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Hybridization, Mitochondrial DNA Phylogeography, and Prediction of the Early Stages of Reproductive Isolation: Lessons from New Zealand Cicadas (Genus *Kikihia*)

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Abstract.—One of the major tenets of the modern synthesis is that genetic differentiation among subpopulations is translated over time into genetic differentiation among species. Phylogeographic exploration is therefore essential to the study of speciation because it can reveal the presence of subpopulations that may go on to become species or that may already represent cryptic species. Acoustic species-specific mating signals provide a significant advantage for the recognition of cryptic or incipient species. Because the majority of species do not have such easily recognized premating signals, data from acoustically signaling species can serve as a valuable heuristic tool. Acoustic signals are also convenient tools for recognizing hybridization events. Here, we demonstrate that evidence of hybridization in the form of intermediate song phenotypes is present in many contact zones between species of the New Zealand grass cicadas of the *Kikihia muta* species complex and that recurring mitochondrial DNA (mtDNA) introgression has created misleading patterns that make it difficult to identify certain taxa using song or mtDNA alone. In one case, introgression appears to have occurred between allopatric taxa by dispersal of introgressed populations of an intermediary species ("hybridization by proxy"). We also present a comparison of mtDNA-tree– and song–based taxonomies obtained for the *K. muta* complex. We find that 12 mtDNA candidate species are identified using shifts in phylogenetic branching rate found by a single-threshold mixed Yule-coalescent lineage model, while only 7 candidate species are identified using songs. Results from the Yule-coalescent model are dependent on factors such as the number of modeled thresholds and the inclusion of duplicate haplotypes. Genetic distances within song species reach a maximum at about 0.028 substitutions/site when likely cases of hybridization and introgression are excluded. Large genetic breaks or "gaps" are not observed between some northern (warmer climate) song clades, possibly because climate-induced bottlenecks have been less severe. These results support ongoing calls for multimarker genetic studies as well as "integrative taxonomy" that combines information from multiple character sources, including behavior, ecology, geography, and morphology. [Barcoding, Cicadidae, cryptic species, introgression, premating isolation, sexual signals.]

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Genetic studies that sample extensively within species often reveal geographically structured phylogenetic patterns suggesting the existence of morphologically cryptic taxa (e.g., Thomas and Hedin 2008; Vogler et al. 2008). These kinds of comprehensive genetic surveys also reveal hidden instances of hybridization and introgression between species (Good et al. 2003, 2008; Berthier et al. 2006). Such cryptic population structure is likely of interest for the examination of genetic, behavioral, and ecological processes operating during the earliest stages of speciation. Furthermore, because so many species remain to be described, there is recent interest in identifying repeatable means of using genetic patterns for the delimitation of new species (DNA taxonomy) as well as the assignment of unknown specimens to known taxa (e.g., Sites and Marshall 2004; Hebert and Gregory 2005; Pons et al. 2006; Shaffer and Thompson 2007; Vogler and Monaghan 2007). Applied methods range from the use of pairwise genetic distance thresholds to the detection of branching-rate shifts on time-calibrated trees. Many of these techniques involve the use of rapidly evolving DNA segments such as mitochondrial DNA (mtDNA), but most also emphasize the need to integrate independent morphological, molecular, and other data sets (Rubinoff and Holland 2005; Will et al. 2005; Carstens and Knowles 2007a; Knowles and Carstens 2007) in part because individual gene trees and/or phenotypic character patterns do not always match the history of the underlying species lineage (Mayden 1999; de Queiroz 2007). The advantages and limitations of these approaches are frequently debated, especially the degree to which species possess genetic characteristics amenable to diagnosis using short mtDNA segments (e.g., Hebert et al. 2003; Tautz et al. 2003; Tavares and Baker 2008; Trewick 2008).

In this paper, we examine a related question—the degree to which clades identified using mtDNA exhibit divergence in phenotypic traits functionally connected to reproductive isolation, a central process for many species concepts. In one of the few studies directly addressing this question, Monaghan et al. (2006) found that morphological species of *Rivacindela* beetles diagnosed using male genitalic traits were only loosely correlated with a classification obtained by DNA barcoding and that several morphological species were incongruent with the mtDNA phylogeny. Pons et al. (2006) also compared results from DNA taxonomy (based on analysis of branch length patterns) in beetles to results obtained from morphological analyses that included genitalia, and their results suggest that

mtDNA-based barcoding may be conservative compared with morphology-based methods. In a related analysis, Hickerson et al. (2006, their fig. 5) summarized a range of empirical studies and found that pairwise mtDNA genetic distances were poor predictors of the degree of reproductive isolation.

When mate-attracting signals can be easily measured (mainly acoustic, vibratory, visual—and typically of males), the characters comprising these signals often distinguish closest relatives more effectively than do genitalia or other morphological attributes, an observation that may be related to (1) greater evolutionary lability of behavioral traits and (2) the fact that sexual signals function earlier in the mating sequence (Alexander 1964; Alexander et al. 1997). These qualities make sexual pair-forming signals attractive as taxonomic indicators and potentially useful for examining the correspondence of mtDNA phylogeography with early stages of phenotypic divergence and reproductive isolation (e.g., Salzburger et al. 2002).

Here, we examine the correspondence between mtDNA population-genetic structure and song divergence in the New Zeland grass-and-shrub cicadas of the *K. muta* complex. Previous research has shown this group to contain cryptic taxonomic diversity correlated with song and habitat divergence (Fleming 1984; Arensburger et al. 2004; Marshall et al. 2008). Additionally, numerous contact zones with evident hybridization have been suggested based mainly on song phenotype observations, and we examine likely cases of hybridization and introgression—these processes suggest difficult challenges for sorting out the species history within this group (see also Funk and Omland 2003; Mallet and Willmott 2003). We also discuss the significance of these findings for methods of DNA taxonomy.

Grass Cicadas of the K. muta *Complex*

New Zealand has experienced large recent radiations of two of its five endemic cicada genera (Fleming 1975b, 1984; Dugdale and Fleming 1978; Arensburger et al. 2004; Buckley and Simon 2007; Marshall et al. 2008; Simon 2009). One of these, the genus *Kikihia*, was the subject of intensive but unfinished work by New Zealand naturalists John Dugdale and Charles Fleming during the 1960s to 1980s. This work and recent efforts (Arensburger et al. 2004; Marshall et al. 2008, this paper), employing a combination of behavioral (acoustic mate-attracting signals), morphological, genetic, and ecological characters, suggest that the widespread, mainly grass-inhabiting cicada *K. muta* is a complex of up to seven morphologically cryptic taxa with distinctive songs. Other than *K. muta*, these have not yet been described, but we refer to the others in this paper using the following nicknames: Aotea East, Aotea West, Tuta, Nelsonensis, Westlandica North, and Westlandica South (see Fig. 1). Unambiguous song differences have not yet been identified for two additional proposed taxa, Astragali and Muta East, discussed by Fleming and Dugdale and by Marshall et al. (2008), but they are discussed briefly later in this paper. As we discuss here, Westlandica North and Westlandica South belong to a separate mitochondrial clade that includes the described tussock specialist *K. angusta* and the putative scrub-inhabiting species nicknamed Tasmani and Murihikua (Fig. 1). Exemplars of these putative species were included in phylogenetic analyses that found most *Kikihia* diversification, including that of the *K. muta* complex, to have occurred in the Pleistocene (Marshall et al. 2008). (Note that the designation of taxa in this paper as "putative species" does not imply complete absence of hybridization or gene flow.)

The *K. muta* complex offers an opportunity to examine the relationship between mitochondrial phylogeography and behavioral evolution. *Kikihia* songs are conspicuous to human ears, structurally complex, and easily recorded and measured. The mode of pair formation characterizing Cicadettini genera like *Kikihia*, in which females do not sing but respond to male song components with precisely timed wing flicks, facilitates the collection of targeted specimens because males can be attracted with finger snaps (Gwynne 1987; Lane 1995; Marshall et al. 2008; Marshall and Hill 2009). Life-history characteristics of cicadas imply low population vagility (de Boer and Duffels 1996) leading to high phylogeographic structure, especially in the tribe Cicadettini. For example, all species have nearly sessile multiple-year juvenile stages spent entirely underground (approximately 3 years for *Kikihia*; Cumber 1952; Fleming and Scott 1970; Logan 2006) and brief adult stages of one to several weeks. Body sizes are small in Cicadettini, and cicadas are not known to migrate. Pair formation in most Cicadettine species, including the New Zealand genera, involves females assuming a stationary role and "calling in" singing males with wing-flick responses—suggesting that net mtDNA dispersal could be especially low. Field observations by the authors and by John Dugdale and Charles Fleming (New Zealand Arthropod Collection, Auckland, New Zealand) suggest the existence of numerous *Kikihia* contact zones with song hybrids. Finally, New Zealand itself has proven a fertile laboratory for studies of phylogeography and hybridization (Beheregaray 2008; Goldberg et al. 2008; Morgan-Richards et al. 2009; Wallis and Trewick 2009), in part because of its dramatic and well-studied geological and climatic history (Cooper and Millener 1993; Alloway et al. 2007).

Materials and Methods

Specimen and DNA Data Collection

One hundred sixty-two specimens were sequenced from 88 sites (Table 1) selected from over 550 Global Positioning System–identified locations obtained for the *K. muta* complex during our surveys (1993 to present), covering all of mainland New Zealand except southern Fiordland and western Stewart Island. *Kikihia muta* complex cicadas are found mainly in low- to mid-elevation

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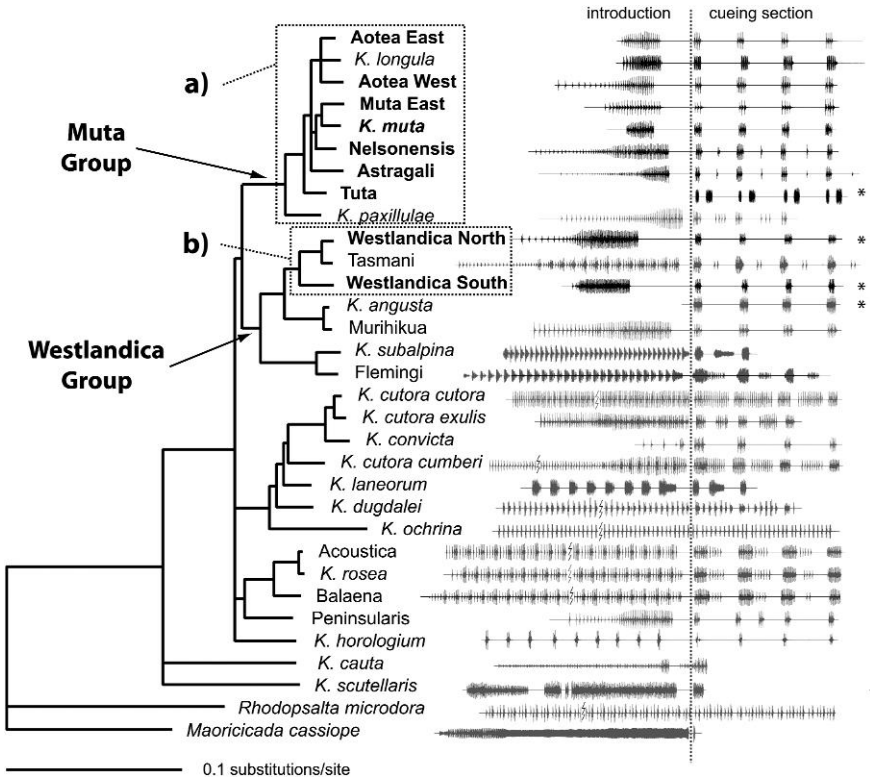

FIGURE 1. *Kikihia* mtDNA phylogeny from Marshall et al. (2008) showing phylogenetic relationships of *Kikihia muta* complex specimens to other species in the *Kikihia* phylogeny. The two boxed clades are shown in detail in Figure 2. *Kikihia* oscillograms showing example songs of each species are aligned structurally with up to the first four cues (song elements eliciting female replies) of the cueing section aligned vertically. Some songs (e.g., Nelsonensis) have minor-cues that alternate with the song cues but do not trigger female replies. Asterisks denote some songs in which the cueing section is either continuously produced (*K. angusta*, Tuta) or extended in duration compared with other *Kikihia*. Diagnostic songs for Astragali (discussed by Fleming and Dugdale and referenced in Marshall et al. 2008) and Muta East remain uncertain so these putative species are only briefly discussed here (see text). A specimen from Westlandica North was included in the *Kikihia* study of Arensburger et al. (2004) and labeled "NWCM."

regions, so our mainly road-based surveys were able to thoroughly sample the entire distribution for these insects. Choice of sampling locations was guided by preliminary phylogenetic analyses and song phenotype distributions and was intended to maximize geographic sampling of each suspected cryptic taxon. Cicadas were preserved either whole in 95% ethanol (EtOH) or by storing one to three legs in 95% EtOH with the rest of the specimen pinned. Later, DNA samples were stored at −20 to −80 °C. Because Arensburger et al. (2004) and Marshall et al. (2008) found deep mitochondrial divergences within the *K. muta* complex (Fig. 1), sequences from 26 additional specimens from the remaining 21 *Kikihia* species were included in our analyses (sequences from Marshall et al. 2008). Again, the capitalized identifiers used here for undescribed taxa (e.g., Aotea West) are intended solely as nicknames until the taxa are formally described. Outgroup taxa were selected from the endemic New Zealand genera *Maoriciada* (*Maoriciada cassiope*) and *Rhodopsalta* (*Rhodopsalta microdora*), which form an unresolved monophyletic group together with the genus *Kikihia* (Buckley et al. 2002).

