

# The usefulness of amplified fragment length polymorphism markers for taxon discrimination across graduated fine evolutionary levels in Caribbean *Anolis* lizards

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## Abstract

Fine-level taxon discrimination is important in biodiversity assessment and ecogeographical research. Genomic markers are often required for studies on closely related taxa, however, most existing mitochondrial and nuclear markers require prior knowledge of the genome and are impractical for use in small conservation projects. This study describes the application of amplified fragment length polymorphism (AFLP) to discriminate at four progressively finer evolutionary levels of Caribbean *Anolis* lizards from the central Lesser Antilles. AFLP is shown to be a rapid and effective method for discriminating between species. Separation increases with primer pair number and choice of primer combination appears to be noncritical. Initial population-level results show markedly less discriminatory power. A screening technique for the identification of population informative markers combining principal component and principal coordinate analyses is presented and assessed. Subsequent results show selected conspecific AFLP data to be remarkably congruent with those of mitochondrial DNA, microsatellite and morphological markers. The use of AFLP as a low-cost nuclear marker in species-level taxon discrimination is supported, whereas population level application demands further consideration.

*Keywords:* AFLP, *Anolis*, biodiversity assessment, genetic differentiation, population discrimination, species discrimination

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## Introduction

Genetic identification of discrete taxa is important in the detection and conservation of biodiversity. Highly speciose genera often present systematicists with difficulty in identifying individual species, because of either strong morphological similarity between taxa or marked morphological differences within taxa, necessitating the use of molecular markers. *Anolis* lizards in the Caribbean are one such group, exhibiting rapid geographical and adaptive radiations throughout the region (Roughgarden 1995). The ability to distinguish between *Anolis* taxa is important at an interspecific level in assessing genetic diversity particularly on satellite islands, and at a population level in the study of evolutionary processes on small islands. The four neigh-

bouring islands of Dominica, Martinique, St. Lucia and Barbados in the central Lesser Antilles typify the pattern of *Anolis* phylogeography, each supporting an endemic species that displays distinct morphological and molecular intra-specific diversity. In addition, phylogenetic studies of the relationships between species show that a large range of interspecific diversification exists within this island group. As such, it provides an excellent model for an assessment of the emerging molecular technique, amplified fragment length polymorphism (AFLP; Vos *et al.* 1995), in a novel application to taxonomic discrimination in reptiles.

Originally restricted to use in the separation of microbial and plant species, AFLP has recently been extended for use in vertebrates such as snakes (Giannasi *et al.* 2001), herring gulls (de Knijff *et al.* 2001), domestic cattle (Nijman *et al.* 1999), cichlid fish (Albertson *et al.* 1999) and catfish (Liu *et al.* 1998, 1999). The technique produces large numbers of reproducible markers for assessing diversity across the

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nuclear genome and provides several advantages over microsatellite and mitochondrial DNA (mtDNA) approaches. AFLP analysis does not require prior knowledge of the genome, thereby eliminating the developmental time, efforts and costs required for specific locus markers. It also allows rapid interspecies discrimination, whereas taxon-specific nuclear markers, e.g. microsatellites, often do not. These factors potentially promote AFLP as a key molecular tool in modern research, particularly in application to small-scale conservation projects (Mueller & Wolfenbarger 1999). At an intraspecific level, AFLP has successfully differentiated between vertebrate populations, for example between Iberian pig breeds (Ovilo *et al.* 2000), however, the dominant nature of AFLP markers and the resulting loss of heterozygotic detection weakens its utility in population dynamic studies. Despite the development of dominant marker estimators for genetic drift and effective population size (Jorde *et al.* 1999), the theoretical usefulness of AFLP in population genetics is currently limited, however, identification of co-dominant AFLP markers and techniques, such as 'microsatellite AFLP', may soon overcome this problem (Mueller & Wolfenbarger 1999).

