Molecular Ecology (2004) **13**, 3787–3796 doi: 10.1111/j.1365-294X.2004.02375.x

Testing geographical pathways of speciation in a recent island radiation

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Abstract

Determining the mode, or geographical context, of speciation is a critical first step to understanding the evolutionary mechanisms that cause new species to arise. In this study, we estimated phylogenetic relationships in the *cerasina* species group of the Hawaiian cricket genus *Laupala* (Orthoptera: Gryllidae) to test competing phylogeographical hypotheses and thus infer the mode of speciation. A previous phylogenetic result based on nuclear sequence data suggested that populations of *L. cerasina* on the Big Island of Hawaii are the result of two independent colonizations from Maui, implying parallel speciation and convergent song evolution, and contradicting systematic hypotheses based on behavioural and morphological data. We used amplified fragment length polymorphisms to investigate further the relationships among species and populations in the *cerasina* species group. Results of these analyses provide a robust estimate of phylogenetic relationships and support the phylogeographical history indicated by behavioural and morphological data.

Keywords: acoustic communication, Hawaii, *Laupala*, mode of speciation, phylogeography

Received 19 May 2004; revision received 13 August 2004; accepted 9 September 2004

Introduction

Modes of speciation typically refer to the geographical context in which speciation has occurred, whereas mechanisms of speciation typically refer to the evolutionary processes that act within the geographical context of speciation. Determining the mode of speciation is a critical first step to understanding the mechanism because it places the events in an appropriate historical context. The number, sequence and geographical context of speciation events in a given group of organisms are now generally inferred by estimating phylogenetic relationships among populations and species in that lineage (e.g. Lynch 1989; Barraclough & Vogler 2000). These historical insights in turn facilitate further hypothesis testing about which processes are likely to have caused speciation (see Taylor & McPhail 1999; Allender *et al*. 2003).

In this study, we examined geographical pathways of speciation in a species group of Hawaiian crickets belonging to the genus *Laupala*. *Laupala* is a genus of small flightless crickets endemic to the Hawaiian Islands that has experienced repeated speciation in recent evolutionary history. Evidence to date indicates that the genus consists of three main species groups — the *kauai*, *pacifica* and *cerasina* groups — including a total of 38 described species (Otte 1994; Shaw 2000). The majority of these species have evolved within the last 5 million years (Shaw 2002).

Laupala cerasina is a member of the *cerasina* species group (Otte 1989, 1994), endemic to the windward, rain-forested slopes of the Big Island of Hawaii, distributed from the Kohala Mountains at the northern tip to Kilauea in the southeastern corner (Fig. 1). Otte (1989, 1994) hypothesized that *L. cerasina* was a single species on the basis of similarity in both the male calling song and male genitalia across populations. Regardless of location, males of *L. cerasina* all sing within a limited range [2.5–3.0 pulses per second (pps)] and share genital morphology typical of the *cerasina* species group. Other evidence, however, calls into question the close relationship of *L. cerasina* populations and their common evolutionary history. For example, there is statistically significant geographical variation in male calling song and female acoustic preference (Shaw, unpublished data) as well as metric variation in male genitalia (Otte 1994). In addition, an analysis based on an anonymous region of nuclear DNA [nDNA; 1049 base pairs (bp)] revealed a phylogeographical break separating northern and southern populations of *L. cerasina* (Shaw 2002). These nDNA data further suggested that the two groups were derived from different Maui ancestors.

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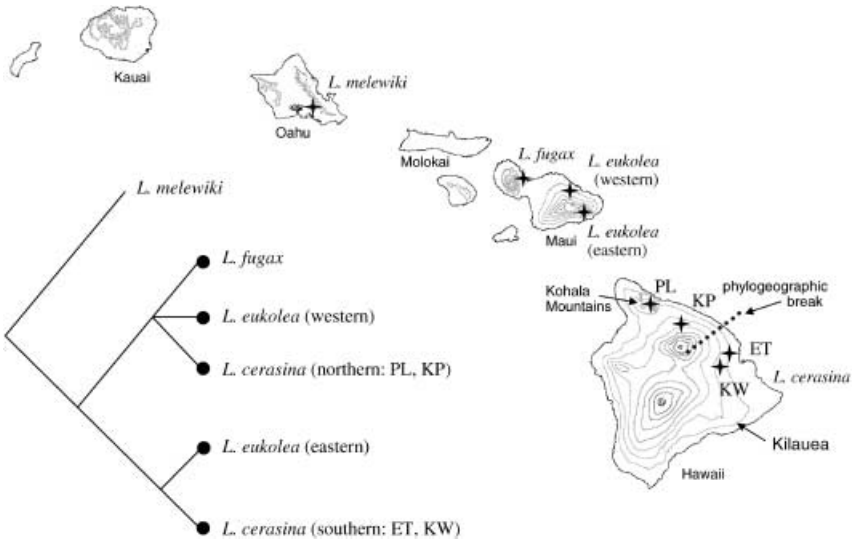

Fig. 1 Locations of sampled populations of *Laupala cerasina* (Hawaii Island), *L. eukolea*, *L. fugax* (Maui), and *L. melewiki* (Oahu) are indicated by black crosses. The phylogeographical break between northern and southern populations of *L. cerasina* evident from nuclear sequence data (Shaw 2002) is also shown. The phylogeny illustrates species and population relationships based on maximum likelihood analysis of a single nuclear marker (1049 bp, Shaw 2002), suggesting that northern and southern populations of *L. cerasina* do not share an exclusive common ancestor.