Genomic DNA was extracted from one to two legs or from thoracic muscle tissue using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) or the Clontech Nucleospin Tissue Kit (Clontech, Mountain View, CA) following the manufacturers' instructions, except that the proteinase K digestion was conducted over 3–18 h. The mitochondrial cytochrome oxidase subunit I (COI; approximately 790 bp of the 3′ end) and subunit II (COII; approximately 680 bp) genes were amplified using the polymerase chain reaction and the Takara ExTaq kit using the following primers: COI, C1-J-2195 and TL2-N-3014 and COII, TL2-J-3034 and TK-N-3786 (Simon et al. 1994), with annealing temperatures 57 °C and 53 °C, respectively. Amplified products were cleaned using the Qiagen PCR Purification Kit or Clontech Extract Kit (see manufacturers above). Cycle sequencing was conducted using the Applied Biosystems Big Dye Terminator v1.1 cycle sequencing kit at 1/4- to 1/2-scale reaction volume, and the product was cleaned by Sephadex (GE Healthcare, Piscataway, NJ) filtration and visualized on an Applied Biosystems ABI 3100 capillary sequencer.

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Sequence Alignment and Model Selection

All sequences were aligned in Sequencher v3.1 (Gene Codes Corp., Ann Arbor, MI) and checked by eye. No length variation was observed. The *K. muta* complex sequences were combined with the sequences from the remaining 21 *Kikihia* species and the two outgroup taxa to construct a single phylogenetic data set. We concatenated the COI and COII data sets because of their common mitochondrial inheritance. Because some authors have suggested that mitochondrial recombination may be widespread and confound concatenation (Tsaousis et al. 2005), we tested for recombination using Genetic Algorithms for Recombination Detection (GARD), an online tool at http://www.datamonkey.org (Kosakovsky, Pond, and Frost 2005), with the data set broken into two parts alphabetically by specimen code to accommodate limits on the maximum number of sequences. For these tests, the GTR model of sequence evolution was assumed (following results given below), along with a four-category discrete gamma approximation of among-site rate variation (ASRV). Homogeneity of base composition, across all sites and across variable sites only, was tested separately for each data partition (see below) with the chi-square statistic in PAUP*4.0b10 (Sinauer Associates, Sunderland, MA).

Alternative models of sequence evolution for likelihood-based phylogenetic analyses were evaluated following Frati et al. (1997) according to the Akaike information criterion (Akaike 1973), which affords an estimate of uncertainty in model selection (Burnham and Anderson 1998). Model fitting was conducted separately on two data set partitions, one including the variable third codon position sites and the other containing the first- and second codon position sites. Preliminary trees for model fitting were obtained in PAUP*4.0b10 (Swofford 1998) using a heuristic parsimony search with 10 random addition sequence replicates and TBR branch swapping. We compared the JC69 (Jukes and Cantor 1969), K80 (Kimura 1980), HKY85 (Hasegawa et al. 1985), and GTR (Yang 1994a) models with no ASRV, with an invariant sites parameter (Hasegawa et al. 1985), with ASRV approximated by a discrete gamma distribution (Yang 1994b), or with both invariant sites and gamma-distributed ASRV (Gu et al. 1995).

Phylogenetic Analysis

Phylogenetic analysis was conducted using maximum likelihood (ML) because of the need for good branch-length estimates. Maximum parsimony methods do not correct for multiple hits, and branch lengths in Bayesian likelihood–based analyses can be strongly affected by choices of prior probability distributions (e.g., Marshall et al. 2006; Brown 2010; Marshall 2010). We used Garli-part version 0.97 (see Zwickl 2006) to obtain our phylogenetic tree. Garli-part allows data partitioning, which has been shown to lead to improvements in phylogenetic inference in Bayesian likelihood-based analyses (e.g., Nylander et al. 2004; Brown and Lemmon 2007) as well as in genetic distance estimates (Papadopoulou et al. 2010).

In the ML analysis, five independent partitioned Garli runs from random starting trees, using the best-fit model for each data partition, were conducted to verify repeatable convergence on a single best topology and branch length solution. Node support was estimated by a nonparametric bootstrap of 100 pseudoreplicates, implemented in a separate Garli analysis.

Song Analyses

Field recordings of cicada song were made using a SONY TCD-D8 DAT digital recorder, a Marantz PMD-660 compact flash recorder, or a Marantz PMD-670 compact flash recorder, combined with a Sennheiser ME-62 omnidirectional microphone mounted in a SONY PBR-330 parabolic reflector. Songs were sampled at 44.1 or 48 kHz. Canary version 1.2.1 (Cornell Bioacoustics Laboratory, Ithaca, NY) was used to visualize song patterns in the form of oscillograms (plots of sound intensity over time or waveforms).

Recordings and notes from over 550 sites where we found *K. muta* complex cicadas were used to identify different male song phenotypes, some of which were originally identified by Fleming and Dugdale (see also Marshall et al. 2008). Specifically, male song patterns were examined for discrete (presence/absence) characters that were recognizable by ear in the field and that consistently distinguished cicadas from different regions. Song qualities that may depend on body temperature (e.g., repetition rates of particular song elements) were avoided because the body temperatures of singing cicadas could not be measured or controlled during field recordings. Oscillograms were used to confirm and illustrate song character states and their basis in timbal contraction patterns (Fleming 1975a). Song types of all collected specimens were noted in the field and, when possible, digitally recorded before specimen collection. In some cases, males were recorded singing in small (approximately 1–2 L) net cages after capture.

Homology assessments of song characters are facilitated by knowledge of which male song element elicits female replies (song structure across the genus is shown in Fig. 1). Ad hoc trials using simulated female wing-flick replies (e.g., finger snaps) and free-flying males allowed us to determine the correct placement of the female reply for each of the song types. Males of all taxa flew readily to the observer when simulated wing flicks were made only after the correct cueing component of the song (see the caption of Fig. 1). In many species, the cue is repeated several times in series and/or alternated with an additional song element (minor-cue) that does not elicit female replies.

The statistical correlation between song phenotype and mtDNA haplotype was examined using Arlequin v 3.1.1 (Excoffier et al. 2005). Individual sequences were grouped according to song phenotype (as coded in Fig. 2 of Results), and an analysis of molecular variance was

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TABLE 1. Locality data and Genbank accession codes for the *Kikihia muta* complex samples used in this study, sorted by mtDNA clade of Figure 2 in alphabetical order