Here, the four islands under study are situated across the centre of the Lesser Antilles archipelago at the point at which northward and southward radiations of *Anolis* meet (Thorpe *et al.* 2002). *Anolis oculatus*, from the northernmost island of Dominica, is a member of the *bimaculatus* series, originating from the Greater Antilles, whereas *A. roquet*, *A. luciae* and *A. extremus* from Martinique, St. Lucia and Barbados, respectively, represent the furthest reach of the *roquet* series which originated from South America (Fig. 1). Mitochondrial cytochrome *b* studies estimate a maximum mtDNA divergence of 26% between these two series (*A. oculatus* and *A. roquet*) and 21% maximum divergence within the *roquet* series (*A. luciae* to *A. extremus*) (Giannasi 1997). Population-level studies on Dominica combining mtDNA, microsatellite and morphological data have discriminated between populations along a west coast transect (Malhotra & Thorpe 1994; Stenson 2000), whereas strong morphological population differentiation within the mtDNA haplotype has been found in southern Martinique. These results provide the framework for a multilevel investigation of taxonomic separation using AFLP. A combination of between- and within-island studies and comparison with existing molecular and morphological data enable the investigation of discrimination at progressively finer systematic levels.

The objective of this study therefore, is to examine the levels at which AFLP can discriminate between taxonomic groups of *Anolis* species. Successful application of AFLP to *Anolis* systematics would enable rapid assessment of genetic diversity and conservation value within the genus while simultaneously verifying its use in population level evolutionary studies.

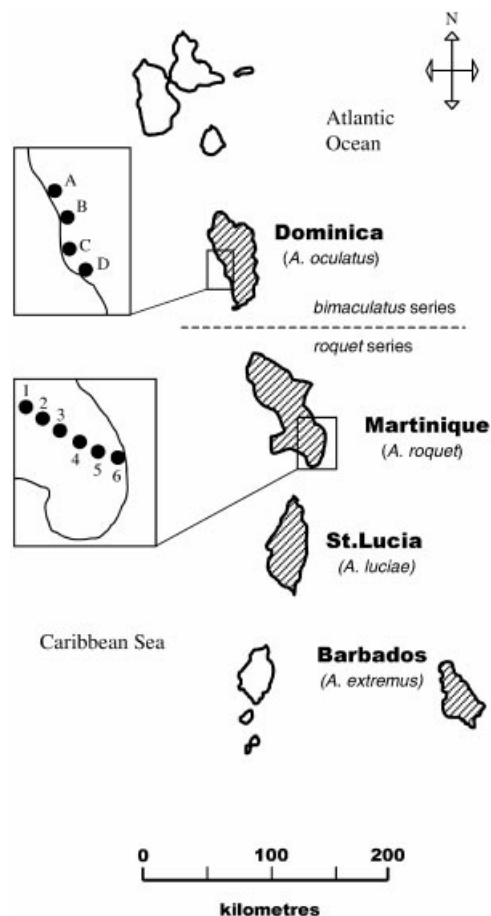


Fig. 1 The central Lesser Antilles, with detail of the sample transects on Dominica and Martinique, and the north-south *Anolis* species group divide.

## Materials and methods

### Experimental design and sampling

The work was divided into individual studies producing comparative AFLP data at four graduated taxonomic levels (localities shown in Fig. 1).

*Level 1.* Congeneric species from two distantly related species groups, *Anolis oculatus* (*bimaculatus* series, Dominica) and *A. roquet* (*roquet* series, Martinique).

*Level 2.* Congeneric species within the *roquet* series, *A. luciae* (St Lucia) and *A. extremus* (Barbados).

*Level 3.* Conspecific, parapatric populations of *A. oculatus*, from four sample sites along an established west coast transect on Dominica. A 6.5-km long transect section displays DNA change in microsatellite and mtDNA markers congruent with morphological variation.

Level 4. Conspecific populations of *A. roquet* from six sample sites along a transect in southern Martinique. This 3.5 km long transect displays morphological variation only.

DNA was sampled by taking tail tip biopsies from lizards collected on the four islands. In total, 48 *A. oculatus*, 90 *A. roquet*, 15 *A. luciae* and 15 *A. extremus* individuals were sampled. The tail tips were stored in 75% ethanol prior to DNA extraction.

