Molecular Ecology (2001) **10**, 1765–1772

Species boundaries and genetic diversity among Hawaiian crickets of the genus *Laupala* identified using amplified fragment length polymorphism

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Abstract

Crickets of the genus *Laupala* represent one of the many morphologically cryptic groups of insects, with the most closely related species distinguished only by the male calling song. Cryptic groups provide a challenge in determining the genetic boundaries between closely related populations and species. We have addressed the question of species boundaries in the Hawaiian cricket, *Laupala*, using nuclear DNA patterns sampled by the amplified fragment length polymorphism (AFLP) technique. This method has been used widely by plant researchers to facilitate the rapid assessment of genetic diversity in very closely related species and varieties. The AFLP technique is simple and robust, can be applied to any organism, and overcomes problems associated with cost, development time, information content and reproducibility that can plague other marker systems. Our results support previously hypothesized taxonomic relationships among sympatric populations and suggest close genetic relationships among allopatric, conspecific populations.

Keywords: AFLPs, courtship song, cryptic species, taxonomy

Received 31 October 2000; revision received 11 March 2001; accepted 15 March 2001

Introduction

Morphologically similar populations that are reproductively isolated, so called cryptic or 'sibling' species (Walker 1964), pose problems for evolutionary biologists trying to determine species boundaries and species diversity. Cryptic species have been identified in many diverse taxa but are especially prevalent in those insects where mating behaviour is often the most distinctive feature among closely related populations (Ritchie *et al*. 1996; Wells & Henry 1999). Taxonomic hypotheses based on morphology alone in these groups have often failed to reflect evolutionary partitions and new species descriptions have resulted when behavioural traits have been included (Henry 1985; Otte 1994; Shaw 2000). In the case of crickets, male calling songs attract females and are thought to promote homogamy, and thus variation in the calling song is sometimes used in taxonomic studies at the specific level (e.g. Otte 1994). The reliability of species level taxonomies based on behaviour remains controversial however, and researchers can now employ molecular assays to test hypotheses of species boundaries (Avise 1994; Shaw 1999).

Hawaiian crickets of the genus *Laupala* (family Gryllidae), with 37 described species, is one group of cryptic species in which the taxonomic recognition of behavioural traits has led to a significant increase in the number of species hypothesized (Otte 1989, 1994; Shaw 2000). These small, forest-dwelling crickets found throughout the archipelago display diversity in acoustic phenotypes (Otte 1994). In his monograph Otte (1994) recognized species of *Laupala* based on slight differentiation in male genitalia among species groups, and distinct variation in the male calling song within species groups. Unique acoustic patterns were thus hypothesized to reflect species status (Otte 1989, 1994), in some cases where no other variation was apparent. In the present study we investigate the utility of the recently developed technique of amplified fragment length polymorphism (AFLP, Vos *et al*. 1995) to address the question of species boundaries in *Laupala*. We test the hypothesis that morphologically cryptic species of *Laupala* are genetically differentiated.

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Molecular markers used to address genetic differentiation among morphologically cryptic species have typically included allozymes, restriction fragment length polymorphisms (RFLPs), mitochondrial DNA (mtDNA) sequence variation and, more recently, random amplified polymorphic DNA (RAPD) markers (Lu *et al*. 1996; Ritchie *et al*. 1996; Shaw 1996a; Ge *et al*. 1999; Harris & Rogers 1999; McFadden 1999). Each of these techniques requires different levels of expertise and resources and no single approach has proven to be ideal for all studies (Avise 1994; Mueller & Wolfenbarger 1999). Allozyme surveys are straightforward and inexpensive but often fail to detect variation among geographical and/or morphologically distinct populations (e.g. Harris & Rogers 1999; McFadden 1999). RFLP and sequencing studies require more development time and expense and the number of independent loci assayed is often low (reviewed in Avise 1994). RAPD studies generate large numbers of markers but problems with reproducibility and polymerase chain reaction (PCR) artefacts can occur (Mueller & Wolfenbarger 1999).