DNA	Specimen	District	Location	Latitude	Longitude	Elevation	COI	COII
AE1	01.WA.THR.04	Wairarapa	NE of Featherston	−41.0808	175.3656	85	HQ830740	HQ830596
AE1	01.WN.WNU.A	Wellington	Wainuiomata Hill	−41.2493	174.9212	220	EU717570	EU717589
AE1	02.HB.CAB.01	Hawkes Bay	5km NE Wmbleton	−40.4073	176.5309	138	HQ830769	HQ830625
AE1	02.HB.HRD.01	Hawkes Bay	1 km S Wanstead	−40.1465	176.5394	72	HQ830771	HQ830627
AE1	02.HB.HRD.02	Hawkes Bay	1 km S Wanstead	−40.1465	176.5394	72	HQ830772	HQ830628
AE1	02.WA.CHP.01	Wairarapa	Castle Point	−40.8961	176.2180	15	HQ830811	HQ830667
AE1	02.WA.FLP.01	Wairarapa	Flat Point	−41.2533	175.9192	5	HQ830812	HQ830668
AE1	02.WA.NGA.01	Wairarapa	20 km N of Tinui	−40.7542	176.0030	264	HQ830814	HQ830670
AE1	02.WA.SEK.01	Wairarapa	28 km S Eketahuna	−40.8655	175.6433	189	HQ830815	HQ830671
AE1	02.WA.WEB.02	Wairarapa	2 km SE of Weber	−40.4178	176.3281	149	HQ830816	HQ830672
AE1	02.WA.WEB.03	Wairarapa	2 km SE of Weber	−40.4178	176.3281	149	HQ830817	HQ830673
AE1	02.WN.ACC.01	Wellington	Pukeroa Bay	−41.0330	174.8964	66	HQ830820	HQ830676
AE1	02.WA.HAM.01	Wairarapa	28 km S Woodville	−40.5507	175.7498	180	HQ830813	HQ830669
AE2	02.BP.NUK.01	Bay of Plenty	SH2 at Nukuhau R	−38.1016	177.1397	50	HQ830747	HQ830603
AE2	02.BP.WAO.01	Bay of Plenty	SH35 Waiorore R	−37.7758	177.6722	44	HQ830749	HQ830605
AE2	02.BP.WAR.01	Bay of Plenty	7 km S of Wairata	−38.3049	177.3956	230	EF051357	EF051388
AE2	02.BP.WTK.01	Bay of Plenty	30 km S of Opotiki	−38.2275	177.3146	150	HQ830750	HQ830606
AE2	02.GB.NWA.03	Gisbourne	8 km SE Piripaua	−38.8941	177.2624	82	HQ830766	HQ830622
AE2	02.GB.WKH.01	Gisbourne	7 km W Te Karaka	−38.4646	177.7309	100	HQ830767	HQ830623
AE2	02.GB.WMB.02	Gisbourne	Lk Waikeremoana	−38.7490	177.1327	632	HQ830768	HQ830624
AE2	02.HB.GGR.03	Hawkes Bay	NW of Eskdale	−39.3501	176.7369	265	HQ830770	HQ830626
AE2	02.HB.NPU.03	Hawkes Bay	N of Putorino	−39.0822	177.0179	224	HQ830773	HQ830629
AE2	03.BP.HAU.02	Bay of Plenty	NW of Te Araroa	−37.5980	178.3210	98	HQ830826	HQ830682
AE2	03.GB.ARA.02	Gisbourne	Te Araroa	−37.6346	178.3689	11	HQ830828	HQ830683
AE2	03.GB.MAR.03	Gisbourne	1 km S Maraetaha	−38.8382	177.8949	74	HQ830829	HQ830684
AE2	03.GB.SRU.01	Gisbourne	SW of Ruatoria	−37.9157	178.2719	75	HQ830830	HQ830685
AE2	03.GB.TBY.01	Gisbourne	8 km N Tolaga Bay	−38.3183	178.2718	20	HQ830831	HQ830686
AW1	01.RI.VIN.09	Rangitikei	Vinegar Hill	−39.9263	175.6276	266	HQ830729	HQ830585
AW1	01.TK.ERS.01	Taranaki	Mt Taranaki	−39.3126	174.1464	691	EF051358	EF051389
AW1	01.TK.ERS.02	Taranaki	Mt Taranaki	−39.3126	174.1464	691	HQ830735	HQ830591
AW1	01.TK.RWY.01	Taranaki	New Plymouth	−39.0580	174.0580	20	HQ830736	HQ830592
AW1	01.TK.RWY.03	Taranaki	New Plymouth	−39.0580	174.0580	20	HQ830737	HQ830593
AW1	01.TO.RCG.01	Taupo	N of Whakapapa	−39.1919	175.5317	1068	HQ830738	HQ830594
AW1	01.TO.WPF.01	Taupo	12 km N Tarawera	−38.9572	176.5239	690	HQ830739	HQ830595
AW1	01.WI.PUT.02	Wanganui	Putorino	−39.9904	175.5968	141	HQ830745	HQ830601
AW1	02.HB.SSA.01	Hawkes Bay	4 km N Te Pohue	−39.2147	176.6883	700	EU717571	EU717590
AW1	02.TK.ORH.05	Taranaki	Ohura	−38.8479	174.9336	391	HQ830808	HQ830664
AW1	02.TO.KFP.03	Taupo	S of Rangipo	−39.1325	175.8239	640	HQ830809	HQ830665
AW1	02.TO.TAS.08	Taupo	Mt Tauhara	−38.6959	176.1631	1088	HQ830810	HQ830666
AW1	03.RI.NGA.01	Rangitikei	W Kuripapango	−39.4002	176.3124	715	HQ830814	HQ830670
AW1	03.WO.MAR.01	Waikato	SW of Te Kuiti	−38.3890	175.1272	98	HQ830845	HQ830700
AW1	93.TO.RVC.01	Taupo	Mt Ruapehu VC	−39.2050	175.5450		HQ830846	HQ830701
AW1	97.TK.ARA.01	Taranaki	Aaratora Scen. Res	−38.5058	175.2033		HQ830848	HQ830703
AW2	02.BP.CAN.02	Bay of Plenty	15 km S of Waihi	−37.4937	175.9282	45	HQ830746	HQ830602
AW2	02.BP.ROT.01	Bay of Plenty	E of Lk Rotomoa	−38.0589	176.6439	187	HQ830748	HQ830604
AW2	02.BP.WTK.02	Bay of Plenty	30 km S of Opotiki	−38.2275	177.3146	150	HQ830750	HQ830606
AW2	02.CL.TPU.03	Coromandel	Tapu	−37.0039	175.5083	—	HQ830764	HQ830620
AW2	02.CL.WAD.11	Coromandel	S of Whitianga	−36.8431	175.6641	23	HQ830765	HQ830621
AW2	02.ND.MIT.01	Northland	NW of Ruawai	−36.1228	173.9899	34	HQ830781	HQ830637
AW2	02.ND.MIT.02	Northland	NW of Ruawai	−36.1228	173.9899	34	HQ830782	HQ830638
AW2	02.WO.PPA.01	Waikato	S of Tirau	−38.0243	175.7633	131	HQ830824	HQ830680
AW2	03.BP.ETA.01	Bay of Plenty	E of Tauranga	−37.7099	176.2714	17	HQ830825	HQ830681
AW2	03.ND.KAT.01	Northland	N of Kaitaia	−35.0902	173.2551	41	HQ830837	HQ830692
AW3	03.WO.AHU.02	Waikato	SE of Rangitoto	−38.4037	175.3453	270	HQ830844	HQ830699
M1	00.WN.NEV.01	Wellington	Miramar, Wellgton	−41.3020	174.8292	100	HQ830704	HQ830560
M1	01.MC.BPT.02	M Canterbury	Banks Peninsula	−43.7800	172.7882	—	HQ830717	HQ830573
M1	01.MC.BPT.04	M Canterbury	Banks Peninsula	−43.7800	172.7882	—	HQ830718	HQ830574
M1	01.WI.FER.01	Wanganui	Feilding	−40.2299	175.5716	69	HQ830743	HQ830599
M1	01.WI.FER.03	Wanganui	Feilding	−40.2299	175.5716	69	EF051372	EF051403
M1	01.WI.MAR.01	Wanganui	9 km SE Wanganui	−39.9808	175.1300	—	HQ830744	HQ830600
M1	02.KA.SBL.01	Kaikoura	5 km N of Ward	−41.7919	174.1481	34	HQ830775	HQ830631
M1	02.KA.WIL.01	Kaikoura	4km NE Kekerengu	−41.9746	174.0412	0	HQ830777	HQ830633
M1	02.MB.WAA.01	Marlborough	8 km W Tuamarina	−41.4417	173.9083	—	HQ830779	HQ830635
M1	02.NC.NCH.03	N Canterbury	1 km N Cheviot	−42.8065	173.2743	89	HQ830780	HQ830636
M1	02.RI.MWT.01	Rangitikei	E Manawatu Gorge	−40.3359	175.8175	100	HQ830800	HQ830656
M1	02.SD.OPI.01	Mar. Sounds	Opihi Bay	−41.2972	174.1158	30	HQ830806	HQ830662
M1	02.SD.OPI.02	Mar. Sounds	Opihi Bay	−41.2972	174.1158	30	HQ830807	HQ830663
M1	02.WI.EPN.01	Wanganui	Ashhurst	−40.3053	175.7301	86	HQ830819	HQ830675
M1	02.WN.AKS.01	Wellington	Akatarawa Summit	−40.9486	175.1082	465	HQ830821	HQ830677
M1	02.WN.MAS.01	Wellington	1 km S Manakau	−40.7233	175.2125	55	HQ830822	HQ830678
M1	02.WN.MAS.02	Wellington	1 km S Manakau	−40.7233	175.2125	55	HQ830823	HQ830679
M1	03.CO.WKU.01	Centr. Otago	W of Kurow	−44.7013	170.4351	243	HQ830827	—
M1	03.MC.CBG.04	M Canterbury	Christchurch	−43.5335	172.6205	3	HQ830835	HQ830690

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TABLE 1. (Continued)

DNA	Specimen	District	Location	Latitude	Longitude	Elevation	COI	COII
M1	03.NC.JBB.01	N Canterbury	SW Const. Hill	−42.7533	173.0798	393	HQ830836	HQ830691
M1	03.ND.TAN.02	Northland	N Tangaoke Br	−34.6293	172.9673	21	HQ830838	HQ830693
M1	05.AK.NHV.02	Auckland	SH16 N Helensville	−36.6484	174.4465	15	HQ840751	HQ840749
M1	05.AK.NHV.04	Auckland	SH16 N Helensville	−36.6484	174.4465	15	HQ840752	HQ840750
M2	01.HB.ESK.01	Hawkes Bay	Eskdale	−39.3879	176.8219	31	HQ830710	HQ830566
M2	02.GB.NUH.01	Gisbourne	Nuhaka	−39.0442	177.7377	22	EF051371	EF051402
M2	02.HB.GGR.02	Hawkes Bay	NW Eskdale	−39.3501	176.7369	265	EU717578	EU717597
M2	02.HB.OCB.01	Hawkes Bay	Ocean Beach	−39.7429	177.0107	18	HQ830774	HQ830630
N1	01.MB.TWI.A	Marlborough	5 km S Havelock	−41.3378	173.7606	40	HQ830716	HQ830572
N1	01.NN.WHR.02	Nelson	NE of Mangarakau	−40.6296	172.5041	29	HQ830727	HQ830583
N1	01.SD.LIN.13	Marlb Sounds	Linkwater	−41.2917	173.8842	39	HQ830731	HQ830587
N1	01.SD.LWS.01	Marlb Sounds	3 km N Linkwater	−41.2639	173.8636	—	HQ830732	HQ830588
N1	01.SD.QCD.01	Marlb Sounds	Havelock	−41.2886	173.7712	—	HQ830734	HQ830590
N2	01.NN.CLO.01	Nelson	SW of Nelson	−41.2867	173.1208	—	HQ830719	HQ830575
N2	01.NN.COL.04	Nelson	Collingwood	−40.6810	172.6707	5	HQ830721	HQ830577
N2	01.NN.WRR.01	Nelson	SW of Nelson	−41.2950	173.1200	—	EU717579	EU717598
N2	02.NN.ABC.02	Nelson	SW of Kaka	−41.5696	172.6882	393	HQ830783	HQ830639
N2	02.NN.CBR.02	Nelson	Upper Takaka	−41.0319	172.7976	109	HQ830784	HQ830640
N2	02.NN.CBR.03	Nelson	Upper Takaka	−41.0319	172.7976	109	HQ830785	HQ830641
N2	02.NN.JDH.01	Nelson	Atawhai, Nelson	−41.2558	173.3106	29	HQ830789	HQ830645
N2	02.NN.MOT.01	Nelson	1 km N Motueka	−41.0920	173.0044	23	HQ830796	HQ830652
N2	02.NN.MOT.02	Nelson	1 km N Motueka	−41.0920	173.0044	23	HQ830797	HQ830653
N2	02.SD.FRB.01	Marlb Sounds	11km S Frnch Pass	−40.9939	173.8038	361	HQ830801	HQ830657
N2	02.SD.FRE.01	Marlb Sounds	French Pass	−40.9293	173.8442	18	HQ830802	HQ830658
N2	03.NN.HHR.01	Nelson	SH60/Hrwd Hle Rd	−41.0217	172.8950	690	HQ830839	HQ830694
N2	03.NN.PIK.01	Nelson	7.5 km S Hrwd Hle	−40.9970	172.8896	743	HQ830840	HQ830695
N3	01.NN.WCR.01	Nelson	W of Seaford	−40.5799	172.6274	12	HQ830724	HQ830580
N3	01.NN.WCR.04	Nelson	W of Seaford	−40.5799	172.6274	12	HQ830725	HQ830581
N3	02.NN.FAR.02	Nelson	Farewell Spit VC	−40.5235	172.7411	44	HQ830788	HQ830644
N3	01.NN.KNH.01	Nelson	Knuckle Hill	−40.6376	172.5634	519	HQ830722	HQ830578
N3	02.NN.KNH.01	Nelson	Knuckle Hill	−40.6376	172.5634	519	EF051359	EF051390
N3	02.NN.KNH.02	Nelson	Knuckle Hill	−40.6376	172.5634	519	HQ830790	HQ830646
N3	02.NN.KNH.03	Nelson	Knuckle Hill	−40.6376	172.5634	519	HQ830791	HQ830647
N3	02.NN.KPL.02	Nelson	SW Knuckle Hill	−40.6191	172.5492	121	HQ830793	HQ830649
N3	02.NN.MIS.03	Nelson	E of Mt Misery	−40.5358	172.6383	63	HQ830794	HQ830650
N3	02.NN.MIS.04	Nelson	E of Mt Misery	−40.5358	172.6383	63	HQ830795	HQ830651
T1	01.KA.BDS.04	Kaikoura	Blue Duck Sc. Res	−42.2754	173.7712	63	HQ830711	HQ830567
T1	01.KA.BDS.05	Kaikoura	Blue Duck Sc. Res	−42.2754	173.7712	63	HQ830712	HQ830568
T1	01.MB.TAP.01	Marlborough	Canvastown	−41.2921	173.6691	25	HQ830713	HQ830569
T1	01.MB.TAP.02	Marlborough	Canvastown	−41.2921	173.6691	25	HQ830714	HQ830570
T1	01.MB.TWI.03	Marlborough	S of Havelock	−41.3378	173.7606	40	EU717586	EU717605
T1	01.MB.TWI.10	Marlborough	S of Havelock	−41.3378	173.7606	40	HQ830715	HQ830571
T1	01.SD.CUL.01	Marlb Sounds	Cullen Pt	−41.2738	173.7879	178	HQ830730	HQ830586
T1	01.SD.LWS.02	Marlb Sounds	Kenepuru Rd	−41.2639	173.8636	—	HQ830733	HQ830589
T1	02.KA.WBS.04	Kaikoura	Whales Back Sddle	−42.4869	173.2018	542	HQ830776	HQ830632
T1	02.SD.MCC.02	Marlb Sounds	11 km E of Picton	−41.2724	174.0753	10	HQ830803	HQ830659
T1	02.SD.MCC.10	Marlb Sounds	11 km E of Picton	−41.2724	174.0753	10	HQ830804	HQ830660
T1	02.SD.OKI.01	Marlb Sounds	Okiwa Bay	−41.2927	173.8222	7	HQ830805	HQ830661
T1	03.KA.OKI.01	Kaikoura	Okiwi Reserve	−42.2197	173.8586	5	HQ830832	HQ830687
T1	03.KA.WKK.04	Kaikoura	3kmW Swyncombe	−42.3797	173.5219	79	HQ830833	HQ830688
T1	03.MB.HNR.01	Marlborough	S Hanmer Springs	−42.5351	172.8211	345	HQ830834	HQ830689
T1	03.SC.PFR.01	S. Canterbury	Peel Forest Reserve	−43.9027	171.2530	333	HQ830842	HQ830697
T2	01.NN.COL.03	Nelson	Collingwood	−40.6810	172.6707	5	HQ830720	HQ830576
T2	01.NN.KPL.01	Nelson	SW of Knuckle Hill	−40.6191	172.5492	121	HQ830723	HQ830579
T2	01.NN.WCR.02	Nelson	W of Seaford	−40.5799	172.6274	12	EU717587	EU717606
T2	02.NN.KPL.01	Nelson	SW of Knuckle Hill	−40.6191	172.5492	121	HQ830792	HQ830648
T2	02.NN.TTA.04	Nelson	N of Pakawau	−40.5495	172.7216	19	HQ830799	HQ830655
T3	02.NN.DEB.01	Nelson	S of Pepin Island	−41.1798	173.4294	23	EF051384	EF051415
T3	02.NN.RAB.13	Nelson	Rabbit Island	−41.2822	173.1294	13	HQ830798	HQ830654
WN1	01.BR.HWC.01	Buller	Hwk Crag, Buller R	−41.8650	171.7833	—	HQ830705	HQ830561
WN1	01.BR.KIL.02	Buller	Kilkenny Lkt, SH6	−41.8656	171.7819	40	HQ830707	HQ830563
WN1	01.BR.MRF.A	Buller	Maruia Falls Sc Res	−41.8606	172.2532	173	HQ830708	HQ830564
WN1	01.BR.MUR.01	Buller	Murchison	−41.7867	172.3250	—	HQ830709	HQ830565
WN1	02.BR.IRO.14	Buller	Iron Br., New Ck	−41.7867	172.0310	113	EU717584	EU717603
WN1	02.BR.ROB.03	Buller	Mt Robert	−41.8345	172.8105	1435	HQ830759	HQ830615
WN1	02.BR.ROB.10	Buller	Mt Robert	−41.8345	172.8105	1435	HQ830760	HQ830616
WN1	02.MB.SIX.01	Marlborough	Six Mile Creek	−41.7353	173.0289	433	HQ830778	HQ830634
WN1	02.NN.COR.21	Nelson	Cobb Ridge	−41.1071	172.6921	1065	HQ830786	HQ830642
WN1	96.NN.SYL.10	Nelson	Lake Sylvester	—	—	—	EF051383	EF051414
WN2	01.BR.KIL.01	Buller	Kilkenny Lookout	−41.8656	171.7819	40	HQ830706	HQ830562
WN2	01.NN.WHR.01	Nelson	NE Mangarakau	−40.6296	172.5041	29	HQ830726	HQ830582
WN2	02.BR.IRO.10	Buller	Iron Bridge, SH6	−41.7867	172.0310	113	EF051374	EF051405
WN2	02.BR.OCE.02	Buller	Tiromoana	−42.0226	171.3925	15	HQ830756	HQ830612
WN2	02.BR.OCE.06	Buller	Tiromoana	−42.0226	171.3925	15	HQ830757	HQ830613

