealand cicada genera grouped into two well-supported monophyletic clades, one clade included the genera *Amphipsalta* and *Notopsalta*, the other the genera *Kikihia*, *Maoricicada*, and *Rhodopsalta* genera (Fig. 1). The first group had an Australian sister genus (*Cicadetta*), and the second, New Caledonian sister taxa. There was strong phylogenetic signal for this result (discussed below) and this suggests a dual origin for modern New Zealand cicada genera.

The COI + COII data set failed a test of homogeneity of base pairs across taxa, but this nucleotide bias did not appear to cause phylogenetic artefacts because phylogenetic analyses performed using the COI + COII data set produced a topology identical to that obtained using the remaining data sets. Furthermore, analysis of the COI + COII data set using a model with log-determinant corrected distances (LogDet + I) (Lockhart *et al.*, 1994; Steel, 1994) that accommodate nucleotide bias among taxa produced a topology congruent with that in Fig. 1. Therefore, combining all five genes into a single data set was valid.

Combining multiple data sets is justified when the same underlying tree is being reconstructed in each data set and the mode of evolution is appropriate (Bull *et al.*, 1993b; Hillis *et al.*, 1996). This was the case here, where the best trees derived from individual data sets produced essentially the same topology as the tree from the combined data (Fig. 1). The inclusion of additional sequence data increased the bootstrap support for the tree but did not affect the topology as would be expected when compatible genes are combined. This was true of both maximum-parsimony- and maximum-likelihood-based analyses. Combination of these data sets was explored in detail by Buckley *et al.* (2002). Using a Bayesian approach they calculated the maximum likelihood topology from all five genes

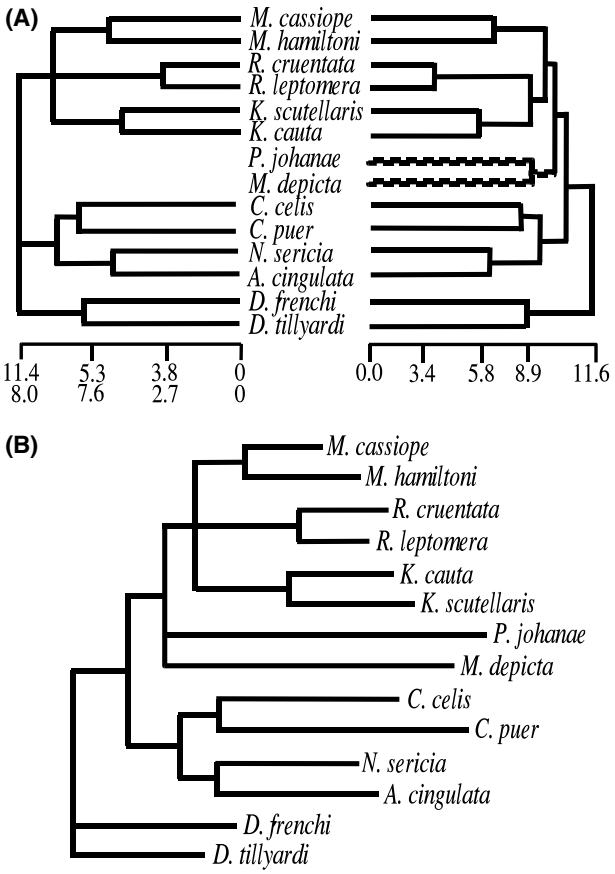

Figure 2 Estimates of divergence between New Zealand, Australian, and New Caledonian cicadas. (a) Phylogenetic trees obtained using the COI + COII data set with branch lengths estimated using the maximum likelihood optimality criterion (left) and using the method of Yoder & Yang (2000) (right). Scales below each tree indicate estimated dates of divergence (Ma) using clock calibrations derived: (1) from the age of the *Kikikhia Rhodopslata Maoricicada* clade (top left scale and right scales) and (2) using the Brower (1994) clock calibration (bottom left scale). The three branches allowed to evolve at different rates from the other branches on the right hand tree are indicated by cross-hatching. (b) Phylogram of the consensus topology in Fig. 1 using the mitochondrial (12S + 16S + COI + COII) and EF1α data sets.

individually, from the combined mitochondrial (12S + 16S + COI + COII) genes, and from all the genes combined. Their results were consistent with the hypothesis that all data partitions had evolved along the same underlying topology, justifying combining these partitions into a single analysis.