In their recent review Mueller & Wolfenbarger (1999) suggest that the AFLP technique approaches the ideal marker system for resolving genetic relatedness among individuals, populations and species. The visible polymorphism of AFLP fragments is primarily generated through variation in restriction enzyme sites, and incorporation of the PCR allows for rapid and efficient marker generation. Set-up costs and development time are low compared to RFLP analysis while the quantity of information obtained is much higher. The AFLP method is less problematic than the RAPD technique, with the high stringency amplification conditions used eliminating nonspecific binding and resulting artefactual 'polymorphism' (Mueller & Wolfenbarger 1999). In addition, extensive screening to identify useful primers is not required, the level of polymorphism identified is generally higher, and the ultimate number of markers that can be generated is virtually unlimited (e.g. Barker *et al*. 1999). Two issues that may be of concern are the mainly dominant nature of AFLP markers and whether same-sized fragments are independent or can be used as homologous character states in estimates of genetic diversity. The large number of potential markers that can be generated across the genome counterbalances the low information content of dominant markers. In addition, more informative co-dominant AFLP markers can sometimes be identified (Lu *et al*. 1998). The question of identity of same-sized bands has been addressed within species (Waugh *et al*. 1997) and can be addressed among species, in principle, by sequencing co-migrating bands.

Although the AFLP technique is widely used in plant research (Travis *et al*. 1996; Barker *et al*. 1999; Miyashita *et al*. 1999) it has only been used infrequently to determine genetic relatedness among animal populations. Recent studies in disparate animal taxa illustrate two of the many possible applications. Albertson *et al*. (1999) demonstrated the efficacy of the AFLP method in estimating the problematic phylogeny of the rapidly evolving Lake Malawi cichlids. McMichael & Prowell (1999), using only a small number of primers, generated AFLP band profiles that successfully distinguished host-related strains of the fall armyworm with 95% concordance. In the study presented here we test the hypothesis that nuclear genetic variation identified with the AFLP technique can distinguish cryptic species of the genus *Laupala*.

Materials and methods

Sample collection

We focused on sympatric, morphologically cryptic species of *Laupala* endemic to the island of Hawaii. The majority of specimens analysed in this study were wild-caught males collected from five localities on the windward side of the Big Island from 1991 to 1998 (Fig. 1). Species sampled included *L. paranigra* (two populations), *L. nigra* (one population) *L. cerasina* (five populations) and *L. kohalensis* (one population). Between six and 10 individuals from these nine natural populations were included in the study (Table 1). *L. cerasina* individuals were sampled from four localities where they co-occur with congeners, and one locality where they occur alone. Species identifications, based on the recorded song (see Shaw 1996b for detailed methods) and genitalia of each mature male, were made according to Otte (1994). Females were included ($n = 9$) only where species identity could be determined unequivocally in their male offspring. Outcrossed nymphs ($n = 3$) bred from previously identified specimens were included to increase sample size for the *L. paranigra* population taken from the northeast forest edge.

Genomic DNA isolation

DNA was extracted from fresh or frozen nymphs or adults. Individuals were ground with a plastic pestle in microcentrifuge tubes in 400 µL extraction buffer [0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 50 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0), 1% sodium dodecyl sulphate (SDS)]. Proteinase K was added to a final concentration of 40 ng/mL prior to incubation for 1 h at 65 °C. DNA was ethanol precipitated following phenol extraction and re-suspended in 50 µL 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). DNA concentrations were determined using UV spectrophotometry and typical yields were 20–50 µg DNA per sample. Samples were diluted to 250 ng/µL for AFLP analysis and 1 µL of each diluted sample was gel electrophoresed to confirm DNA quality and quantity.