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TABLE 1. (Continued)

DNA	Specimen	District	Location	Latitude	Longitude	Elevation	COI	COII
WN2	02.BR.PKP.04	Buller	Punakaiki	−42.1096	171.3369	16	HQ830758	HQ830614
WN2	02.BR.WES.02	Buller	S. of Westport	−41.7733	171.5922	46	HQ830763	HQ830619
WN2	02.NN.FAR.01	Nelson	Farewell Spit VC	−40.5235	172.7411	44	HQ830787	HQ830643
WS1	01.OL.HAC.A	Otago Lakes	6km SW Haast Pass	−44.1460	169.3223	—	HQ830728	HQ830584
WS1	01.WD.FJV.01	Westland	Franz Josef Glacier	−43.3921	170.1808	157	HQ830741	HQ830597
WS1	01.WD.FJV.A	Westland	Franz Josef Glacier	−43.3921	170.1808	157	HQ830742	HQ830598
WS1	02.BR.CAR.01	Buller	3 km SW of Ahaura	−42.3409	171.5724	137	HQ830752	HQ830608
WS1	02.BR.HOP.01	Buller	40km W Hanmer	−42.5911	172.4472	442	HQ830753	HQ830609
WS1	02.BR.HOP.03	Buller	40km W Hanmer	−42.5911	172.4472	442	HQ830754	HQ830610
WS1	02.BR.MRV.04	Buller	2 km E Maruia Spr	−42.3803	172.3146	580	HQ830755	HQ830611
WS1	02.BR.RUN.03	Buller	Runanga	−42.4127	171.2491	40	EF051382	EF051413
WS1	02.BR.RUN.06	Buller	Runanga	−42.4127	171.2491	40	HQ830761	HQ830617
WS1	02.BR.RUN.09	Buller	Runanga	−42.4127	171.2491	40	HQ830762	HQ830618
WS1	02.NC.APV.01	N.Canterbury	Arthur's Pass Vill.	−42.9466	171.5637	760	EU717585	EU717604
WS1	02.WD.SOT.01	Westland	1 km W Jacksons	−42.7454	171.5056	189	HQ830818	HQ830674
WS1	03.WD.LPR.01	Westland	Lake Paringa	−43.7234	169.4115	34	HQ830843	HQ830698
WS1	93.WD.IAN.39	Westland	Lake Ianthe	−43.0583	170.6367	—	HQ830847	HQ830702

Notes: The specimen code contains, in order, the last two digits of the collection year, the two-letter district code (Crosby et al. 1998), a three-letter site code, and a one- or two-character specimen number. Latitude and longitude (rounded to four decimal places) are given using New Zealand Geodetic Datum 1949. Approximate verbal locality descriptions are provided for convenience. Elevation is in meters above sea level. Specimen information for the other *Kikihia* species can be found in Marshall et al. (2008).

conducted to estimate the fraction of genetic variation explained by the resulting song groupings. Significance was assessed using a null distribution of the test statistic obtained using 1000 random permutations of the data.

Species Delimitation: Tree-Based DNA Taxonomy

Chronogram analysis.—Pons et al. (2006) demonstrated that a sharp increase in the rate of accumulation of new haplotype lineages coincided with species boundaries estimated by morphology in the beetle genus *Rivacindela* (see also Fontaneto et al. 2007). They used a ML algorithm (mixed Yule coalescent) to diagnose each species-level clade by identifying the shift in diversification rate associated with the change from reticulate evolution (within species) to divergent evolution (between species). This method was later extended to allow multiple thresholds (generalized mixed Yule-coalescent [GMYC] method, implemented in R; Papadopoulou et al. 2008; Monaghan et al. 2009) to account for variation in the waiting time to speciation (Coyne and Orr 1997, 1989). We applied the GMYC technique to a chronogram (a diagram of lineage splitting relationships over time) generated from our ML tree using penalized likelihood and the TN algorithm as implemented by the program r8s v1.71 (Sanderson 2002). Zero-length branches were added using TreeEdit v1.0a10 (Rambaut and Charleston 2001) to make the topology fully bifurcating. Because the recommended cross-validation method for selecting a smoothing parameter (which adjusts the trade-off between smoothing and fitting) returned frequent errors, we tested a variety of smoothing parameters ranging from 0.1 to 100, and, upon determining that the smoothing parameter had no visible influence on the shape of the resulting chronogram, we used the default value (1).

The GMYC model was fitted to the resulting chronogram in R across the interval range from 0 to 10 (wider interval settings were explored to confirm that this did not change the outcome). Both single- and multiple-threshold models were examined using the log-likelihood test provided in the GMYC package. Because initial results identified nearly all the tip branches as putative species, we re-ran the analyses with identical haplotypes reduced to single exemplars following Ahrens et al. (2007). The GMYC package also produced a lineage-through-time (LTT) plot, which we visually evaluated for changes in branching rate.

Statistical parsimony.—Recent studies (e.g., Monaghan et al. 2005; Hart and Sunday 2007) have applied statistical parsimony using the program TCS (Clement et al. 2000), usually under default settings, as a means to identify candidate taxa. For comparison purposes, we analyzed the complete data set (*K. muta* group unique haplotypes plus single exemplars for other *Kikihia* species) each using TCS version 1.2.1 under the default (95%) reconnection limit. Statistical parsimony (Templeton et al. 1992) uses maximum parsimony to join haplotypes into an unrooted network until the chosen limit of confidence is exceeded ("parsimony connection limit"), depending on sequence length and variability.

Pairwise Distances and DNA Taxonomy

To compare DNA divergence levels of candidate species, pairwise genetic distance values were calculated in PAUP* for all the mtDNA clades identified using the GMYC technique plus three geographically coherent clades identified visually and diagnosed in some preliminary analyses. For each clade, two sequences were selected to represent the maximum

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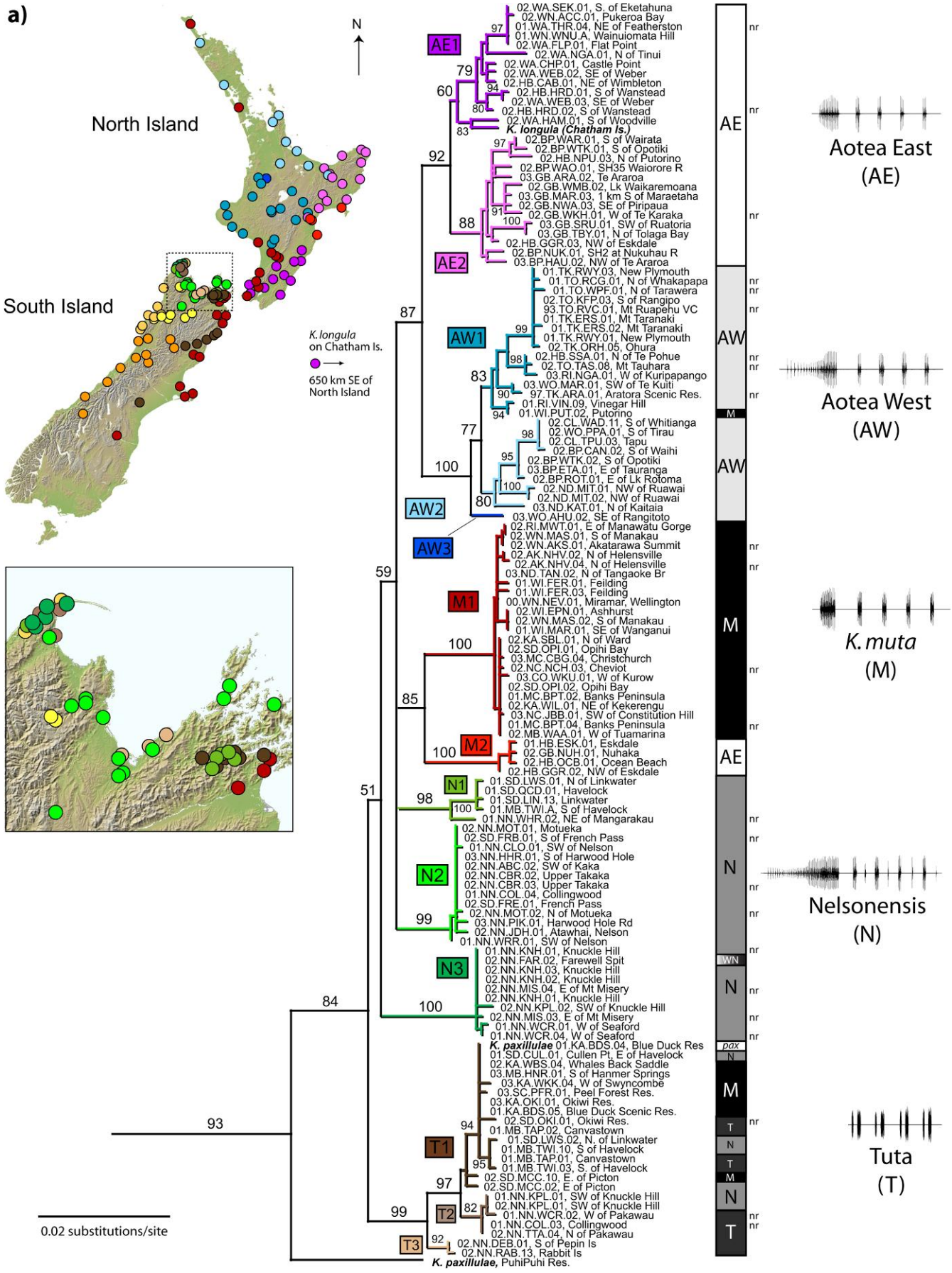