Northern populations of *L. cerasina* formed a sister group to *L. fugax* and a population of *L. eukolea* from western Maui, whereas the southern populations formed a sister group to an eastern population of *L. eukolea* (Fig. 1).

Based on evidence to date, the historical origins of *L. cerasina* must have followed one of three geographical pathways (Fig. 2): (i) a double invasion from Maui to the Big Island, with one colonist arriving in the north and one arriving in the south (Fig. 2A); (ii) a single invasion from Maui to the Big Island in the south, with subsequent northward migration (Fig. 2B); or (iii) a single invasion from Maui to the Big Island in the north, with subsequent southward migration (Fig. 2C). Nuclear DNA sequence data, indicating a clear phylogeographical break and two different ancestral species, are most consistent with a double invasion scenario. In contrast, the original hypothesis of Otte based on shared behaviour and morphology is more consistent with either of the single invasion hypotheses.

The objective of this study was to reconstruct relationships in the *cerasina* species group and thereby determine whether *L. cerasina* is the result of a single or double invasion of the Big Island. Estimating relationships in young species radiations is challenging, however, because of the paucity of accumulated genetic variation. Minimal sequence divergence resulted in the low resolution of the nDNA analysis (Shaw 2002); a phylogenetic analysis with greater resolution would more reliably indicate historical phylogeographical patterns. Here we used amplified fragment length polymorphisms (AFLPs) to estimate phylogenetic relationships in the *cerasina* species group. The AFLP technique (Vos *et al*. 1995; Mueller & Wolfenbarger 1999) has proven especially useful for resolving relationships among closely related species (Allender *et al*. 2003; Beardsley *et al*. 2003; Despres *et al*. 2003; Pelser *et al*. 2003), including species of *Laupala* (Parsons & Shaw 2001; Mendelson & Shaw 2002). By generating hundreds or thousands of neutral markers, AFLPs may be more likely to reveal the recent and rare substitutions that characterize close species relationships, and, by broadly sampling the genome for genetic variation, AFLPs may provide a more accurate reflection of species relationships than sampling variation within a single locus, because the pattern of evolution is averaged across loci (see Barrett *et al*. 1991). We predicted that using AFLPs would increase the resolution of species relationships in the closely related *cerasina* species group and thus would more accurately indicate the geographical pathway of speciation.

The mode of speciation in *L. cerasina* has considerable bearing on mechanistic hypotheses that could explain acoustic evolution in this group. All populations of *L. cerasina* sing significantly more slowly (2.5–3.0 pps) and prefer slower songs (Shaw & Herlihy 2000; Mendelson & Shaw 2002; Grace & Shaw 2004) than their Maui relatives: *L. fugax* and *L. eukolea* sing at roughly 3.6–4.0 pps. A double invasion scenario would therefore imply parallel evolution in acoustical communication features between the northern and southern *L. cerasina* populations. If so, northern and southern populations of *L. cerasina* may be examples of parallel evolution resulting from distinct evolutionary processes with seemingly opposite effects. In the northern part of its range, *L. cerasina* coexists with a faster singing congener (*L. kohalensis*, ~3.7 pps) and in the southern part of its range with slower singing congeners (*L. pruna*, ~2.0 pps; *L. paranigra*, ~1.0 pps; and *L. nigra*, ~0.5 pps). Therefore, in the case of a double invasion, if a fast-singing Maui ancestor first arrived in the north, the slower pulse rate and preference in *L. cerasina* may have resulted from reproductive character displacement in response to contact with *L. kohalensis*, whereby selection favoured divergence between sympatric species with similar mate recognition signals. Character