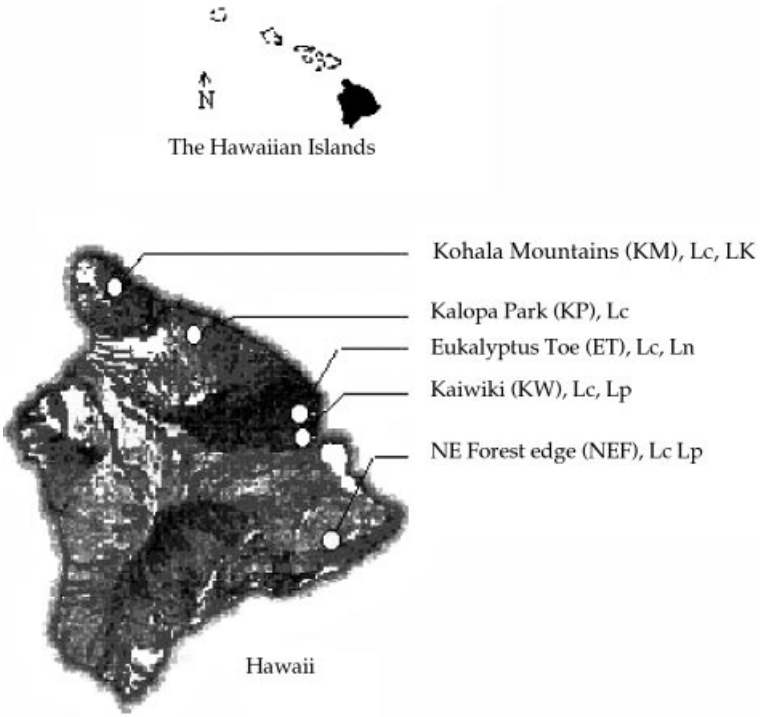

Fig. 1 Geographical locations of populations represented in the study. Specific locality data: northeast forest edge (154°57′ W, 19°26.2′ N), Kawaiki (155°10′ W, 19°46′ N), ET (155°9′ W, 19°48′ N), Kalopa Park (155°27′ W, 20°2′ N), and Kohala Mountains (155°46′W, 20°10′N). Species sampled at each location are shown (Lc, *Laupala cerasina*; Lp, *Laupala paranigra*; Ln, *Laupala nigra*; Lk, *Laupala kohalensis*).

Table 1 Species and populations sampled (m = male, f = female, n = nymph). See Fig. 1 for locality sites

Species	Locality	*n*
Laupala paranigra	Kaiwiki	7m, 1f
	northeast forest edge	3m, 3n
Laupala cerasina	Kaiwiki	10m
	northeast forest edge	10m
	Eukalyptus Toe	10m
	Kohala Mountains	5m, 5f
	Kalopa Park	10m
Laupala nigra	Eukalyptus Toe	10m
Laupala kohalensis	Kohala Mountains	7m, 3f

AFLP assay

The AFLP assay was carried out essentially following Vos *et al*. (1995) and the manufacturer's protocol (Gibco BRL AFLP Starter Primer Kit, Life Technologies) with some modifications. Additional adapters and primers were obtained from Operon Technologies Inc. Assays were conducted using *Eco*RI and *Pst*I restriction digested DNA following ligation of *Eco*RI and *Pst*I adapters. The AFLP protocol includes two rounds of PCR with the ligated adaptors serving as target sites for primer annealing. Selective nucleotides added to the 3′ ends of the AFLP primers act to amplify a subset of the restriction fragments selectively (Vos *et al*. 1995). Round one (preselective) primers have one nucleotide added and round two (selective) primers have a further two nucleotides added. In this study AFLP bands were generated using four primer-pair combinations: *Pst*I: 5′-GACTGCGTACATGCAGAGA-3′ plus *Eco*RI: 5′-GACTGCGTACCAATTCACC/–GC/–CG/–AG-3′ (the underlined primer sequences represent preselective reaction primers and selective reaction primers included the additional two bases). PCR reactions were carried out in a total volume of 10 μL. After round one amplification, preselective reactions were diluted 1 : 40 and 2 μL was used in selective PCR reactions. PCR products were separated by electrophoresis through 5% polyacrylamide gels (SequaGel™, National Diagnostics) using a 40 × 20 cm gel rig. Gels were silver-stained (Silver Sequence™ staining reagents, Promega) following the manufacturer's protocol, air-dried overnight, scanned and computer visualized (Adobe® Photoshop®) for band scoring.

Data analysis

To generate estimates of genetic relationships we employed distance and molecular variance analyses (e.g. Lu *et al*. 1996; Hongtrakul *et al*. 1997; Ge *et al*. 1999), and parsimony analysis for comparison. Presence/absence band data of all polymorphic AFLP bands were compiled for all individuals. The RAPDISTANCE PACKAGE version 1.04 (Armstrong *et al*. 1996) was used to generate distance matrices from the Rogers & Tanimoto (1960) identity metric [$(n11 + n00)/(n11 + 2 * (n10 + n01) + n00)$ where $n11$ = number of positions where a band is present in both individuals, $n00$ = number where absent in both and $n10$ or $n01$ = number where individuals differ in both]. A phenogram was constructed