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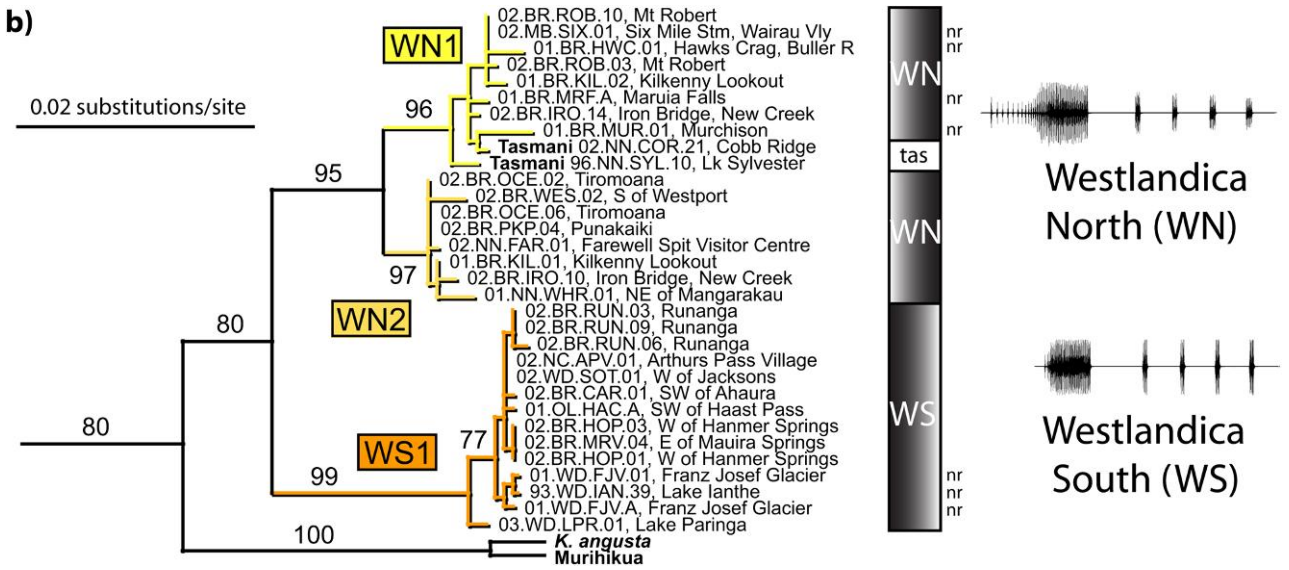

FIGURE 2. a) Song and mtDNA concordance and discordance within the *Kikihia muta* species complex (Muta group section). Node support numbers are bootstrap percentage from partitioned ML analysis in the program Garli.part (see Zwickl 2006). Two nodes with less than 50% support have been collapsed. Colors and letter plus number codes (e.g., AE1) indicate key geographically coherent mtDNA clades identified using song- and mtDNA-based methods (text). Additional clades recognized by eye (AW1–AW3) are noted to visualize additional geographic structure missed by those methods. Male song types are mapped along the vertical bar with abbreviations from Table 3 (e.g., AE = Aotea East). Oscillograms of songs from the *K. muta* complex are also shown. NR = no song recording available, song scored in the field. See text for specimen naming system. b) Song and mtDNA concordance within the *K. muta* species complex (Westlandica group section). See Figure 2a for additional explanation and geographic distribution of haplotypes.

and minimum root-to-tip distance within the group. Then, the distance between two clades was estimated using the average of the four interclade distances measured between the representative sequences. Uncorrected (p) pairwise distances were calculated as well as with model-corrected ML distances using the model parameters estimated from the Garli analysis. Finally, the same pairwise distance relationships were calculated by summing path lengths on the ML tree for comparison with the PAUP* distances. For these comparisons, haplotypes corresponding to isolated cases of hybridization in contact zones (see below) were excluded.

RESULTS

Sequence Alignment and Model Selection

A total of 1467 bases were amplified from most of the *K. muta* complex specimens, 789 from COI and 678 from COII; 107 unique mitochondrial haplotypes were observed in the concatenated *K. muta* complex data set and 209 parsimony-informative sites were found across the entire data set (including the other *Kikihia* taxa and outgroups). The alignment was unambiguous and contained no gaps. The tests of homogeneity of base frequencies were nonsignificant in all four tests ($P = 1$). GARD tests on the two halves of the data set found no evidence of recombination. Genbank accession numbers are given in Table 1, and the data matrix has been deposited with Treebase at the following address: http://purl.org/phylo/treebase/phylows/study/TB2:S11130.

The best-fitting model for the first data partition (first- and second codon positions) was GTR + I + Γ, whereas GTR + Γ was best fitting for the third codon positions. The Akaike information criterion weights for the best-fit model were 0.895 and 0.760, respectively, for the first- and second position sites combined and for the third position sites alone.

Phylogenetic Analysis

ML (Fig. 2) analysis of the extended *Kikihia* mtDNA data set yielded a tree that differed from the previous results of Marshall et al. (2008) mainly at deeper poorly supported nodes joining major species groups. These deeper level relationships were estimated in Marshall et al. (2008) with a combined nuclear plus mtDNA genetic data set and they are not considered further here. The likelihood score of the final ML tree, shown in Figure 2, was −8501.614, and the total tree length was 1.380 substitutions/site (s/s). The estimated substitution model and relative rate parameters are shown in Table 2.

Haplotypes from the *K. muta* complex formed two well-supported clades connected to different parts of the *Kikihia* tree (see Figs. 1 and 2). As in the previous studies of *Kikihia*, the deep genetic divergence within the *K. muta* complex was well supported (ML bootstrap value > 85%). The largest group of *K. muta* complex sequences, including both North Island and South Island samples, formed a monophyletic clade sister to *K. paxillulae* (which is restricted to Kaikoura) (Fig. 2a). This large clade, including *K. paxillulae*, was referred

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TABLE 2. Estimated substitution model parameters[a] from the ML phylogenetic analysis

Partition	m	R_{AC}	R_{AG}	R_{AT}	R_{CG}	R_{CT}	π_A	π_C	π_G	α	p_{inv}
1	0.283	3.929	20.170	2.563	3.678	31.963	0.292	0.148	0.171	0.548	0.758
2	2.434	2.788	29.255	0.515	0.017	32.696	0.474	0.053	0.049	1.838	N/A

[a]Relative rate multipliers (m), relative substitution rates (R), equilibrium base frequencies (π), gamma distribution hyperprior (α), and proportion of invariant sites (p_{inv}).

to as the "Muta group" by Marshall et al. (2008). The remaining *K. muta* complex sequences (Fig. 2b), all from South Island, were most closely related to a set taxa including *K. subalpina*, *K. angusta*, and the putative species Murihikua, Flemingi, and Tasmani (Westlandica group of Marshall et al. 2008). Within these larger groups, the haplotypes clustered into geographically coherent mtDNA clades, most with additional geographic substructure. These were given simple code names reflecting the song type characterizing the clade (see below). Many of these clades were supported by long branches, relative to branch lengths within the clade (e.g., M1, N3, WS1), whereas in other sections of the tree, the geographically coherent clades were not supported by long branches (e.g., AE1–AE2, AW1–AW3, and T1–T3 sections).

Haplotypes representing individuals from three other *Kikihia* species (or putative species) nested within sections of the *K. muta* tree. Specifically, one *K. paxillulae* sequence was found within the T1 clade, the *K. longula* (Chatham Is.) haplotype grouped with the AE1 clade (Fig. 2a), and both Tasmani sequences grouped with the WN1 clade (Fig. 2b).

Species Delimitation: Male Song Characters

Seven discrete (presence/absence) characters were identified (see Table 3 and oscillograms in Fig. 2) that consistently distinguished songs from the main ranges of seven candidate species (Aotea East—AE, Aotea West—AW, *K. muta*—M, Nelsonensis—N, Tuta—T, Westlandica North—WN, and Westlandica South—WS). Four characters (A, B, C, and G) are found in the introductory sections of male songs, which may function in species recognition and/or female activation. Two characters (D and F) are found in the cueing sections of the songs, which contain the repeated elements that cue female wing-flick responses. The remaining song character (E) applies to all song elements and is linked to a morphological difference in the number of timbal ribs (which affect timbal buckling, the basic mechanism of sound production). All these song variations can be visualized on a sonogram and recognized by ear in the field.

TABLE 3. Song characters distinguishing members of the *Kikihia muta* species complex

	Song character						
Taxon	A	B	C	D	E	F	G
True *K. muta* (M)	1	0	0	0	0	0	0
Nelsonensis (N)	1	1	1	1	1	0	1
Aotea West (AW)	1	1	0	0	0	0	1
Aotea East (AE)	1	0	0	0	1	0	0
Tuta (T)	0	N/A	N/A	1	0	0	N/A
Westlandica North (WN)	1	1	0	0	0	1	2
Westlandica South (WS)	1	0	0	0	0	1	0

Notes: Descriptions of characters are as follows: A, introductory section present: 0 = no, 1 = yes; B, introductory section form: 0 = syllable rate uniform ("buzz"), 1 = syllable rate accelerating ("zeet"); C, delay before cueing section: 0 = equal to delay between major song cues, 1 = half as long; D, minor-cue: 0 = absent, 1 = present; E, primary doublet form: 0 = out-click suppressed, much quieter than in-click, 1 = in- and out-clicks both loud, often equal amplitude. This song character is correlated with a morphological difference in the timbal (0 = five ribs, 1 = four ribs); F, extended cueing section: 0 = absent, 1 = present. Species with an extended cueing section produce more than 50 repeated cues in the average cueing phrase; G, amplitude modulation pattern of introductory section: 0 = absent (steady amplitude), 1 = high–low–high, 2 = low–high.

In 15 individuals, song phenotypes were observed that combined character states of two candidate song types (e.g., Fig. 3). These cicadas were all collected in or near locations of geographic contact between song species. For the Arlequin analysis, these individuals were classified with the song phenotype that matched most of the characters in the song (see vertical song phenotype bar in Fig. 2). There is no evidence that cicadas or other "singing insects" learn songs or match songs of other species, so the appearance of song characters from another species in an area of contact is best interpreted as evidence of hybridization (see also Wells and Henry 1994). Although dependence of song phenotype on larval rearing environment has been shown in one recent study (Beckers and Schul 2008), empirical evidence of song plasticity within an adult insect's life is so far limited to minor changes in song rhythm, timing, or diel patterning (e.g., Greenfield 1988), often in the context of species in which males alternate or synchronize their songs (e.g., Shaw 1975). All cases of hybridization between different *Kikihia* taxa found to date, as indicated by intermediate songs in contact zones, are noted in Table 4.