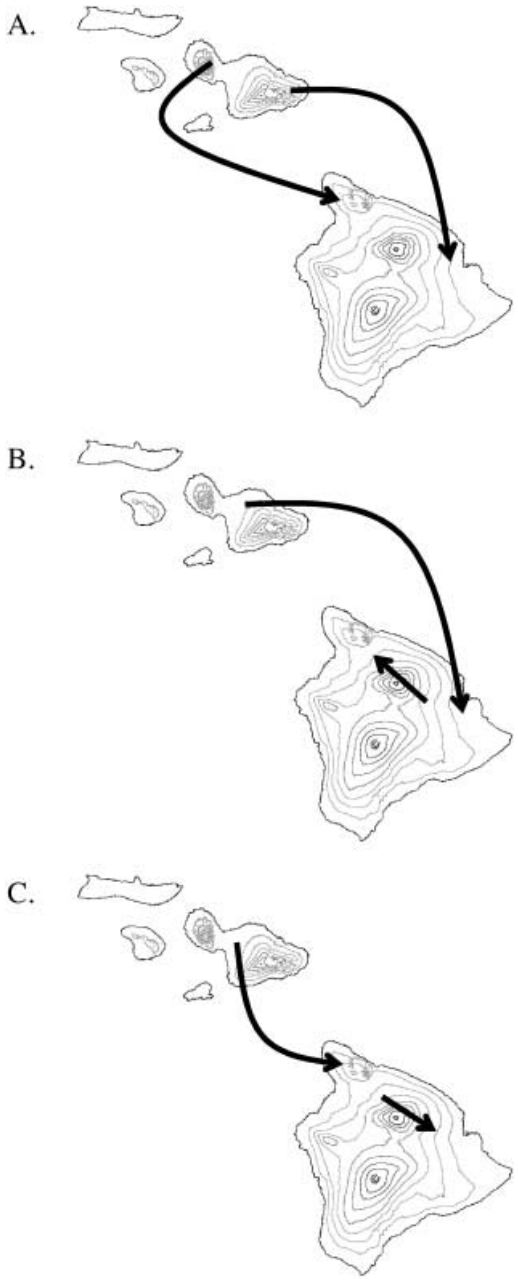

Fig. 2 A schematic of potential demographic histories of *Laupala cerasina*. The northernmost population of *L. cerasina* (PL) is located in the Kohala mountains; the southernmost population is located in the forests of Kilauea volcano. (A) A double invasion, with different Maui ancestors giving rise to northern and southern populations of *L. cerasina*. (B) A single colonization in the south with subsequent northward migration. (C) A single colonization in the north with subsequent southward migration. Potential source populations are found throughout Maui.

displacement has been examined closely as a force in the evolution of acoustic signalling and support for its existence has accumulated in many taxa (Gerhardt & Huber 2002).

A Maui ancestor invading in the south, however, would have come into contact with slower singers, thus excluding a character displacement hypothesis. Rather, hybridization between southern endemics and the Maui ancestor, and subsequent introgression of alleles responsible for slow song, could explain a reduction in pulse rate and preference in southern populations of *L. cerasina*. In the south, then, acoustic evolution would be the result of convergence with, rather than divergence from, sympatric congeners. Indeed, while mitochondrial DNA sequence data (mtDNA) provide little information on species relationships, they do suggest that hybridization has been common in the history of *Laupala* (Shaw 1996, 2002; Mendelson & Shaw 2002).

The nDNA sequence patterns may not represent completely the history of *L. cerasina* populations, and *L. cerasina* may in fact be derived from a single invasion of the Big Island, thus eliminating the need to explain a parallel reduction in pulse rate and pulse rate preferences. The nDNA pattern may instead reflect a history of stochastic lineage sorting of ancestral nDNA copies in a widespread polymorphic population of *L. cerasina*, followed by northern and southern differentiation. In this case, discordance between current nDNA data and additional gene trees is expected. Although a single invasion scenario would allow for phylogeographical discontinuity between northern and southern *L. cerasina* populations, it should nonetheless result in the northern and southern *L. cerasina* populations forming a monophyletic clade with a single Maui ancestor.

Materials and Methods

Sampling

Individuals were collected from eight sites on Hawaii, Maui and Oahu (Fig. 1). Seventy-four individuals representing four species of the *cerasina* species group as described by Otte (1994) and Shaw (2000, 2002) were used in the analysis. *Laupala cerasina* was collected from four sites on the Big Island. Two of these populations, Eucalyptus Toe (ET, $n = 10$) and Kaiwiki (KW, $n = 10$) represent southern populations, and two populations, Kalopa Park (KP, $n = 10$) and Pololu Valley (PL, $n = 10$), the latter a subset of the KM population in Parsons & Shaw (2001), represent northern populations. The southern populations were suggested in the nDNA analysis to be more closely related to an eastern population of *L. eukolea*, whereas the two northern populations were found by nDNA to be more closely related to *L. fugax* and a western population of *L. eukolea* (Shaw 2002). Northern and southern populations are separated by a minimum of approximately 42 km. Most *L. cerasina* individuals sampled were previously analysed in Parsons & Shaw (2001) or Mendelson & Shaw (2002). However, data in the present study were derived

independently from source DNA, using 13 unique AFLP primer pairs.

Individuals identified as *L. eukolea* were collected from two populations on East Maui. Eastern *L. eukolea* was collected in the vicinity of Dog Leg Camp in Kipahulu Valley of the Haleakala National Park (DL, $n = 10$). Western *L. eukolea* was collected from the Hana Road at mile marker 8.1 (HR, $n = 5$). *Laupala fugax* ($n = 10$) was collected from the Waihe'e Ridge trailhead, West Maui.

Laupala melewiki is a member of the *cerasina* species group endemic to the island of Oahu and was used as the outgroup. Individuals ($n = 9$) were collected from Mount Konahuanui (type locality, Shaw 2000) of the Koolau mountain range.