Node linking the genera Kikihia, Maoricicada, and *Rhodopsalta*

The only node in Fig. 1 with less than 50% bootstrap support linked the genera *Kikihia*, *Maoricicada*, and *Rhodopsalta* (hereafter referred to as the KMR clade). The three possible resolutions for the KMR clade were not equally well supported. Parsimony analyses yielded two most parsimonious trees with different resolutions of the KMR clade: one topology had *Rhodopsalta* and *Maoricicada* as sister genera, the other *Rhodopsalta* and *Kikihia*. Estimates of ln-likelihood scores (using maximum likelihood optimality criterion and a data set with all five genes combined) of the three possible topologies of the genera in the KMR clade indicated that the tree with *Rhodopsalta* – *Maoricicada* sister species had the lowest ln-likelihood score (-ln likelihood = 18 433.88). The remaining two topologies were tested against this most likely resolution using both parametric bootstrap and Shimodaira–Hasegawa (SH) (Shimodaira & Hasegawa, 1999) tests (H_0 = topology with *Rhodopsalta* and *Maoricicada* sister genera, H_1 = topology with *Rhodopsalta* and *Kikihia* sister genera, H_2 = topology with *Maoricicada* and *Kikihia* sister genera). These tests indicated that the H_2 topology was significantly different from the H_0 topology ($P > 0.05$ for both tests) but that the H_1 was not ($P < 0.05$ for both tests). These results are consistent with the maximum parsimony results, rejecting the topology with *Maoricicada* and *Kikihia* as sister genera.

Aside from the above molecular data few other characters are available to resolve the KMR clade. Genitalic characters, on which all three genera were mostly described, appear uninformative on this question (Dugdale, 1972), on the contrary, pleisiomorphic similarities among *Rhodopsalta*, *Amphipsalta* and *Notopsalta* genitalia are reflected in the endings of the generic names. Only the absence of an alarm call, a character state shared by *Rhodopsalta* and *Maoricicada* but not *Kikihia* may indicate a possible resolution. However, this is a very weak phylogenetic character since it involves the loss of a single character and is likely to be under strong selection.

The above results lead to the following conclusions regarding the KMR clade: (1) *Maoricicada* and *Kikihia* are not sister genera, and (2) the phylogenetic position of *Rhodopsalta* as the sister genus to either *Maoricicada* or *Kikihia* cannot be resolved using the present data. This conclusion is consistent with the analysis of these data by Buckley *et al.* (2002), who found the individual gene data sets favoring different arrangements of the KMR clade and the combined data result best represented as a KMR trichotomy. These three taxa appear to have arisen very close together in time.

Node linking P. johanae and *M. depicta*

Pauropsalta johanae and *M. depicta* were supported as sister taxa in analyses using all three optimality criteria (bootstrap values ≥68%, Fig. 1). However, both taxa were at the tips of very long branches with a short internal node, suggesting that they potentially fell into the classical 'Felsenstein zone' where long branches are incorrectly united and maximum parsimony is inconsistent (Felsenstein, 1978; Hendy & Penny, 1989). However, the fact that the maximum parsimony and maximum likelihood analyses resulted in the same topology suggests that these taxa were correctly united (Swofford *et al.*, 1996). The node linking these two taxa was the only one that had a lower bootstrap support with maximum likelihood than parsimony (Fig. 1). This was expected because maximum

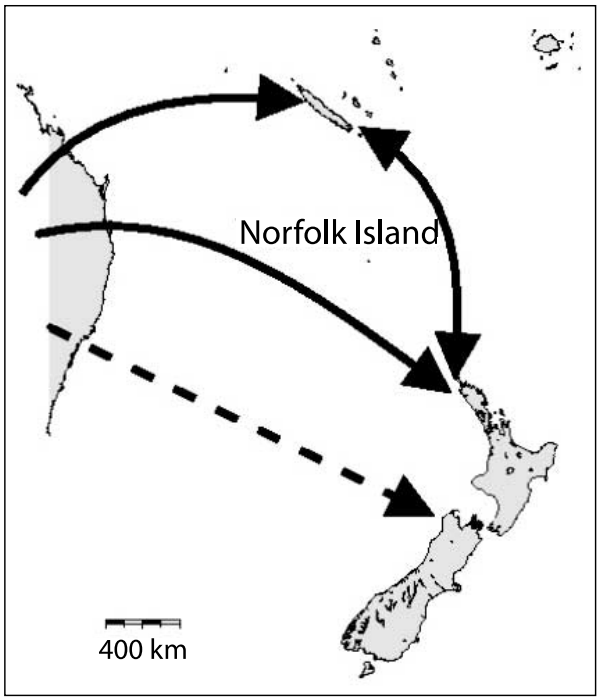

Figure 3 Possible invasion routes of cicadas into New Zealand since the Miocene. Solid arrows indicate possible invasion routes of the *Kikihia-Maoricicada-Kikihia* clade ancestors (see text) either from Australia or via New Caledonia. The dotted arrow indicates ancestors of the *Amphipsalta-Notopsalta* clade.

parsimony will assume that shared parallel changes on long branches are synapomorphies and thus cause inflated bootstrap values (Frati *et al.*, 1997).