Song phenotype groups correlated well with underlying mtDNA genetic patterns (Fig. 2). Overall, group structure determined by song type explained over 67% of the variation in mtDNA ($P < 0.00001$; Table 5). Five song species (Aotea West, *K. muta*, Tuta, Westlandica North, and Westlandica South) corresponded closely to monophyletic groups containing one to three recognizable mtDNA clades. One song species, Aotea East, contained nonsister (or polyphyletic) mtDNA clades (AE1 + AE2 and M2). The remaining song species, Nelsonensis, contained a polyphyletic assemblage of three mtDNA clades (N1, N2, and N3). Three of Fleming's recordings

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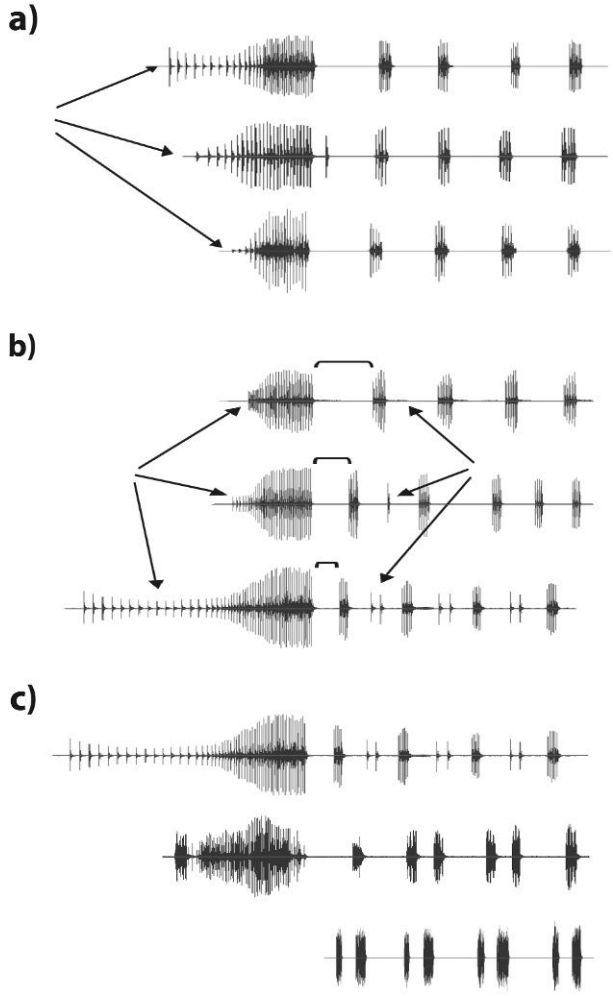

FIGURE 3. Examples of putative hybrid songs observed in contact zone sites, with the two proposed parental species. a) Aotea West (upper song), Aotea East (lower song), and hybrid (middle song). b) *Kikihia muta* (upper song), Nelsonensis (lower song), and hybrid (middle song). Arrows and brackets in A and B highlight characters showing intermediate states and combinations. c) Nelsonensis (upper song), Tuta (lower song), and hybrid (middle song). Note that Tuta lacks an introductory section. The introductory section of the putative hybrid combines qualities of the Nelsonensis introduction and the two-part song cue of Tuta, and the putative hybrid produces the two-part cue of Tuta inconsistently.

of *K. longula*, which grouped with clade AE1, showed no obvious differences from the Aotea East song type (recordings deposited at Te Papa, the National Museum of New Zealand; see Discussion section); however, additional recordings should be examined to exclude the possibility of minor differences.

In addition to the four congeneric specimens that grouped within the *K. muta* complex, several cases occurred in which one or more individuals with an unambiguous song phenotype of one *K. muta* species grouped phylogenetically with haplotypes of a different *K. muta* song species. Two of these cases, involving single individuals (01.WI.PUT.02 and 02.NN.FAR.02; Fig. 2), occurred in populations located at the boundary between two song species, and in both instances, the conflicting song phenotype corresponded to that of the other species in the contact zone, suggesting hybridization (see Table 4 for a list of putative song-hybrids we have found in *Kikihia*). More significantly, 13 specimens with song (and color pattern) phenotypes indicating Nelsonensis or Muta species identity (plus the *K. paxillulae* specimen mentioned earlier) grouped with the Tuta song species (Clade T1; Fig. 2a). Although some of these individuals were found in contact zones with the alternative song species, six specimens possessing clade T1 mtDNA, but with Muta song, were found at six locations extending along inland eastern South Island as far as Peel Forest, hundreds of kilometers from the nearest Tuta song population. This last case may involve secondary contact and introgression followed by dispersal and a second introgression event involving a third species (see Discussion section).

Species Delimitation: mtDNA

Chronogram and GMYC analysis.—The LTT plot (Fig. 4) showed an approximately steady increase in lineage accumulation with a sharp increase in diversification rate toward the present. The GMYC algorithm, when applied with the single-threshold assumption (and duplicate haplotypes removed), recognized 12 putatively intraspecific clades arising at about the time of this apparent rate shift (Fig. 4). The likelihood-ratio test statistic for this outcome was 20.5988 ($P = 0.00013$). Four of the mtDNA clades identified using the GMYC algorithm corresponded to taxa distinguished using song. The multiple-threshold GMYC model also found a highly significant result (likelihood-ratio statistic was 29.19675, $P = 0.00005$) with three inferred rate shifts, one at the same time as the single-threshold model and two more recent shifts (one very close to the present). These yielded 37 *K. muta* mtDNA clades, with all but one of the additional clades nesting within the AW, AE1, AE2, and T clades from the single-threshold model (results not illustrated). When splits corresponding to song changes were identified on this plot, the graph showed that song change, on average, only slightly lags (i.e., falls to the left of) the branching-rate inflection point identified by the single-threshold analysis (Fig. 4b).

Additional GMYC analyses conducted with congeneric sequences removed (all *K. muta* complex data only, Muta group only, and Westlandica group only) did not give the same results as the whole-genus analysis. Some tests failed to find a statistically significant shift in branching rate, and all yielded different sets of diagnosed mtDNA clades (data not shown).

Statistical parsimony.—The TCS analysis diagnosed 9 geographically coherent mtDNA clades (Fig. 5), 5 of them matching song-defined groups (note that the clade containing groups M1 and M2 is geographically coherent when the distribution of alluvial lowlands is considered). Six of these (N1, N2, N3, AW, T, and WS1)

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TABLE 4. Cases of putative song hybrids in the genus *Kikihia* (e.g., see Fig. 3) observed at locations of geographic contact or overlap between the proposed parental species

	Aotea W (AW)	Aotea E (AE)	Muta (M)	Nelsonensis (N)	Tuta (T)	Westlandica N (WN)	Westlandica S (WS)	*K. angusta*	*K. c. cutora*	*K. c. cumberi*	Flemingi	*K. horologium*	Murihikua	*K. paxillulae*	Peninsularis	*K. rosea*	Acoustica	Tasmani
Aotea W (AW)	=																	
Aotea E (AE)	X	=																
Muta (M)	X	X	=															
Nelsonensis (N)			X	=														
Tuta (T)			X	X	=													
Westlandica N (WN)			X	X		=												
Westlandica S (WS)			X			X	=											
K. angusta			X					=										
K. cutora cutora									=									
K. cutora cumberi									X	=								
Flemingi											=							
K. horologium												=						
Murihikua							X	X			X		=					
K. paxillulae			X											=				
Peninsularis			X												=			
K. rosea													X			=		
Acoustica																X	=	
Tasmani												X						=

Notes: "X" indicates crosses for which intermediate song phenotypes have been observed and recorded. *Kikihia* species for which putative hybrids have not yet been identified are not included in the table. Boxes shaded in gray indicate taxa within the *Kikihia muta* species complex.

corresponded to clades diagnosed using the GMYC method.

Pairwise Distance Data

Uncorrected mtDNA sequence divergence (concatenated COI + COII) within the *K. muta* complex ranged up to 6.7% for comparisons between individuals of the most divergent *K. muta* groups (Table 6). This maximum corresponds to an ML model–corrected distance of 0.104 expected substitutions/site. Pairwise distances inferred by summing branch lengths on the ML tree were larger than the PAUP*-corrected pairwise distances, with the maximum contrast reaching 0.116 s/s (data not shown), presumably because of the influence of topological information on model parameter estimates.

When individual cases of likely hybridization and introgression were excluded from consideration (see below), average uncorrected distances between mtDNA clades within individual song species ranged from 0.7% to 2.4% in most cases (0.008–0.028 s/s, model-corrected distance) (see gray cells in Table 6). Contrasts involving likely hybridization in contact zones (not shown, and excluded from outlined cells in Table 6), involved much larger pairwise distances ranging up to 0.060 uncorrected s/s (0.087 s/s corrected distance). No genetic "gap" or break-separated values of within song–species contrasts from between song–species contrasts, and identical values were observed in comparisons of some within- and between-species contrasts (e.g., N1 or N2 vs. T3 in Table 6).

DISCUSSION

Introgression and Reconstruction of Complex Species Histories in Kikihia

A review by Funk and Omland (2003) proposed that a high observed frequency of species-level paraphyly and polyphyly (23% of assayed species) is caused by introgression and incomplete lineage sorting events following recent speciation. Hybridization in the form of intermediate songs at contact zones is commonly observed in *Kikihia* (Fig. 2 and Table 4), and the strongest examples of discordance between song phenotype and mtDNA phylogeny are likely due to recent and/or ongoing hybridization and, in some cases, introgression. Interestingly, these cases contrast with results from the other large New Zealand cicada radiation, the genus *Maoricicada*, where hybridization appears to have taken

TABLE 5. Analysis of molecular variance results showing partitioning of mtDNA genetic variation within and among groups defined by song phenotype

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among populations	6	2572.067	18.60399 (Va)	67.45
Within populations	155	1391.599	8.97806 (Vb)	32.55
Total	161	3963.667	27.58205	

Note: Fixation Index (F_{st}) = 0.67450.

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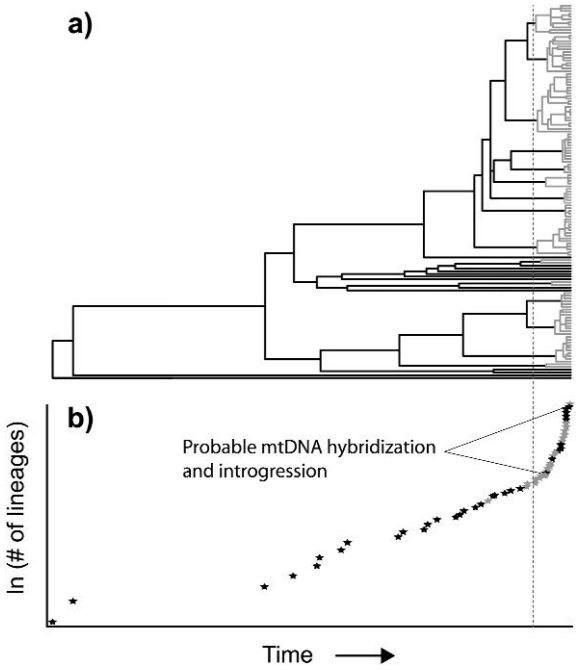

FIGURE 4. a) Chronogram of *Kikihia* ingroup phylogeny with *Kikihia muta* complex haplotypes included (indicated by gray bars). Order of taxa is the same as the phylogram in Figure 2. b) LTT plot for chronogram in A. Dotted line highlights apparent recent shift in the rate of lineage splitting expected to correspond to the transition from between- to within-species coalescence patterns (see Methods section). This shift corresponds to lineages that diverged during the middle Pleistocene (ca. 1 Ma) according to the divergence time analysis of Marshall et al. (2008). Black stars indicate splits that correlate with species-specific song differences. Text discusses evidence that all splits younger than the recent diversification rate shift that correlate with song differences are due to hybridization and mtDNA introgression.

place more anciently (Buckley et al. 2006). This section reviews three examples from the *K. muta* complex in detail to illustrate the need to combine information from multiple sources (behavior, ecology, geography, morphology, and DNA) to formulate effective historical hypotheses.

Tuta: simple songs, nondiscriminating females, and "hybridization by proxy."—Data from multiple character sets show what appears to be an ongoing case of mtDNA introgression from the Tuta song species to the true *K. muta* species, made more striking because the introgressed *K. muta* populations have transferred the Tuta mtDNA to a hybrid zone with a third *Kikihia* species. In Figure 2a, all specimens with Tuta songs possess mtDNA haplotypes from clades T1, T2, or T3, suggesting that the combined mtDNA clade corresponds to a species represented by this song type. However, many individuals of clades T1 and T2 bear true *K. muta*, Nelsonensis, or *K. paxillulae* songs instead. The cicadas with Nelsonensis song and T1 or T2 mtDNA were all observed at contact zones between Nelsonensis and T1 or T2 populations of Tuta, making it likely that those individuals were interspecific hybrids. However, many of the specimens bearing clade T1 mtDNA and *K. muta* song, as well as the individual with *K. paxillulae* song and T1 clade mtDNA, were found hundreds of kilometers from the nearest Tuta population (note the dark brown circles extending down the eastern South Island in Fig. 2a).