AFLP analysis

Generating AFLP band profiles. Genomic DNA was extracted using standard phenol/chloroform extraction procedures (see Parsons & Shaw 2001). Procedures for generating AFLP band profiles generally followed Vos *et al*. (1995). Briefly, restriction enzymes *Eco*RI and *Pst*I were used to digest 250 ng genomic DNA. Ligation of *Eco*RI and *Pst*I adapters to restriction fragments took place concurrently with restriction digestion. A 1 : 10 dilution of restriction–ligation product (2 μL) was used as template in a pre-amplification polymerase chain reaction (PCR). Primers for the preamplification were *Eco*RI and *Pst*I primers with one additional selective nucleotide (*Eco*RI: 5′-GACTGCGTACCAATTC + A; *Pst*I: 5′-GACTGCGTACATGCAG + A). A second, selective amplification was conducted using 2 μL of a 1 : 400 dilution of preamplification product. Primers were the same as in preamplification, but with two additional selective nucleotides. PCR conditions for selective amplification entailed 35 cycles beginning with 95 °C for 2 min, 65 °C for 30 s, and 72 °C for 1 min. Subsequent cycles entailed a denaturing step of 95 °C for 30 s, with annealing temperature reduced by 1 °C each cycle and held at 56 °C for the remaining 27 cycles. Data were initially collected using manual silver staining techniques and subsequently using automated fragment analysis on an ABIPrism 3100 DNA Analyser (PE Applied Biosystems, Inc.), to increase the rate of data collection. All individuals were analysed for all primer pairs (i.e. using both methods), thus no artefacts should result. Three different *Eco*RI primers, two of which were fluorescently labelled (Qiagen, Inc.) for automated fragment analysis, were combined with various *Pst*I primers to yield 15 unique combinations of primer pairs. The primer *Eco*RI + ACG (unlabelled) was paired with *Pst*I + AGA/+ ATA for manual scoring; for automated fragment analysis, *Eco*RI + AAC (labelled) was paired with *Pst*I + ACG/+ ACT/+ AGA/+ ATA/+ AAG/+ AAC/+ AAA, and *Eco*RI + AGC (labelled) was paired with *Pst*I + AGA/+ ACG/+ AAA/+ AAC/+ AGG/+ AAG.

For manual scoring, selective PCR products were electrophoresed through 5% polyacrylamide gels (SequaGel™, National Diagnostics) which were then silver-stained (Silver Sequence™ staining reagents, Promega, Inc.), dried, scored and scanned into ADOBE® PHOTOSHOP® for permanent storage. For automated fragment analysis, selective PCR products were injected with an internal size standard (GS-500 ROX, PE Applied Biosystems, Inc.) and run on the ABI 3100. Fragments were sized using GENESCAN® ANALYSIS v3.7 (PE Applied Biosystems, Inc.).

Data analysis. Scoring of AFLP data entailed noting the presence or absence of same-sized fragments, or bands. Bands used in scoring ranged in size from 75 to 680 bp. For manual scoring, two bands were assumed to be homologous if they appeared on the gel to be the same molecular weight (silver staining). Band sizes were estimated using a 100-bp DNA Ladder size standard (Gibco™, Invitrogen Corporation). Bands that varied slightly in size were excluded from the analysis, as we could not be confident in our homology assessment. For automated fragment analysis, homology was assessed using GENOTYPER® 2.5 software (Perkin-Elmer Corp.). Bands that differed by less than 1 bp were considered homologous. Although the assumption that comigrating AFLP bands represent homologous genetic regions is not universally upheld (Robinson & Harris 1999; Mechanda *et al*. 2004), we were reasonably confident in the homology of same-sized bands in this analysis, for two reasons. First, we used two six-base ('rare') cutters, which yielded fewer total bands and therefore fewer bands per size class. Second, the homology of comigrating bands in this genus has been supported by direct sequencing of bands in previous studies (Parsons & Shaw 2001; Mendelson & Shaw in press, supporting material online).

A presence/absence matrix ('1' for present, '0' for absent) was generated for all scorable loci for all individuals. A distance matrix, representing genetic distances among individuals, was generated in PAUP* (Swofford 2000) using the Nei–Li equation for genetic distances (Nei & Li 1979). A phylogram based on this distance matrix was generated using the neighbour-joining algorithm of tree construction. The tree was rooted using *L. melewiki* as the outgroup, which was constrained to be a monophyletic sister group to the ingroup. Bootstrap support for each node was estimated from 1000 replicates using the neighbour-joining algorithm.

The presence/absence matrix was also used to generate estimates of polymorphism and expected heterozygosity (H_E) for each population in AFLP-SURV (Vekemans 2002). Allele frequencies used to estimate these parameters were derived using a Bayesian estimator based on a nonuniform prior distribution (Zhivotovsky 1999) and assuming Hardy–Weinberg equilibrium.