from these distance measures using the random addition neighbour-joining method of Saitou & Nei (1987). Maximum parsimony analyses were conducted using the heuristic search option in PAUP* version 4.0b1 (Swofford 1998). Ten thousand replicates were conducted to search the tree-space for minimal length tree-islands. Initially no more than 10 trees were saved in each replicate to avoid memory depletion (i.e. the 'out of memory' problem encountered when the program is unable to swap the starting tree to completion; Giribet & Wheeler 1999). Trees saved from each minimal length tree-island were then swapped until 10 000 trees of minimal length were obtained. Maxtrees (= 10 000) and the tree bisection–reconnection (TBR) branch-swapping algorithm were in effect. Bootstrap support was evaluated through 1000 bootstrap replicates via a random addition heuristic search with maxtrees set to 100.

Population structure and variability were determined through an analysis of molecular variance (AMOVA) using WINAMOVA version 1.55 (Excoffier *et al*. 1992), partitioning the variation among localities (irrespective of species), among individuals within populations, among populations within species, and among species. The AMOVA-PREP program version 1.1 (Miller 1998) was employed to read the raw data sets and prepare all input files needed for the AMOVA analysis. In addition, genetic diversity at the population and species level was measured by dividing the number of polymorphic bands by the total number of bands assayed (percentage of polymorphic bands, Ge *et al*. 1999).

Sequencing of AFLP bands

We used direct sequencing to determine the identity of same-sized AFLP fragments within and among species. DNA from co-migrating AFLP products of different individuals was extracted, re-amplified and sequenced as follows. Bands to be sequenced were chosen arbitrarily from those displaying among-species polymorphism. Gels were re-hydrated in distilled water for 15 min and individual bands were then excised, placed in 500 μL extraction buffer (0.5 M $NH_4C_2H_3O_2$) and incubated for 1 h at 55 °C. Samples were centrifuged at 1400 ***g*** for 15 min and the supernatant was removed to a fresh tube. DNA was recovered following ethanol precipitation overnight at 4 °C and re-suspended in 50 μL 1 × TE buffer. Re-amplification was performed in a total volume of 30 μL using 3–6 μL recovered DNA solution and the relevant primer pair. PCR reactions were electrophoresed through 1.5% low melting agarose gels, and product DNA was extracted using Geneclean® (Bio 101). Using the *Pst*I selective AFLP primer, purified bands were sequenced by dideoxy-terminated cycle sequencing (ABI Ready Reaction kit and ABI 373 DNA sequencer) and resulting sequences were assembled using Sequencher™ computer software.

Results

AFLP patterns

The number of observed AFLP bands per primer-pair varied between 70 and 100. Thirty-three per cent (n = 109) of all bands observed displayed variation among or within populations and/or species. Ninety-five of these bands were observed in at least some *Laupala cerasina* individuals and, of these, 11 were invariant in one or more *L. cerasina* population. A similar pattern was observed in the remaining four populations comprising *L. kohalensis*, *L. paranigra* and *L. nigra* individuals. All populations exhibited similar levels of variability, as determined by the percentage of polymorphic bands, ranging from 28 to 43% (χ^2 test = 4.82 (d.f. 8) $P > 0.1$). For 10% of the bands scored, a unique pattern of band presence/absence (i.e. fixed presence, fixed absence, or polymorphic) was observed in single populations. In addition, patterns resulting from 19% of the total bands scored could be used to differentiate *L. cerasina* from all others in the study. Overall 46% of the polymorphic AFLP bands exhibited fixed differences among species and only one marker was variable within all populations. Much of the divergence among species can therefore be attributed to bands exhibiting fixed differences rather than to bands of variable frequency. However, species appear to share polymorphism and the frequency variation remains a significant source of differentiation.