We suggest that these geographic patterns fit a double-introgression scenario (see numbered stages in Fig. 6) in which postglacial secondary contact between true *K. muta* populations dispersing from the north and T1 clade–bearing Tuta populations led to hybridization and introgression of T1 clade mtDNA into phenotypically *muta* populations. Then, introgressed *muta* populations continued their southward dispersal, eventually hybridizing with *K. paxillulae* and passing the invasive Tuta mtDNA to populations of that species—in effect, "hybridization by proxy." The low overall haplotype diversity of the M1 clade suggests only one recent glacial refugium, although the fact that one or more (poorly supported) fixed differences apparently exist between North Island and South Island M1 clade haplotypes (Fig. 2a) could indicate either a brief isolation or a founder event associated with postglacial range expansion.

The Tuta song species is unusual because its song appears to lack an introductory section (Fig. 1). In *Kikihia*, such simplified songs are found in species that occupy habitats with few, if any, congeneric species co-occurring (e.g., the tussock-grass *K. angusta* and, to a certain extent, the two Westlandica species, whose songs have greatly extended cueing sections). The introductory portion of the song is also sporadically omitted by males of *K. convicta*, which probably arrived on otherwise cicada-free Norfolk Island <1 Ma (Marshall et al. 2008). If the introductory song component contributes to species recognition, then Tuta females (whose males lack this

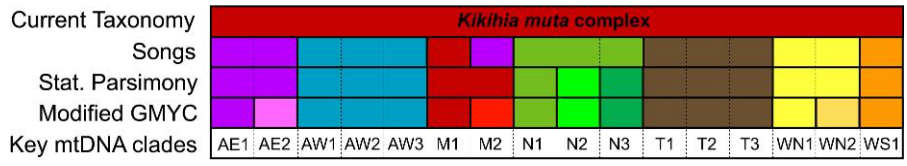

FIGURE 5. Current taxonomy and putative species diagnosed by DNA-based methods (see text) contrasted with putative species diagnosed by male song characters. Letter plus number codes refer to mtDNA clades shown in Figure 2. Type locality information suggests that mtDNA clade M1 corresponds to true *Kikihia muta*. GMYC analysis of mtDNA branching-rate patterns (Monaghan et al. 2009). Statistical parsimony = method of Templeton et al. (1992) with a 95% reconnection limit.

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TABLE 6. Average pairwise genetic distances for *Kikihia muta* mtDNA clades

	AE1	AE2	AW1	AW2	M1	M2	N1	N2	N3	T1	T2	T3	WN1	WN2	WS1
AE1	—	0.013	0.022	0.021	0.027	0.028	0.022	0.020	0.028	0.028	0.028	0.027	0.066	0.062	0.061
AE2	0.014	—	0.022	0.019	0.030	0.029	0.024	0.023	0.030	0.029	0.029	0.027	0.057	0.064	0.062
AW1	0.024	0.024	—	0.012	0.028	0.027	0.025	0.021	0.027	0.032	0.032	0.029	0.062	0.058	0.058
AW2	0.024	0.024	0.013	—	0.027	0.026	0.024	0.020	0.028	0.029	0.028	0.027	0.064	0.059	0.061
M1	0.033	0.035	0.033	0.031	—	0.021	0.023	0.023	0.031	0.030	0.027	0.026	0.067	0.063	0.060
M2	0.032	0.034	0.031	0.031	0.023	—	0.023	0.020	0.026	0.030	0.030	0.028	0.063	0.059	0.059
N1	0.025	0.027	0.029	0.028	0.026	0.026	—	0.016	0.024	0.026	0.025	0.020	0.060	0.056	0.058
N2	0.022	0.024	0.025	0.029	0.026	0.022	0.018	—	0.020	0.024	0.024	0.020	0.062	0.056	0.057
N3	0.032	0.035	0.031	0.033	0.036	0.030	0.028	0.023	—	0.029	0.028	0.025	0.060	0.057	0.057
T1	0.033	0.034	0.037	0.034	0.035	0.035	0.029	0.027	0.034	—	0.007	0.011	0.062	0.060	0.057
T2	0.033	0.034	0.038	0.034	0.031	0.036	0.028	0.028	0.033	0.008	—	0.010	0.060	0.059	0.056
T3	0.030	0.031	0.033	0.032	0.029	0.032	0.023	0.022	0.028	0.011	0.011	—	0.058	0.057	0.055
WN1	0.097	0.100	0.092	0.097	0.104	0.096	0.087	0.090	0.087	0.091	0.087	0.082	—	0.014	0.031
WN2	0.090	0.095	0.083	0.087	0.095	0.086	0.080	0.079	0.081	0.086	0.086	0.080	0.015	—	0.029
WS1	0.088	0.091	0.083	0.091	0.088	0.084	0.082	0.080	0.081	0.080	0.080	0.076	0.037	0.033	—

Notes: ML (GTR + *I*+ Γ) pairwise distances (substitutions/site) from the partitioned Garli analysis are below the diagonal and *p* distances (uncorrected pairwise percentage/100) are above. Cells in gray correspond to comparisons of clades with the same song phenotype predominating. Outlined cells indicate contrasts for which haplotypes were excluded due to hybridization in contact zones. Note that the WN1, WN2, and WS clades are only distantly related to the remaining *K. muta* complex clades in the *Kikihia* mtDNA tree (Fig. 1).

component) may be more prone to interbreeding than females of other *Kikihia* species, perhaps explaining the disproportionate frequency with which cicadas are found in Tuta contact zones with Tuta mtDNA and a song phenotype of a different song species (Fig. 2a). A second possibility is that an as-yet-unrecognized selective advantage favors introgression of Tuta mtDNA (Ballard and Whitlock 2004) or that Tuta mtDNA survives better in a hybrid nuclear background than the mtDNA of other species, a phenomenon that has been documented in a number of other taxa (Arntzen et al. 2009). Asymmetric gene introgression is also expected to be more likely to occur when one species is rare and dispersing into the range of another (Excoffier et al. 2009), and when the introgressing marker is uniparentally inherited (Chan and Levin 2005), a situation that has been observed in other species radiations involving island hopping (e.g., Shaw 2002; Mendelson et al. 2004).

Eastern Muta—ghost of hybridization past?—As in the Tuta case, song and mtDNA discordance involving the Aotea East song species suggests recent hybridization and introgression, but in this scenario, one of the partners—a population originally defined by the M2 haplotype—is in danger of extinction by "genetic swamping" (cf. Roberts et al. 2010). The Aotea East song species is polyphyletic for mtDNA, containing clades AE1 and AE2 and a separate, unrelated, clade M2. Importantly, the M2 clade haplotypes have been found only in coastal populations along and south of Hawkes Bay in eastern North Island where alluvial lowlands are found. In western North Island, similar alluvial plains are occupied by true *K. muta* cicadas bearing mtDNA clade M1—the sister clade to M2. An ancestral southern North Island *K. muta* population was likely subdivided by the rise of the southern axial North Island mountains during the last 1–1.5 myr (Raven 1973; Ghani 1978; Rogers 1989), after which hybridization between Aotea East and "Eastern Muta" created a broad hybrid zone that was eventually all but lost, along with the parental M2 populations, as tectonic and sea level changes eliminated most low-lying *K. muta* habitat on the eastern North Island. Divergence time estimates (Marshall et al. 2008) are broadly consistent with the above hypothesis that late Pleistocene tectonic uplift has played a role in isolating these and other *K. muta* complex populations.

The Aotea East case contains another difficult taxonomic issue. We have sequenced mtDNA from five individuals of the Chatham Islands cicada *K. longula*, all of which group closely with clade AE1 of Aotea East. In Figure 2, one of these haplotypes is shown with a corrected mtDNA distance of just 0.006 s/s from a specimen of Aotea East taken near Woodville, North Island. Previous studies (Arensburger et al. 2004; Marshall et al. 2008) based on smaller data sets found greater genetic distances to the nearest mainland relative of *K. longula* (0.05 s/s for Arensburger et al. 2004), illustrating how important complete taxon sampling and within-species genetic sampling can be for divergence time studies. The low mtDNA distance found in this study implies that *K. longula* colonized the Chatham Islands in the Late Pleistocene, on the recent end of the scale of estimates found by studies of other invertebrate taxa, which range up to around 4 Ma (Trewick 2000; Chinn and Gemmell 2004; Paterson et al. 2006). Hill et al. (2005) raised the possibility of human transplantation for this species because cicada nymphs can survive in root balls of live plants. Alternatively, the low genetic distance could reflect ongoing recurrent dispersal and gene flow to the Chathams, as suggested by Shepherd et al. (2009) for the fern *Asplenium hookerianum*, although this seems unlikely given the fine-scale phylogeographic structure observed within the *K. muta* complex over much shorter distances on the mainland. Because no song differences from Aotea East are apparent in a handful of

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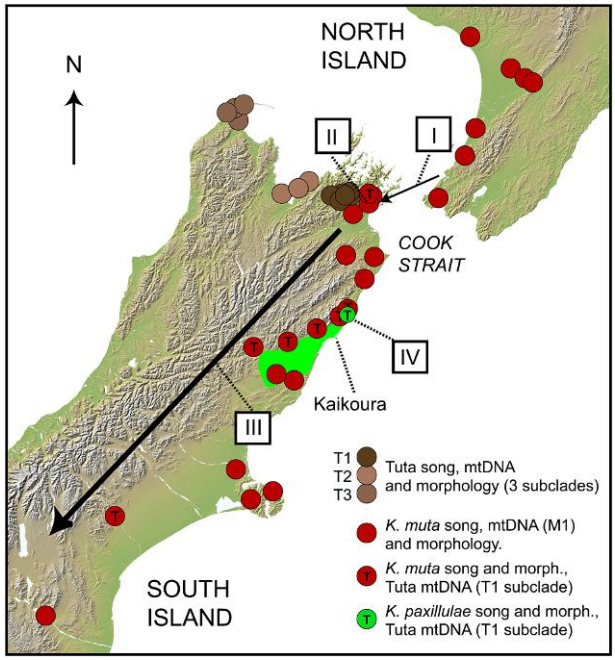

FIGURE 6. "Hybridization by proxy" scenario explaining discordance between biogeographic mtDNA, morphology, and song patterns of Tuta, true *Kikihia muta*, and *K. paxillulae* (see Fig. 2a). Roman numerals indicate the following sequence of hypothesized events: I), Postglacial southward range expansion of *K. muta* populations (clade M1 mtDNA), probably from a refugium on North Island, brings populations into contact/hybridization with Tuta populations bearing mtDNA clade T1 before rising sea levels form Cook Strait. II), Hybridization between *K. muta* and Tuta leads to introgression of T1 haplotypes into populations bearing genes for *muta* song and morphology. III), Southward range expansion continues for both introgressed and nonintrogressed populations. IV), Secondary contact between T1-introgressed *muta* populations and the related *K. paxillulae*, forming hybrid zones observed today near Kaikoura in northeastern South Island, allows the passing of T1 clade Tuta haplotypes from phenotypically *muta* cicadas to populations of cicadas that otherwise look and sound like *K. paxillulae* near Kaikoura—in effect, hybridization by proxy. Note that Tuta contains three distinct mtDNA clades and that the symbol color code is somewhat different from that of Figure 2. The green-shaded region is the approximate geographic distribution of *K. paxillulae*.

archived recordings of *K. longula*, the best approach may be to revise the species, which was named for a difference in wing length, to include at least the Aotea East cicadas.