Results

The two primer pairs used for manual scoring yielded an average of 20.5 (range = 13–28) scorable loci per primer pair ranging in size from 100 to 680 bp. Automated fragment analysis yielded an average of 45.4 (range = 18–79) scorable loci per primer pair; scored bands ranged in size from 75 to 490 bp.

In total, 631 loci were used in the analysis; of these, 494 (78%) were polymorphic. Of the 494 polymorphic loci, 219 (44.3%) had character states (presence or absence) that were unique to one of the 13 nodes, or clades, in the final tree (eight populations plus five internal nodes). Of these clade-specific loci, 33 were fixed for one character state in one clade (and fixed for the alternate character state in all other populations), and 186 loci (the remainder) were polymorphic within a single clade only (Table 1).

Phylogenetic analysis

Estimated phylogenetic relationships among the four species examined are presented in Fig. 3. The four populations of *Laupala cerasina* (Big Island) formed a monophyletic clade with 100% bootstrap support. This group formed a sister clade to the eastern population of *L. eukolea* with 88% bootstrap support. That clade, including *L. cerasina* and eastern *L. eukolea*, was sister to the strongly supported clade including the western population of *L. eukolea* and *L. fugax*. Finally, the Maui and Big Island species formed a strongly supported monophyletic clade relative to the outgroup, *L. melewiki*. Although the phylogram pictured in Fig. 3 was rooted with *L. melewiki*, the same result was obtained with midpoint rooting.

Bootstrap values were generally high, with an average of 92.2% across the 13 nodes in the final tree (Table 1). At the population level, the average bootstrap value was 88.75%. Internal nodes were also well supported, with an average bootstrap value of 97.6%.

Both the number of resolved nodes and the average bootstrap value increased as data were added to the analysis (Fig. 4). Of the 13 nodes of interest, 10 received greater than 50% bootstrap support within the first five primer pairs (189 characters). The number of resolved nodes (i.e. those with greater than 50% bootstrap support) increased to 12 within 11 primer pairs (432 characters) but did not increase with additional data because of the low resolution of the Eucalyptus Toe population of *L. cerasina*, for which bootstrap support was 34% in the final analysis.

The average bootstrap value for the 13 nodes of interest, including values less than 50%, increased steadily with increasing data but appeared to level off within 13 primer pairs (549 characters, Fig. 4B). Any noticeable increase in average bootstrap value will depend on increased resolution of the Eucalyptus Toe population.

Table 1 Number of clade-specific loci and associated bootstrap support for the 13 nodes of interest

Node	Total no. clade-specific loci	No. of fixed clade-specific loci
1. *Laupala cerasina* (KP)	26	3
2. *L. cerasina* (PL)	23	1
3. Northern *L. cerasina* (KP & PL)	67	2
4. *L. cerasina* (ET)	17	0
5. *L. cerasina* (KW)	12	2
6. Southern *L. cerasina* (ET & KW)	44	0
7. *L. cerasina* (All populations)	141	2
8. *L. eukolea* (eastern)	33	4
9. *L. cerasina* and *L. eukolea* (eastern)	204	2
10. *L. eukolea* (western)	23	3
11. *L. fugax*	33	3
12. *L. eukolea* (western) and *L. fugax*	71	1
13. *L. melewiki*	52	17

First column indicates the total number of loci with character states (presence or absence) unique to that particular clade. Second column indicates the number of those loci that were shared by all individuals in that clade (i.e. fixed).
ET, Eucalyptus Toe; KW, Kaiwiki; KP, Kalopa Park; PL, Pololu Valley.

Gene diversity within populations

Estimates of the percentage of polymorphic loci (P) and expected heterozygosity (H_E) for each population are shown in Table 2. Estimates of P ranged from 15.7 to 46.3% (average 22.7%). Estimates of H_E ranged from 0.0860 to 0.1147 (average 0.1001). With the exception of the western population of *L. eukolea*, the most genetically diverse group, populations did not appear to vary dramatically in either polymorphism or heterozygosity estimates, although a global analysis of variance indicated significant heterogeneity in H_E among populations ($\chi^2 = 22.57$, d.f. = 7, $P < 0.005$). We tested the hypothesis that this variation is explained by a reduction in genetic diversity upon invasion of the Big Island. A reduction in heterozygosity might be expected if, for example, the founding populations of *L. cerasina* were very small. After correcting for multiple comparisons, we found no significant differences between the putative source population, eastern *L. eukolea*, and any of the four *L. cerasina* populations.