Population structure

Two distinct groups of populations were suggested by both the distance and parsimony analyses, assuming a midpoint root. Unfortunately, we lacked samples from a suitable outgroup, although in principle, outgroup rooting should offer an alternative rooting option. The distance measure tree (Fig. 2) shows all *L. cerasina* populations in one group and the *L. paranigra*, *L. kohalensis* and *L. nigra* populations in the other. Within the *L. cerasina* group only the Kohala Mountain and Kalopa Park populations form exclusive groups. In the second group the *L. kohalensis*, *L. nigra* and *L. paranigra* northeast forest edge populations form exclusive groups while the *L. paranigra* Kaiwiki population does not. The maximum parsimony results were similar. The heuristic search with 10 000 replicates found minimum length trees of 659 steps in 123 tree islands. The 1230 trees saved were swapped until 10 000 trees of minimal length were obtained. On the basis of 106 parsimony-informative characters, the topology of the strict consensus parsimony tree approximates the neighbour-joining tree with the exception that the *L. kohalensis* population could not be resolved as an exclusive group. Bootstrap support for the branch dividing *L. cerasina* populations and all others was 100% (Fig. 3).

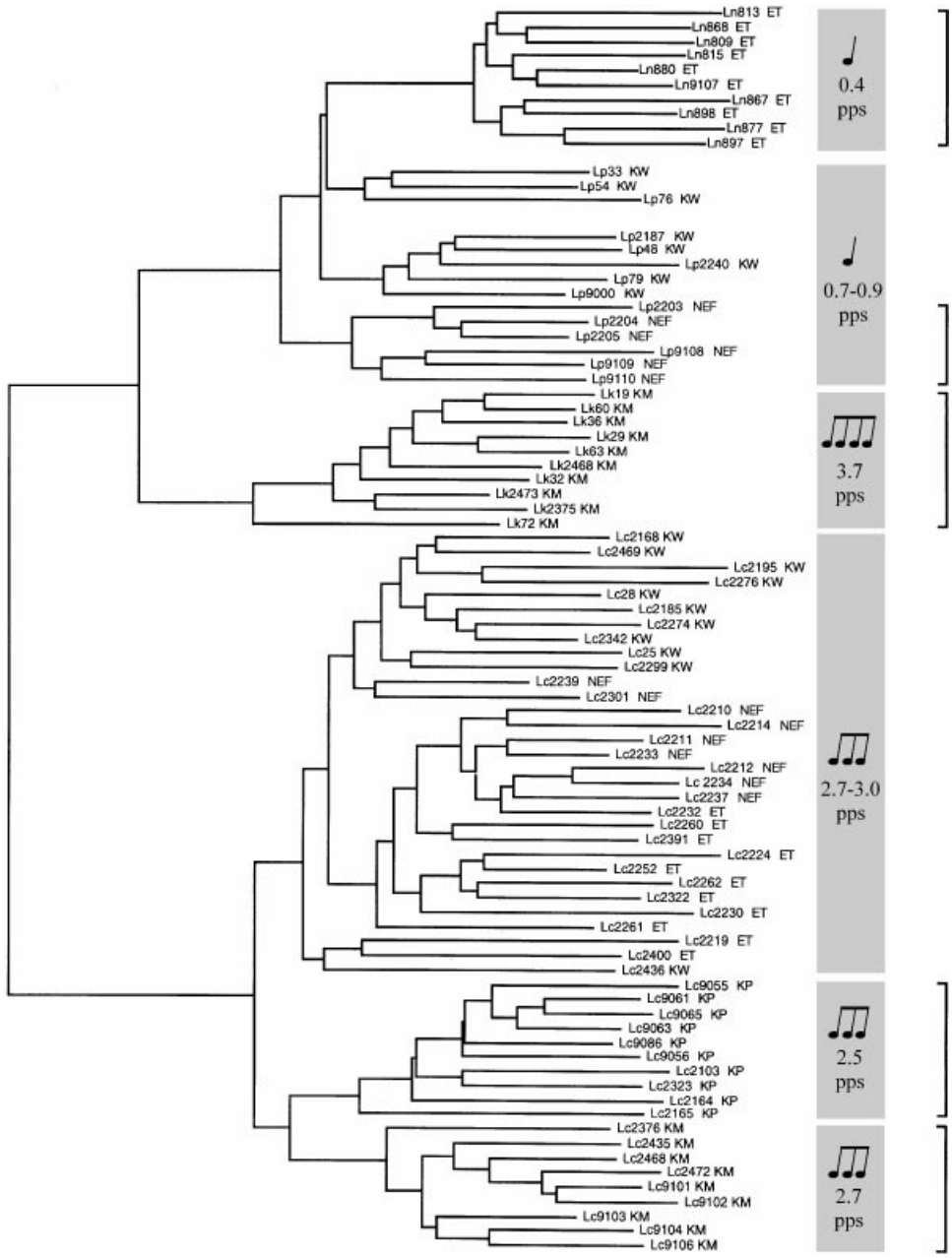