These data strongly support a recent common ancestor of the KMR clade and New Caledonian cicadas. Topologies with several alternative placements of the New Caledonian taxa were tested using parametric bootstraps and SH tests (data not shown). These alternative topologies included placing the New Caledonian taxa in different combinations at all possible positions inside the KMR clade. All alternative topologies tested were rejected by parametric bootstrap tests ($P < 0.01$, topology in Fig. 1 used as a the null hypothesis), further supporting a recent common ancestor of the KMR clade and New Caledonian cicadas. The same conclusion was reached by Buckley *et al.* (2001c) using the same data set and Bayesian and maximum likelihood techniques. Therefore, since it appears that the KMR clade is more closely related to New Caledonian species rather than to other New Zealand species, the KMR clade must have either: (1) originated from a New Caledonian ancestor, (2) given rise to one or several New Caledonian clades, or (3) have arisen from an Australian ancestor that colonized both New Zealand and New Caledonia (Fig. 3). Examination of more taxa in the future, especially from Australia, will be necessary to rigorously infer the monophyly of the KMR-New Caledonia clade. However, based on morphological characters no Australian species appears to be an obvious candidate.

Origin of New Zealand taxa

The significance of the above phylogenetic analyses on New Zealand cicada origins should be examined in the context of prior hypotheses of modern New Zealand taxa origins. The geological history of New Zealand since the late Mesozoic suggests three possible origins of the New Zealand fauna: (1) vicariance events dating back to its separation from Gondwanaland, (2) subsequent colonization via island arcs and land bridges, and (3) long-distance dispersal.

New Zealand has been isolated from other major land masses for the last 82 Myr (Cooper & Millener, 1993). It has long been hypothesized that the origin of much of its biota could be explained by vicariance events following the breakup of Gondwanaland (e.g. Brundin, 1966; Edmunds, 1981; Stary & Block, 1998). However, to date no Cicadettini (the tribe to which all New Zealand cicadas belong) have been described from South America as would be expected from an ancient Gondwanan distribution (Metcalf, 1963a,b; Duffels & van der Laan, 1985). Therefore, the hypothesis of an old Gondwanan-wide Cicadettini would require either that the Cicadettini have gone extinct in South America or that the South American Cicadettini have not yet been described (not an impossible scenario given the little attention South American cicadas have received to date).

There appears to be little evidence that a volcanic arc or oceanic hot spot have provided even a discontinuous land bridge between New Zealand and Australia during the last 82 Myr (Cooper & Millener, 1993). Such a connection has been suggested (e.g. Linder & Crisp, 1995) but the evidence remains very poor. However, several workers have explored the possibility of a discontinuous land bridge directly between New Caledonia and New Zealand. Dettman & Jarzen (1990) suggested that organisms may have been able to island hop between New Caledonia and New Zealand via the Kermadec and Colville ridges until *c.* 40 Ma. A different route is suggested by the geological data of Herzer *et al.* (1997) who found evidence for a series of long low islands connecting New Caledonia and New Zealand that disappeared in the mid-Miocene (but see McLoughlin, 2001). This idea suggests a possible one-way migration from north to south by progressive colonization of the Norfolk Ridge in the Oligocene and the Reinga Ridge in the early Miocene, with back migration discouraged by subsequent subsidence.

There is little doubt that long-distance dispersal into New Zealand has occurred as evidenced by the large number of continental (Australian) species now found on oceanic islands surrounding New Zealand (i.e. Norfolk Island, Lord Howe, Fiji, and the Kermadec Islands, reviewed in Pole, 1994). Examples of dispersal prior to the establishment of the circum-Antarctic current (and associated west wind drift) at the end of the Oligocene (23.5 ± 2.5 Ma) are scarce (examples in Fleming, 1962; Darlington, 1965). However, after the end of the Oligocene there is abundant evidence of dispersal into New Zealand by many groups including plants (reviewed in Pole,