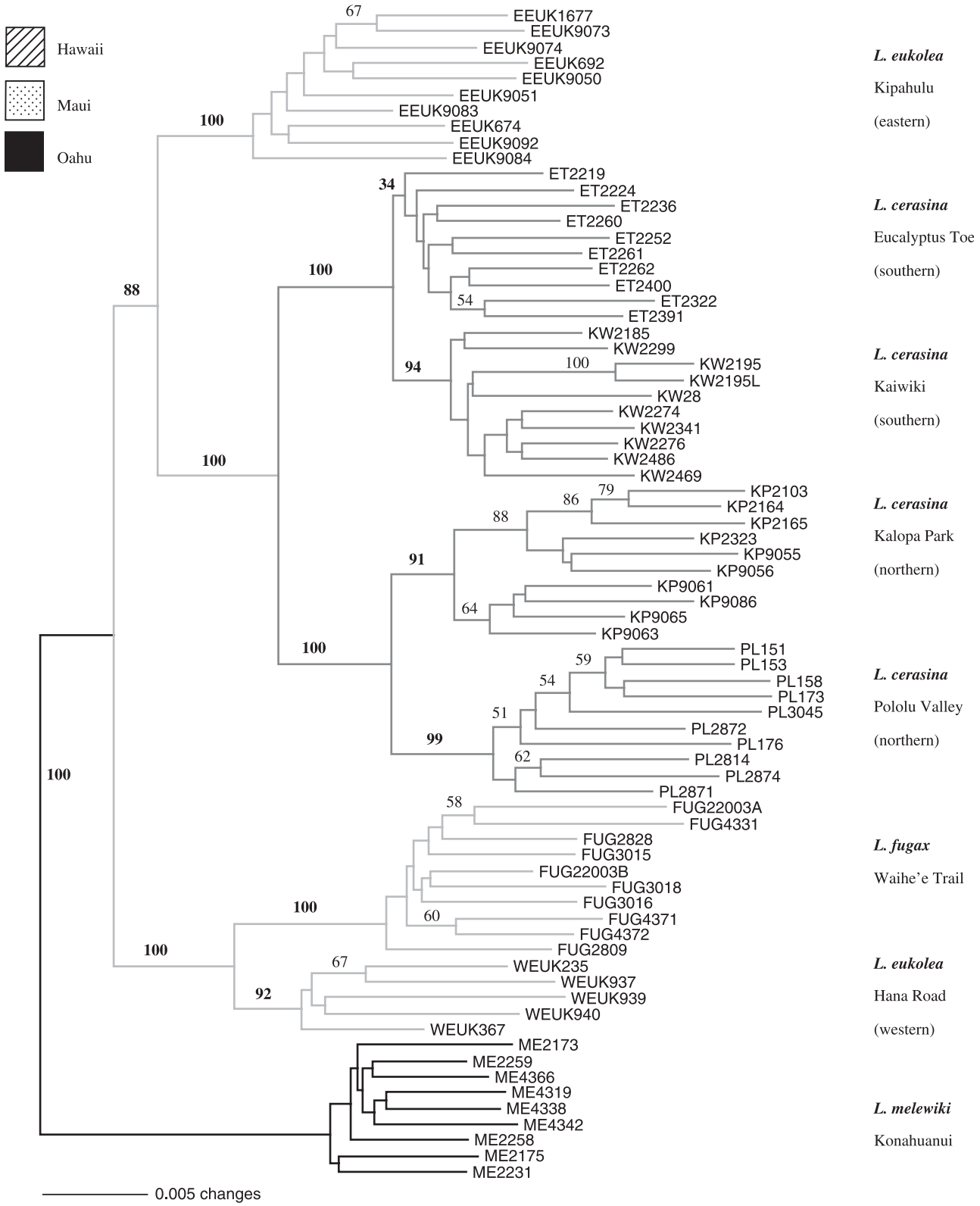

Fig. 3 Phylogeny estimate based on 631 AFLP characters. The tree was constructed using the neighbour-joining algorithm based on Nei–Li genetic distances among individuals. The tree is rooted with the outgroup, *Laupala melewiki*. Bootstrap values, calculated based on 1000 replicates using the neighbour-joining algorithm, are presented for the 13 nodes of interest in bold. Operational taxonomic units (OTUs) are individuals. Bootstrap values greater than 50% are shown for groups of individuals within terminal taxa.