Fig. 2 Neighbour-joining tree from AFLP profiles of all individuals in the study. Individuals are designated by species (Lc, *Laupala cerasina*; Lp, *Laupala paranigra*; Ln, *Laupala nigra*; Lk, *Laupala kohalensis*) and sample number followed by locality (KM, Kohala Mountains; KP, Kalopa Park; ET, Eukalyptus Toe; KW, Kaiwiki; NEF, northeast forest edge). Brackets indicate populations forming exclusive groups. Mean pulse rate for each population, or pulse rate ranges for groups of populations, is indicated to the right of resolved clades. Pulse rate data are from Shaw (1996a,b, 1999; and unpublished results).

Genetic diversity

The AMOVA results corroborate the assessment of population structure above. Variation among species and populations is significantly higher ($P < 0.001$) than that among individuals within populations. Of the total genetic diversity almost half (47.5%) can be attributed to variation among species, 21.5% to variation among populations within species and the remainder (30.0%) to variation among individuals within a population (Table 2). By comparison, analysis of populations grouped within localities rather than species resulted in a complete reversal with almost all the variation (84.4%) residing among individuals. This is indicative of the high degree of genetic differentiation among the species that co-occur in sympatry at each location.

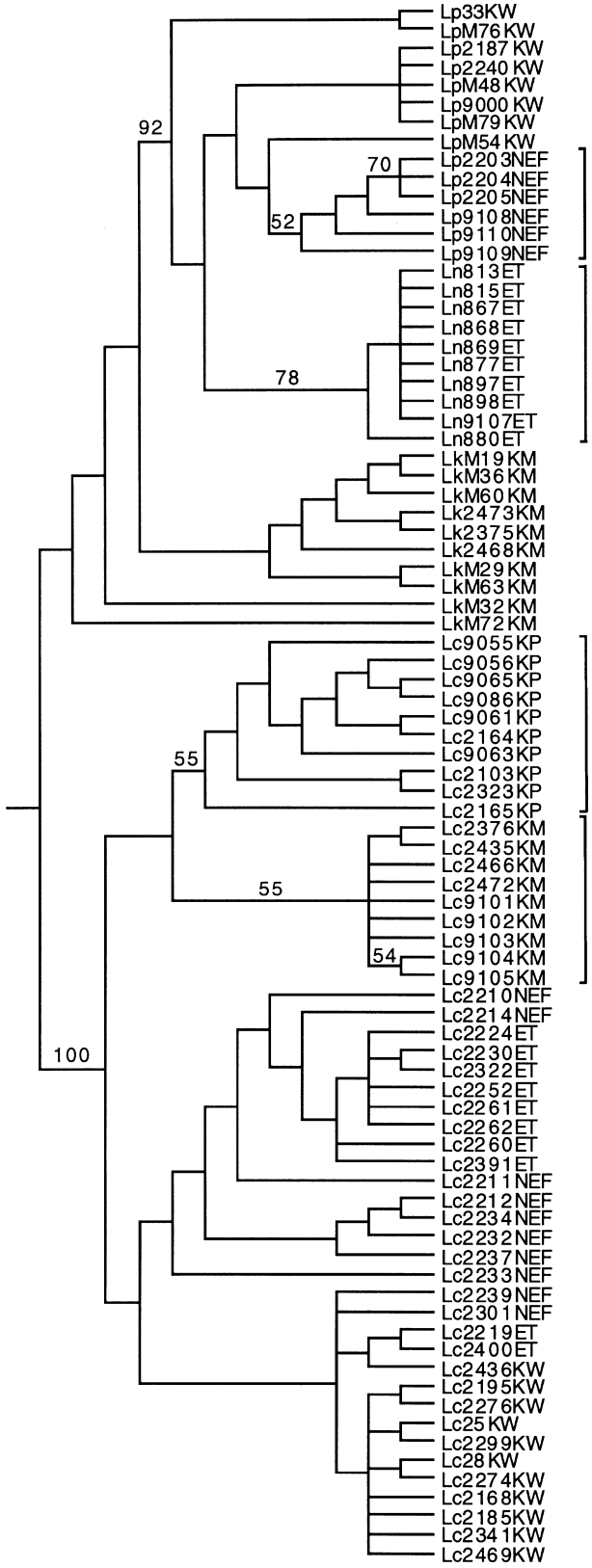

Fig. 3 Strict consensus of 10 000 trees of minimal length illustrating those nodes supported in > 50% bootstrap replicates. Individual designations as in Fig. 2.