#### DNA extraction and AFLP procedure

DNA was extracted from the tail tips using the NucleoSpin C + T extraction kit (Macherey-Nagel GmbH & Co.). AFLP was then performed using the AFLP Analysis System 1 kit (Gibco BRL, cat. no. 10544-013, <http://www.invitrogen.com>), developed from work by Vos *et al.* (1995). The protocol followed the manufacturer's instructions with three S3-nucleotide selective primer extension combinations used for the forward and reverse primers. Each primer combination comprised of an *EcoRI* radiolabelled (<sup>33</sup>P) primer, E-AGG, with one of three *MseI* primers (M-CTT, M-CAT, M-CTA). These will subsequently be referred to as follows: primer pair A (E-AGG + M-CTT), primer pair B (E-AGG + M-CAT) and primer pair C (E-AGG + M-CTA). Following the prescribed series of polymerase chain reactions (PCRs) and acrylamide gel electrophoresis (6% polyacrylamide, 55 W, 2.5 h), the gels were dried and exposed to X-ray film (Kodak XLS-1) for 72 h.

#### Primary data analysis

AFLP markers were scored by hand and coded as 1/0 for presence/absence for fragments ranging from ≈ 100–280 bp in length. Data from the three primer pairs were analysed both individually and collectively by combining matrices. With AFLP data, eigenvectors can be extracted from a correlation matrix by principal components analysis or a dissimilarity matrix by principal coordinates analysis. These give similar or mathematically dual results as discussed in Thorpe (1980). Using *MVSP* Version 3.11c (Kovach 1999), a dissimilarity matrix was generated using Gower's general similarity coefficient. As only binary data were being used in this analysis, Gower's general similarity coefficient ( $GGSc_{ij}$ ) was equivalent to Jaccard's coefficient ( $J_{ij}$ ):

$$GGSc_{ij} = J_{ij} = a / (a + b + c)$$

where  $a$  is the number of bands shared by individuals  $i$  and  $j$ ,  $b$  is the number of bands present in  $i$ , but not in  $j$  and  $c$ , the number in  $j$  but not in  $i$  (Sneath & Sokal 1963). A Q-mode principal coordinate analysis (PCOA) was subsequently performed on the dissimilarity matrix. Resulting principal coordinate scores were then plotted to assess the usefulness of AFLP in discriminating between species or

populations. To measure any increase in species discrimination with an increase in the number of primer pairs used, PCOA scatter plots from one, two and three primer pairs were compared. Pairwise Mantel tests on three matrices based on the single primer pairs (A vs. B, A vs. C, B vs. C) and three matrices based on two primer pairs (A & B vs. C, A & C vs. B, B & C vs. A) were then applied to test for association between primer pair data (TFPGA Version 1.3, 10 000 randomizations).