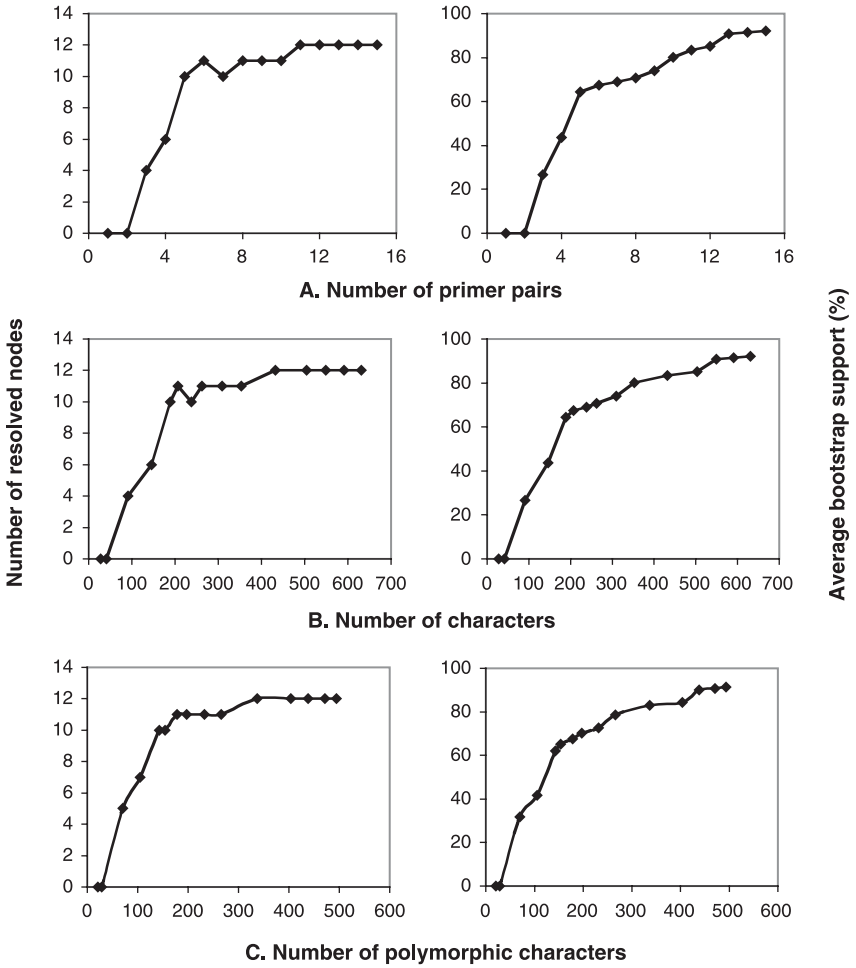

Fig. 4 (A) Relationship between the number of primer pairs used in the analysis (as compiled chronologically) and two estimates of tree resolution: the number of nodes supported at the > 50% bootstrap level out of a possible 13 (left panel) and the average bootstrap support across all 13 nodes of interest (right panel). (B) Relationships between the number of characters used in the analysis and tree resolution. (C) Relationships between the number of polymorphic characters and tree resolution. Both estimates of resolution increased as data were added but appear to have levelled off within 13 primer pairs (549 characters, 438 polymorphic characters).

Table 2 Per cent polymorphism (P) and expected heterozygosity (H_E) for each sampled population (n indicates number of individuals analysed per population)

Species	Island	Site	n	P (%)	$H_E \pm$ SE
Laupala cerasina	Hawaii	Kalopa Park (N)	10	19.3	0.1069 ± 0.0060
L. cerasina	Hawaii	Pololu Valley (N)	10	21.2	0.1038 ± 0.0056
L. cerasina	Hawaii	Eucalyptus Toe (S)	10	19.5	0.0961 ± 0.0055
L. cerasina	Hawaii	Kaiwiki (S)	10	17.0	0.0877 ± 0.0054
L. eukolea	Maui	Dog Leg (E)	10	22.2	0.1056 ± 0.0056
L. eukolea	Maui	Hana Road (W)	5	46.3	0.1147 ± 0.0059
L. fugax	Maui	Waihe'e Trail (W)	10	20.3	0.1057 ± 0.0060
L. melewiki	Oahu	Konahuanui	9	15.7	0.0860 ± 0.0053

Discussion

Geographic pathways of speciation

Identifying modes of speciation in a given lineage is a critical first step to inferring underlying evolutionary mechanisms. For example, in the three-spine stickleback species complex *Gastorosteus aculeatus*, evidence suggests that populations in different glacial lakes are derived independently from separate oceanic to fresh water invasions, and that lakes containing two morphological forms have undergone double invasions (McKinnon & Rundle 2002). The evolutionary mechanisms required to explain divergence between morphological forms within a single lake depend greatly on the mode of speciation, that is, whether divergence occurred *in situ* or began prior to contact between

sympatric forms. The sticklebacks therefore provide a clear example of how focus on ancestry directs attention to the historical sequences of change and lends insight into the evolutionary mechanisms underlying those changes.

In the case of *Laupala cerasina*, previous work left unclear whether populations that are recognized as a single species are the result of a single invasion of a newly emerged island, or instead are the result of independent invasions by distinct source populations. Early hypotheses based on morphological and behavioural similarities suggested that populations of *L. cerasina* form an exclusive group derived from a single colonization of the Big Island from Maui (Otte 1994). The most recent nDNA phylogeny, however, implied a double invasion of the Big Island, with northern and southern *L. cerasina* populations derived from different Maui ancestors (Shaw 2002). To distinguish between these hypotheses, we examined phylogenetic relationships among four populations of *L. cerasina* and several of its putative sister species.

Results of our analyses of AFLP data support Otte's original hypothesis that *L. cerasina* is derived from a single invasion of the Big Island of Hawaii from Maui. A single clade consisting of northern and southern populations of *L. cerasina* received 100% bootstrap support. Moreover, an eastern Maui population of *L. eukolea* was found to be the single closest relative of *L. cerasina*. We therefore reject the hypothesis suggested by the single locus nDNA data that *L. cerasina* is derived from two separate source populations (Fig. 2A).