Table 2 Results of AMOVA analyses. Variation was partitioned [1] among individuals nested within populations nested within species; [2] among individuals within populations; [3] among individuals within species; and [4] among individuals

Source of variation	d.f.	SSD	MSD	Variance component*	*P*
[1]Species	3	828.85	276.28	13.03 (48.50)	< 0.001
Populations	5	310.84	62.17	5.77 (21.49)	< 0.001
Individuals	75	604.48	8.06	8.06 (30.01)	
[2]Populations	8	1139.69	142.46	14.44 (64.17)	< 0.001
Individuals	75	604.48	8.06	8.06 (35.83)	
[3]Species	3	716.30	238.77	13.86 (58.27)	< 0.001
Individuals	80	794.31	9.93	9.93 (41.73)	
[4]Localities	4	251.99	63.00	2.86 (15.62)	< 0.001
Individuals	79	1222.05	15.47	15.47 (84.38)	

*Data in parentheses are % total variance.

Sequencing analysis

A total of 33 separate AFLP bands corresponding to 10 same-sized fragments observed in at least two species were sequenced (Table 3). One to two individuals from populations that exhibited each targeted band were included. The sequenced length ranged from 260 to 509 nucleotides per fragment and the overall degree of sequence similarity between each of the same-sized bands was between 97 and 100% at both the population and species levels. Insert/deletion variation among species was observed in two pairs of closely migrating bands generated with the same primer combination.

Discussion

The AFLP technique proved to be an efficient tool for corroborating relationships within and among species of Hawaiian *Laupala*. Over 100 informative markers were generated with just four primer-pair combinations, and both distance and parsimony analyses resulted in substantial resolution at the lowest detectable hierarchical level (i.e. of natural populations). The high degree of resolution can be explained, in part, by the large percentage of fixed differences exhibited among species included in the study.

Genetic relationships among conspecifics suggested by the distance analysis of the AFLP data support the taxonomic relationships hypothesized by Otte (1989, 1994), indicating an association between genetic variation and song variation among distinct populations (Fig. 2). Furthermore, the AMOVA among localities corroborates the neighbour-joining topology, suggesting a history of reduced gene flow between *L. cerasina* and sympatric or allopatric congeneric populations. As sympatric congeners can be identified by their distinctive mating call, song phenotypes appear to correspond to species boundaries (see also Shaw 1999).

Table 3 AFLP same-sized gel bands sequenced. Species and population designations as for Fig. 1

Primers	Band	Species	Population	*n*	Bases sequenced
*Pst*I/E-ACC	c	Lp	NEF	2	350
			KW	2	
		Ln	ET	1	
	d	Lc	KW	1	509
		Lp	NEF	1	
			KW	2	
		Lk	KM	1	
	n	Lc	KP	2	
			KW	1	
		Lp	KW	2	
	y/z*	Lc	Kp	1	260
			KW	1	
		Lp	NEF	1	
			KW	2	
		Ln	ET	1	
		Lk	KM	1	
*Pst*I/E-AGC	b	Ln	ET	1	290
		Lk	KW	1	
	g	Ln	ET	2	470
		Lk	KW	1	
	m	Ln	ET	2	463
		Lk	KW	1	
	q/r*	Ln	ET	2	260
		Lk	KW	1	

*Band pairs exhibiting insert/deletion variation among species.

Assuming a midpoint root, the parsimony analysis yielded conclusions consistent with the AMOVA and neighbour-joining analyses, particularly with respect to the branch dividing the *L. cerasina* group from all others. The strict consensus phylogeny shows *L. kohalensis* and *L. paranigra* to be paraphyletic, however. Along with *L. nigra*, these species are each allopatric with respect to one another (each co-occurring with *L. cerasina* across their respective ranges). Thus, paraphyly may be due to shared polymorphisms inherited from the most recent common ancestor of *L. paranigra* and *L. kohalensis*, or low levels of current gene flow. While all our analyses lead to the conclusion of genetic differentiation among these taxa, quantitative assessment of ancestral polymorphism and/or current gene flow will require larger sample sizes. One consequence of small sample size is that low-frequency polymorphisms may have gone undetected. While the detection of low-frequency polymorphisms in future studies should not alter our conclusion of genetic differentiation, it may prove quite informative for estimating levels of recent gene flow.