1994; Swenson & Bremer, 1997; Heenan, 1998; von Hagen & Kadereit, 2001; Winkworth *et al.*, 2002), mollusks (reviewed in Beu, 1998), mosses (Vitt, 1995), birds (Trewick, 1997), fish (Waters & White, 1997; Waters & Burridge, 1999) and insects (Gibbs, 1961; Close *et al.*, 1978; Adrian *et al.*, 2002). The great majority of these examples, including all insect dispersals, suggest colonization of New Zealand from Australia, presumably via the west wind drift. Direct evidence of long-distance dispersal from New Caledonia to New Zealand is more difficult to find [examples are found in Vitt (1995) and McDowall *et al.* (1998)]. However, there is considerable evidence of close relationships between New Zealand and New Caledonian species, some of which must be the result of long-distance dispersal [e.g. herpetofauna (Bauer & Vindum, 1990), Lepidoptera (Holloway, 1979; Holloway, 1993), Coleoptera (Matthews, 1998), Ephemeroptera (Chazeau, 1993)].

Phylogenetic analysis of New Zealand cicadas of the genus *Kikihia* (P. Arensburger and C. Simon, pers. comm.) demonstrate that they have shown incredible dispersal abilities by colonizing the Kermadec Islands (1000 km northwest of New Zealand, volcanic origin, Pliocene to Recent in age; Myers, 1929; Dugdale, 1972; Watt, 1975), the Chatham Islands (650 km southeast of New Zealand, separated from Gondwanaland 82 Ma; Cooper & Millener, 1993), Stewart Island (off the south tip of the South Island, connected to the South Island during the Pleistocene; Fleming, 1979), and even Norfolk Island (1000 km northwest of New Zealand, volcanic, 3.05–2.3 Myr in age; Pole, 1994).

Dates of divergence

Dates of divergence were estimated using two data sets: COI + COII and COII alone. The calibration based on the divergence date of the genera *Kikihia Rhodopsalta Maoricicada* (C. Simon *et al.*, pers. comm.; based on COI, COII, and ATPase six and eight mtDNA and two independent geological calibrations) resulted in higher divergence dates than the Brower (1994) calibration (Fig. 2b). The effect of allowing different evolutionary rates for the New Caledonian taxa was examined using the local clock method of Yoder & Yang (2000). The resulting divergence estimates were very similar to dates estimated from the calibration based on the divergence of the genera *Kikihia Rhodopsalta Maoricicada*. Regardless of which calibration was used, the results of the molecular clock analyses indicated that all taxa in this study diverged within the last 11.6 Myr. Furthermore, deep level divergences were very difficult to resolve (short branches for deep level nodes in Fig. 2). This might be indicative of a short burst of evolution sometime around 10 Ma giving rise to several modern cicada genera.

Hypotheses of New Zealand cicada origins

Several hypotheses of New Zealand cicada origins have been suggested above. Here we examine each hypothesis in light of the results of this study.

Hypothesis 1: New Zealand cicadas are Gondwanan in origin

A Gondwanan origin requires that New Zealand cicadas evolved in isolation for the last 82 Myr. The molecular clock results do not support such an ancient divergence among Australian, New Zealand, and New Caledonian cicadas. Instead, all the present taxa appear to have diverged much more recently, only within the last 11.6 Myr (Fig. 2b). Therefore, the origin of the two groups of extant New Zealand cicadas, one closely related to Australian taxa, the other to New Caledonia is more likely explained by long-distance dispersal rather than by a previously undetected vicariance event in Gondwanaland (82 Ma) or by island hopping (20–40 Ma).

Hypothesis 2: New Zealand cicadas are a monophyletic group (Myers, 1929)

Myers (1929) hypothesized a single origin for all New Zealand cicadas. Several alternative topologies in which New Zealand cicada genera were constrained to be monophyletic were tested against the topology in Fig. 1. All were strongly rejected by parametric bootstrap and SH tests (P of all tests < 0.01). Myers (1929) also hypothesized that all New Zealand cicadas were descended from an ancestor closely related to the genus *Notopsalta*. This was also unsupported by this study where the genus *Notopsalta* did not appear to be related to the common ancestor of all New Zealand cicada genera (although the ancestor could have been '*Notopsalta*-like' in appearance, we have no way of testing that hypothesis).

Hypothesis 3: New Zealand cicadas are derived from multiple invasions from Australia (Dugdale, 1972)

Two New Zealand genera, *Amphipsalta* and *Notopsalta* grouped with two of the Australian taxa sequenced in this study. The monophyly of the genera *Cicadetta*, *Amphipsalta*, and *Notopsalta* was strongly supported in this analysis by individual data sets and when all data sets were combined (Fig. 1). The corrected genetic distance based on the combined data (using GTR + G + I model) between *Amphipsalta* and *Notopsalta* was only 0.06 (data not shown). This is the lowest pairwise level of divergence between any two genera in this study and is close to the level of divergence among species in the genus *Kikihia* (e.g. corrected distance between *K. cauta* (Myers) and *K. scutellaris* (Walker, 1850) is 0.054 based on these same genes). A broader study of Australian cicadas may find species that group within the *Amphipsalta-Notopsalta* clade, but these two genera certainly have a very recent common ancestor as shown by the molecular clock analysis. Therefore, it appears likely that the *Amphipsalta-Notopsalta* clade originated from a single Australian ancestor.