The AFLP analysis does, however, corroborate evidence of a phylogeographical break between northern and southern populations observed in the nDNA sequence data. This phylogeographical discontinuity corresponds to populations inhabiting mid-elevation forests of the Kohala mountains (the oldest of the Big Island volcanoes, ~430 000 years old) and the far northern slopes of Mauna Kea (400 000 years old; Clague & Dalrymple 1987) on the one hand, and populations inhabiting mid-elevation forests on the southern slopes of Mauna Kea on the other. The results also suggest additional subdivision of varying degrees within *L. cerasina*. Geographic features of the island may explain this level of structuring; for example, the Kohala population is separated from the others by deep, sea-level valleys, which is likely to reduce the probability of recurrent migration and gene flow between this population and others. However, limited sampling across the species range could have contributed to the appearance of phylogeographical divisions, especially if populations are characterized by isolation by distance. Increased sampling across the range of *L. cerasina* will be needed to assess the evolutionary reality of these phylogeographical breaks.

Our results also provide compelling support for the hypothesis that speciation in *Laupala* has proceeded through a series of invasions from older to younger islands (the 'progression rule'; Funk & Wagner 1995). An older to younger biogeographical pattern was suggested at the inter-island level by previous phylogenies based on nucleotide sequences — basal lineages are found on the oldest island of Kauai, and the more derived lineages on the youngest island of Hawaii (Shaw 1996, 2002). However, by providing a finer resolution of species relationships, AFLPs indicate that even within islands, in this case Maui, the progression rule is evident. Based on outgroup rooting with a taxon from Oahu, the oldest and most western island sampled in this study, the AFLP tree suggests an invasion from Oahu onto West Maui, a subsequent invasion to the geologically younger East Maui, and finally a colonization of the youngest island of Hawaii from East Maui. Our data also therefore indicate that populations of *L. eukolea* do not share a common evolutionary history and suggest a need for further investigation of the status of this species.

Our analyses do not indicate a significant loss of genetic diversity upon invasion of the Big Island, as estimates of H_E did not differ significantly between eastern *L. eukolea* and any of the *L. cerasina* populations. We interpret these results with caution, however, as the validity of assuming Hardy–Weinberg equilibrium in these populations, an assumption that is required when estimating H_E from dominant markers such as AFLPs, is currently unknown. Future studies examining haplotype or microsatellite data may provide a better sense of the validity of Hardy–Weinberg equilibrium and will have a bearing on the accuracy of gene diversity estimates derived from AFLP data.

The question remains as to whether initial colonization of the Big Island occurred in the north or in the south (Fig. 2B,C). Results of the AFLP analysis do not allow us to address this question; however, sampling additional populations across the range of *L. cerasina* may determine the region of colonization. A greater sampling effort may indicate whether populations from one region are nested within populations of another, indicating a more derived origin of the former. Once the source of the invasion is determined, more directed hypotheses concerning the causal mechanisms of evolutionary change can be pursued.

Modes and mechanisms

Compared to a double invasion scenario, a single origin of *L. cerasina* leads to a more parsimonious explanation for the evolution of acoustic communication in this species. Males in all populations of *L. cerasina* sing significantly slower than any member of the *cerasina* group on Maui (Otte 1994), and females in all examined populations of *L. cerasina* preferentially respond to pulse rates of their own species over those of the Maui taxa (Shaw & Herlihy 2000; Mendelson & Shaw 2002; Grace & Shaw 2004). A double invasion would therefore imply two evolutionary changes representing independent reductions in pulse rate and

pulse rate preferences upon migration to the Big Island. In contrast, the AFLP data, by indicating the exclusive common ancestry of northern and southern *L. cerasina* populations, require only one evolutionary reduction in male pulse rate and female preference.

The causal mechanisms underlying these changes in acoustic signalling behaviour are currently unknown. Hypotheses proposed to explain the reduction in pulse rate and pulse rate preferences in *L. cerasina* relative to its ancestor will depend on whether the source population invaded the Big Island in the north or in the south. If colonization occurred in the north, acoustic signalling features may have evolved as a consequence of interactions with *L. kohalensis*, a fast-singing congener endemic to that region. In this case, reproductive character displacement and/or reinforcement (Howard 1993) may have led to divergence in pulse rate and pulse rate preference and the evolution of the slower singing *L. cerasina*. Subsequent southward migration of *L. cerasina* would explain the broad distribution of the slower song on the Big Island. If the initial invasion occurred in the south, hybridization with *L. pruna*, *L. paranigra*, or *L. nigra*, all slower singing congeners endemic to the southern region, may have led to introgression of alleles contributing to slow pulse rates and preferences, thereby causing a reduction in these features in *L. cerasina*. Subsequent northward migration would then explain the distribution of slower song across the range of *L. cerasina*. These mechanistic hypotheses remain to be tested.

Acknowledgements

We thank S. Lesnick for assistance. Support for this research was provided by a grant from the Howard Hughes Medical Institute through the Undergraduate Biological Sciences Education Program to A.M.S. and in part by NSF grant no. BIR-9602266 to T.C.M. and NSF grant no. DEB-9729325 to K.L.S.

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