Both inter- and intraspecific gene flow may be a feature of the *Laupala* system. The study by Shaw (1996a) of *Laupala* based on mtDNA sequence data suggested a lack of congruence between Otte's species-level taxonomy and mtDNA genealogy at broad geographical scales. Further investigation (Shaw 1999) showed that the mtDNA haplotypes from three acoustically similar populations of *L. cerasina* were each more closely related to the mtDNA haplotypes of their sympatric congeners than to each other. Shaw (1999) hypothesized that mtDNA gene flow between sympatric congeners may have occurred and investigations based on nuclear data were suggested as a means to test this hypothesis. The data presented here argue that allopatric populations of *L. cerasina* comprise a closely related group in contrast to the mtDNA genealogy as presented by Shaw (1996a). This corroborates a conclusion drawn from nuclear sequence data (K. Shaw unpublished) and suggests that interspecific mtDNA gene flow has occurred extensively throughout the genus.

We addressed the question of homology of same-sized AFLP fragments among species of Hawaiian *Laupala* through direct sequencing of recovered gel fragments. Ten co-migrating AFLP bands sampled from at least two species exhibited close to identical nucleotide sequence. This evidence of band homology suggests that same-sized AFLP bands can confidently be used as homologous character states and that the estimated measure of diversity obtained accurately reflects the genetic relationships among the populations studied. Of the fragments sequenced two pairs were identified as length variants of the same genetic locus, indicating that the assumption of strict dominance of AFLP bands is sometimes violated. However, given the large numbers of useful markers that can be produced with the technique any resulting bias is likely to be small. Although the results presented here suggest a high incidence of co-dominant AFLP bands, analysis of a larger data set for a quantitative trait locus (QTL) mapping study (Y. Parsons & K. Shaw unpublished) indicates that the frequency of co-dominant bands is less than 10%. The resolving power of the AFLP technique together with the high degree of putative homology exhibited among bands in Hawaiian populations of *Laupala* indicate the suitability of this technique for a broader study to investigate the biogeography of the remaining species on the island of Hawaii and, ultimately, the entire genus.

In this study we have demonstrated the power of AFLPs to determine species boundaries in morphologically cryptic, sympatric species. The AFLP method was originally developed for genetic mapping in plants (Vos *et al*. 1995) and has since been widely applied by plant researchers to measure genetic diversity (Lu *et al*. 1996; Travis *et al*. 1996; Hongtrakul *et al*. 1997; Barker *et al*. 1999). Studies in plants have shown that the AFLP technique can reveal more genetic diversity and is less problematic than RAPDs (Barker *et al*. 1999), can distinguish closely inbred lines (Hongtrakul *et al*. 1997), and is an invaluable tool for assessing genetic diversity in endangered species (Travis *et al*. 1996). The results of the present study, and recent studies in cichlids and armyworms, show that the AFLP technique can be equally applied to animal taxa. Together they support the claim that the AFLP method is cost efficient, reliable and

repeatable and has the capacity to resolve closely related lineages and estimate genetic relatedness among cryptic species.

Acknowledgements

We thank Joshua Mell for technical assistance and Gonzalo Giribet, Steve Palumbi, David Hawthorne and Richard Highton for helpful comments regarding the manuscript. This research was funded by NSF DEB-9729325 awarded to KLS.

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This work was conducted in Kerry L. Shaw's laboratory, now Associate Professor in the Department of Biology at the University of Maryland. This research laboratory investigates the nature and origin of species, focusing on genetic and phylogenetic behavioural changes that diverge early in speciation. Research effort focuses on studies of reproductive behaviour and evolution among closely related species. Investigations of speciation in *Laupala* include analyses of species boundaries (through analysis of DNA sequence and courtship variation), microevolutionary divergence through sexual selection, and the genomic and phylogenetic consequences of speciation (through studies of the genetic architecture and phylogenetic patterns of character evolution).