The remaining three New Zealand genera (*Kikihia*, *Maoricicada*, and *Rhodopsalta*) do not appear to have as clear-cut an origin. The very small genetic distances separating the three New Zealand genera from each other (all less than 0.082 corrected genetic distance) suggest a single recent invasion

followed by adaptive radiation. As mentioned above, this analysis supports the existence of a recent common ancestor of the KMR clade and the two New Caledonian cicada taxa examined in this study. However, because both groups are monophyletic with respect to each other it is unclear which one is ancestral. Our molecular data therefore support at least two invasions of New Zealand *c.* 10 Ma (Fig. 2) followed by speciation.

Dugdale (1972), based primarily on his examination of genitalic characters, suggested that the New Zealand genera could be divided into two groups, each sharing morphological similarities with Australian genera. The first group with small pygofers, short aedeagi, long-dorsal parameres, and short ventral supports included the New Zealand genera *Amphipsalta*, *Notopsalta*, and *Rhodopsalta*, as well as the Australian genera *Frogattoides*, *Kobonga*, and *Urabunana*. The molecular data presented here do not support a close relationship between the *Amphipsalta-Notopsalta* group and the genus *Rhodopsalta*. Furthermore, a section of the COII gene was sequenced from the Australian genus *Urabunana* in the course of a preliminary study (see materials and methods). It was found to be very distant (22% uncorrected sequence divergence) from the New Zealand genera suggesting it is either a long branch or not a close relative. Dugdale's second group with large male pygofers and long aedeagi included the New Zealand genera *Kikihia* and *Maoricicada* and the Australian species *C. arenaria*, *C. denisoni*, *C. circumdata* (Walker), *C. incepta* (Walker), *C. murrayensis* (Distant), *C. landsboroughi* (Distant), and *C. tristrigata*. As indicated above, the molecular data did not support a sister relationship between *Kikihia* and *Maoricicada*. Furthermore, three of the proposed Australian *Cicadetta* species were partially sequenced for the COII gene (*C. arenaria*, *C. denisoni*, and *C. tristrigata*) and none were found to have low genetic distances to New Zealand genera (however, they could be close relatives with many autapomorphies).

CONCLUSIONS

The analysis of molecular sequence data resulted in well-supported phylogenetic trees that were consistent with at least two invasions of New Zealand. One invasion was from Australia leading to the New Zealand species in the genera *Amphipsalta* and *Notopsalta*. The second invasion was associated with New Caledonia (either an invasion of New Zealand from New Caledonia or an Australian migrant invading both New Zealand and New Caledonia at about the same time; Fig. 3) and gave rise to the genera *Kikihia*, *Maoricicada*, and *Rhodopsalta*. These invasions occurred within the last 11.6 Myr, well after New Zealand became isolated from other land masses, indicating that both invasions must have occurred through long-distance dispersal.

These results strongly reject Myers' (1929) hypothesis of a single invasion of New Zealand from a New Caledonian ancestor. However, our results are consistent with the hypothesis of multiple Australian invasions (Dugdale, 1972). Only two invasions of New Zealand could be detected here, but this was based on a small sample of Australian cicadas. Future studies may detect Australian or New Caledonian taxa that break up the monophyletic *Kikihia-Maoricicada-Rhodopsalta* clade, since cicada taxonomy of neither country is as well described as that of New Zealand.

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BIOSKETCHES

Peter Arensburger, postdoctoral researcher at the University of California, currently works on the evolution of transposable elements. His doctoral research focused on the evolution of New Zealand cicada genera and on species in the New Zealand genus *Kikihia*. **Thomas Buckley**, a researcher at Landcare Research, New Zealand, studies methods of phylogenetic analysis. His doctoral research also focused on the evolution of New Zealand cicada genera and on the New Zealand genus *Maoricicada*. **Chris Simon**, professor at the University of Connecticut, studies the systematics, evolution, and life histories of cicadas. **Max Moulds**, Research Fellow at the Australian Museum, studies the taxonomy, systematics, and acoustical mechanisms of Australian cicadas. **Kent Holsinger**, professor at the University of Connecticut, studies various aspects of conservation biology with an emphasis on population genetics.