Mitochondrial capture of divergent taxa.—In Figure 2b, both mtDNA sequences from the undescribed species Tasmani are nested within clade WN1 of the nearby Westlandica North song species. (Three additional Tasmani specimens have been sequenced, one from a more distant location—Mt. Arthur—with the same result.) Tasmani, a high-elevation taxon, is divergent from Westlandica North in song, morphology, and habitat preference, and it more closely resembles another subalpine taxon, *K. horologium*, with which it hybridizes at Mt. Murchison. Tasmani and *K. horologium* appear closely related in the EF-1α tree of Marshall et al. (2008). Minimal evolutionary divergence of mtDNA along with deeper divergence in other character sets is consistent with the hypothesis that recent hybridization and mtDNA introgression has led to the "mitochondrial capture" of Tasmani by haplotypes from Westlandica North, and the pattern is not predicted by the hypothesis of differential lineage sorting (see Holder et al. 2001). Similar reasoning has been used to infer an introgression event in the *Emys* complex of turtles (Spinks and Shaffer 2009) and repeated mtDNA introgression in *Etheostoma* darters (Bossu and Near 2009). The phylogenetic position of most taxa in the mtDNA Westlandica clade could be due to introgression, as this diverse group contains grass (Westlandica North, Westlandica South, and *K. angusta*), scrub (Tasmani and Murihikua), and tree (*K. subalpina* and Flemingi) *Kikihia* taxa that each look and sound more like cicadas from other clades. Other clades of similar age in the *Kikihia* mtDNA tree contain more ecologically and morphologically similar species. Perhaps one introgression event can make further hybridization and introgression with other species more likely? Together, the Tuta, Tasmani, and Westlandica clade introgression scenarios suggest that mitochondrial introgression has been a recurring feature of *Kikihia* diversification (cf. Grant et al. 2005).

mtDNA Phylogeography and Song Evolution

Although it is widely appreciated that purely mtDNA-based estimates generally fall short of those that integrate multiple morphological, ecological, or behavioral information sources (see also Rubinoff and Holland 2005; Trewick 2008; Padial et al. 2009), mtDNA data can yield reasonable initial estimates of population lineages in the progress of evolving reproductive isolation. When cases involving likely hybridization are set aside, patterns of song divergence in a species complex of New Zealand grass cicadas closely track mitochondrial phylogeographic patterns such that all "song species" consist of assemblages of recognizable mtDNA phylogroups (Avise and Walker 1999) identified by one or more DNA taxonomic methods and in most cases form monophyletic clades. Baker et al. (2009) also discuss the utility of single-gene data sets in DNA taxonomy when used alone for efficient exploration and when used in combination with other markers in coalescent-based analyses.

Comparatively few species have been described solely on the basis of sexual mate attracting signals. The taxonomic significance of songs in the "singing insects" (crickets, katydids, and cicadas) was not recognized until the early 20th century (e.g., Fulton 1915; Davis 1922; Alexander 1964), and the use of songs in taxonomic studies has been steadily increasing (e.g., Lloyd 1990; Henry 1994; Otte 1994; Marshall and Cooley 2000; Percy et al. 2006; Sueur and Puissant 2007; Sueur et al. 2007; Gogala et al. 2008; Tobias et al. 2010). Our results validate the anticipated utility of sexual signals for identifying clades in the earliest stages of population differentiation.

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If comparisons between the unrelated Muta and Westlandica clades of Figure 1 are excluded, the candidate species diagnosed by song differ by uncorrected mtDNA genetic distances ranging from 1.9% to 3.2% (Table 6) similar to or slightly lower than that observed across a range of vertebrate and invertebrate species pairs (Johns and Avise 1998; Avise 2000; Buckley et al. 2001; Hebert et al. 2004, but see Pereira and Baker 2006). The corrected distances (0.022–0.036 s/s) obtained from the ML analysis (summed branch lengths) are greater, as expected. Distance estimates are expected to increase as more sensitive corrections for multiple substitutions are applied—from p distances to pairwise model-corrected distance estimates to summed branch lengths from partitioned likelihood-based phylogenetic analyses. A recent attempt to re-calibrate an insect COI molecular clock (Papadopoulou et al. 2010), using a suite of co-occurring species and a common geological calibration, emphasized the importance of data partitioning for estimating branch lengths and obtained a rate of 0.0354 s/s/myr, considerably higher than the "classical" clock rate of 0.023 (Brower 1994). Note that Carstens and Knowles (2007b) have also warned that large errors in clock rate estimates can also occur when ancestral polymorphism is not taken into account.

The genetic distance estimates suggest no clear genetic "break," or bimodal distribution, to support a threshold that could be applied to distinguish "intraspecific" and "interspecific" comparisons in our mtDNA trees (see also Mallet et al. 2007). Early studies suggesting that such gaps are prevalent may have suffered from insufficient geographic or population sampling within species (Moritz and Cicero 2004), so there may be little hope for automated species delimitation based on such thresholds (see also Meyer and Paulay 2005; Vogler and Monaghan 2007).

Molecular techniques used for investigating within-species or cryptic population structure often involve attempts to diagnose shifts in DNA lineage-splitting rates (diversification rates). The use of a pairwise distance threshold such as the "ten times rule" (Hebert et al. 2004), for example, would require that clades contain closely related haplotypes joined to a common ancestor and supported by a comparatively long branch (again, a gap). The same pattern is important for recent approaches that fit ML models of lineage branching rates to gene trees (Pons et al. 2006; Fontaneto et al. 2007; Papadopoulou et al. 2008; Monaghan et al. 2009) without requiring the assumption of a particular threshold divergence value. Although the *K. muta* complex mtDNA tree does contain several clades with long branches supporting sets of closely related haplotypes, the branches supporting song diagnosable clades do not share a consistent length relative to within-clade divergence, although a visual inspection of the LTT plot suggests a reasonably clear shift in diversification rate associated with initial changes in song (Fig. 4b). Consequently, the GMYC method recognized only four of the seven clades suggested by song, and it identified additional clades that showed no song divergence.

The GMYC method was developed on the premise that diversification rate shifts in phylogeographic data indicate the change from within- to between-species population-genetic dynamics, originally with the limitation that the shift would take place at the same time in each lineage (Pons et al. 2006). However, extrinsic processes like climate-induced range expansion and contraction events (Jansson and Dynesius 2002) also structure genetic variation of clades independent of the degree of reproductive isolation or time since isolation. Differences in the severity of Pleistocene range contractions across the range of the *K. muta* complex may account for the fact that the clades on the upper half of North Island and parts of northern South Island, where climates have been historically more moderate, are the least easily distinguished (i.e., have comparatively short branches subtending each clade compared with those within the clades), whereas southern North Island and central South Island clades, which experienced more severe climate fluctuations, are most easily recognized (Fig. 2). The GMYC method returned a highly significant result under the multiple-threshold model, as expected under such a scenario, although the result (21 clades diagnosed within the AE/AW section of Fig. 2a, not shown) remains unsatisfying. Other papers have suggested that the method may be dependent on levels of gene flow (Papadopoulou et al. 2008) and on population-genetic structure (Lohse 2009, but see Papadopoulou et al. 2009). The need for removal of duplicate haplotypes might be explained by violations of model assumptions caused by recent intraspecific range expansions.

Interestingly, the GMYC single-threshold model also grouped six of the non-*K. muta* congeners into three clusters of two species each. One of these splits, *K. cutora exulis* and *K. cutora cutora*, involves a Kermadec Islands subspecies with minimal, if any, song divergence from its North Island relative and another, *K. rosea rosea* and Acoustica, involves two taxa with minimal song divergence. The third, Murihikua and *K. angusta*, is a suspected case of recent mtDNA introgression (see above). Although additional refinements of the GMYC technique are apparently needed before it can be applied in a repeatable manner across data sets, it does appear that the single-threshold method can serve as a useful heuristic tool for detecting independent lineages when used in combination with other information.

Statistical parsimony recognized five of the seven clades diagnosed using song. This method has been applied in several DNA taxonomy studies (e.g., Hart and Sunday 2007; Chen et al. 2010) (but see Bond and Stockman 2008; Cardoso et al. 2009), and it is sometimes used to identify prospective clades that are then subjected to additional tests (Monaghan et al. 2005). However, unlike the GMYC method, statistical parsimony lacks an explicit conceptual justification for why it should be expected to identify the species threshold. A 95% reconnection limit is commonly assumed, but different thresholds are probably required depending on the evolutionary rate of the marker used

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(e.g., Geml et al. [2008] suggested a 98% reconnection limit for an internal transcribed spacer data set in *Agaricus* fungi); in one study, analysis of COI and cytochrome-b data sets yielded different results (Richards et al. 2009). The fact that no accounting is made for ASRV is also a concern, at least when more distantly related haplotypes are being studied. Additional study could be focused on the possibility that many newly divergent taxa share commonalities in their histories (e.g., Pleistocene divergence times and recent bottlenecks) that, in combination with ordinary substitution dynamics, often happen to create mtDNA branching patterns that are well diagnosed with a 95% parsimony reconnection limit.

Concluding Remarks

The Utility of Sexual Signals in Evolutionary Studies and Taxonomy

Evolutionary changes in mate recognition signals, such as those observed in the *K. muta* complex, are important for taxonomic considerations under several species concepts because of their close functional connection to mating systems and interbreeding. However, this does not mean that sexual signals unambiguously indicate reproductive isolation or evolutionary divergence. We know from the existence of so many hybrid zones that the minor song differences observed in this study do not always completely isolate the populations, and the isolating effects may still be minor in some cases. The comparative speed with which behaviors like songs change in diagnosable ways, compared with "slower" morphological traits, probably means that sexual signals will tend to diagnose populations at earlier evolutionary stages—stages that will sometimes be ranked at subspecific level when species status is decided based on complete or near-complete prezygotic reproductive isolation. Nonetheless, it is likely that the song changes observed within *K. muta* represent the early stages of a process that has yielded the larger differences observed between more deeply divergent taxa, species that are commonly sympatric and isolated in part by their songs.

Although songs in the *K. muta* complex seem to change reliably soon after lineages begin to diverge (Fig. 4b), we know from other groups that song evolution does not always precede significant morphological and/or ecological divergence. For example, *R. leptomera*, a beach-grass cicada of North Island, and *R. microdora*, a shrub cicada of the eastern North and South Islands, are morphologically and ecologically distinctive, yet their complex songs are all but identical (see also *Maoricicada iolanthe* and *M. campbelli*—Buckley et al. 2006). Failure of advertisement signals to distinguish some morphologically identifiable species was also noted in a recent study of Neotropical frogs (Padial et al. 2009). Such examples are rare; however, we are aware of fewer than 10 cases out of the hundreds of morphologically distinctive species that we have recorded.

We suggest that uncertainty over the ecological and reproductive "status" of the *Kikihia* song species, and over the functional significance of their differences in mating signals, should not retard their description (see also Winker et al. 2007). Hybridization, introgression, and even horizontal gene transfer are now accepted to occur between species of widely varying degrees of relatedness (McKinnon et al. 2003; Grant et al. 2005; Álvarez and Wendel 2006; Berthier et al. 2006; Arntzen et al. 2009). Hybrid zones, while problematic for the identification difficulties they present, represent one end of a continuum of interbreeding situations, and they (and, perhaps, the consequent gene flow) are often geographically restricted in comparison to the ranges of the main populations involved. Whether the *K. muta* complex taxa should be identified as species or assigned subspecific rank is a question meriting more thorough treatment than we can offer here, but their description at either level will facilitate their ongoing study.

DNA Taxonomy

Two challenges for DNA taxonomy are posed by radiations like the *K. muta* complex, and both will likely be difficult to solve. First, we lack a reliable threshold or standard for operationalizing distinguishable DNA clades that accurately approximate the species level. Multimarker analyses (Nielsen and Matz 2006; Carstens and Knowles 2007a) will improve estimates of actual lineage diversification times, but they will not likely solve the problem of deciding how much divergence is "enough." The second challenge, detecting misleading lineage-sorting and introgression events (perhaps especially involving mtDNA—Ballard and Whitlock 2004), is difficult to accomplish through DNA sequences alone, although recent papers suggest new approaches for these challenges (Holder et al. 2001; Carstens and Knowles 2007a; Joly et al. 2009). Nonetheless, we think that it would be a mistake to refuse the opportunity to consult a geographically well-sampled genetic data set (especially mtDNA) when conducting alpha-level taxonomic investigation, an approach similar to what has been termed "reverse taxonomy" (Blaxter 2004; Markmann and Tautz 2005; Rubinoff and Holland 2005; Vogler and Monaghan 2007).

Supplementary Material

Supplementary material, including data files and/or online-only appendices, can be found at http://www.sysbio.oxfordjournals.org/.

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