#### Secondary data analysis

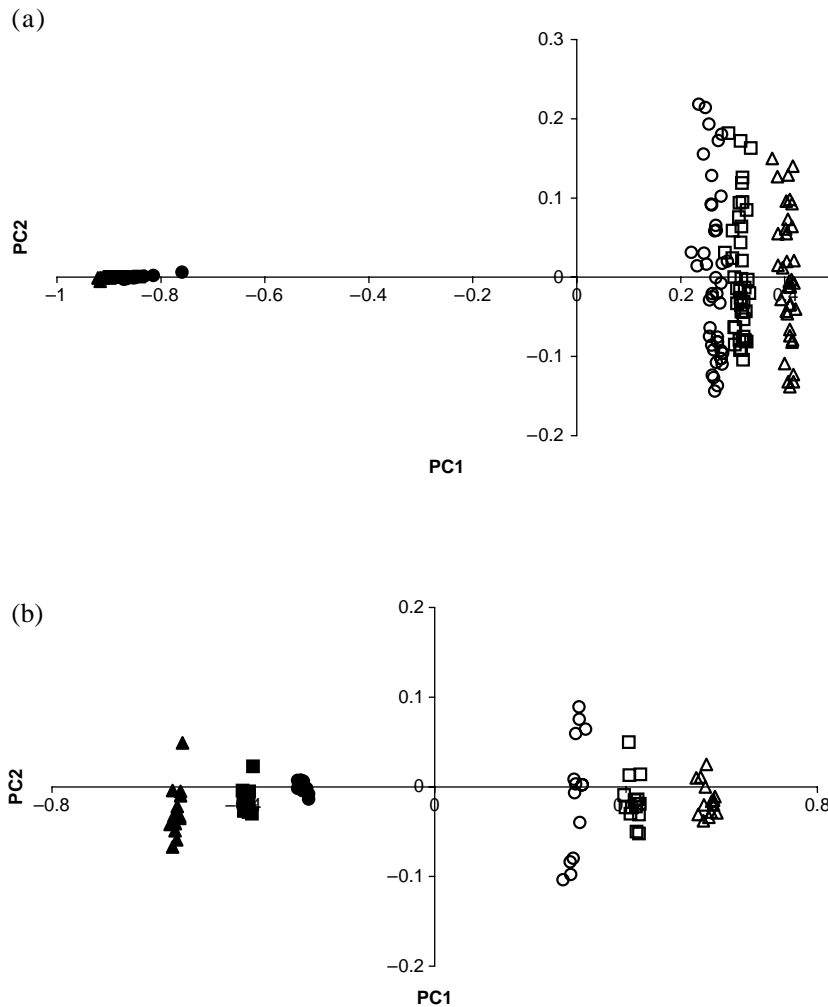
A secondary analysis was employed at the finer evolutionary levels (3 and 4) to test whether relatively high within-locality variation produced a lower signal-to-noise ratio. The technique aimed to increase the signal strength by selecting population-informative AFLP bands; a method traditionally used in morphological analyses that overtly or covertly select characters to optimize the study. Multivariate discrimination between morphotypes using a specific set of characters is widely used, for example in the identification of Colorado fish species (Douglas *et al.* 1998) and in the separation of Californian Channel Island deer mouse populations (Pergams & Ashley 1999); a study that also demonstrates how the optimal set of selected characters can change over small evolutionary time. Hence, in this study we sought to use a selective approach with AFLP data in order to isolate a suite of characters (genetic markers) from the total character set that contain the clearest population signal. The following rationale was designed to identify such a subset of markers that produced between-locality differentiation. An additional marker was incorporated into the data representing perfect binary discrimination between the two outermost localities on each transect. An R-mode principal component analysis was then conducted using only these two localities. The resulting similarity matrix allowed the identification of bands that correlated strongly with the artificial marker. The bands were ranked in order of decreasing correlation and a fixed proportion (the top 20%) was selected as a subset of population informative markers. These markers were then used in a principal coordinate analysis conducted using all localities to test whether an improved population signal could be extracted from the data.

#### Results

The three primer pairs produced over 300 DNA fragments (bands) at each of the four taxonomic levels studied. The percentage of polymorphic bands decreased with taxonomic level from 93.0% between species groups to 61.9% between populations of *Anolis oculatus*, indicating a reduction in the potential capacity for discrimination at the population level (Table 1).

**Table 1** Amplified fragment length polymorphism (AFLP) results from the four individual studies. Percentage polymorphism decreases with systematic level

| Systematic level          | <i>Anolis</i> species           | Sample origin                | No. of AFLP bands (markers) |          |          | Polymorphic bands (%) |
|---------------------------|---------------------------------|------------------------------|-----------------------------|----------|----------|-----------------------|
|                           |                                 |                              | Primer 1                    | Primer 2 | Primer 3 |                       |
| Species (between species) | <i>oculatus</i> , <i>roquet</i> | Dominica/Martinique          | 85                          | 120      | 100      | 93.0                  |
| Species (within series)   | <i>luciae</i> , <i>extremus</i> | St. Lucia/Barbados           | 132                         | 120      | 140      | 84.8                  |
| Population                | <i>oculatus</i>                 | Dominica (4 site transect)   | 100                         | 110      | 100      | 61.9                  |
| Population                | <i>roquet</i>                   | Martinique (6 site transect) | 105                         | 108      | 100      | 62.9                  |

**Fig. 2** Principal coordinate plots based on AFLP data showing separation between: (a) *Anolis oculatus* (open symbols) and *A. roquet* (closed symbols), and (b) *A. luciae* (open symbols) and *A. extremus* (closed symbols). Differentiation increases with primer pair number:  $n = 1$ , circles;  $n = 2$ , squares;  $n = 3$ , triangles.

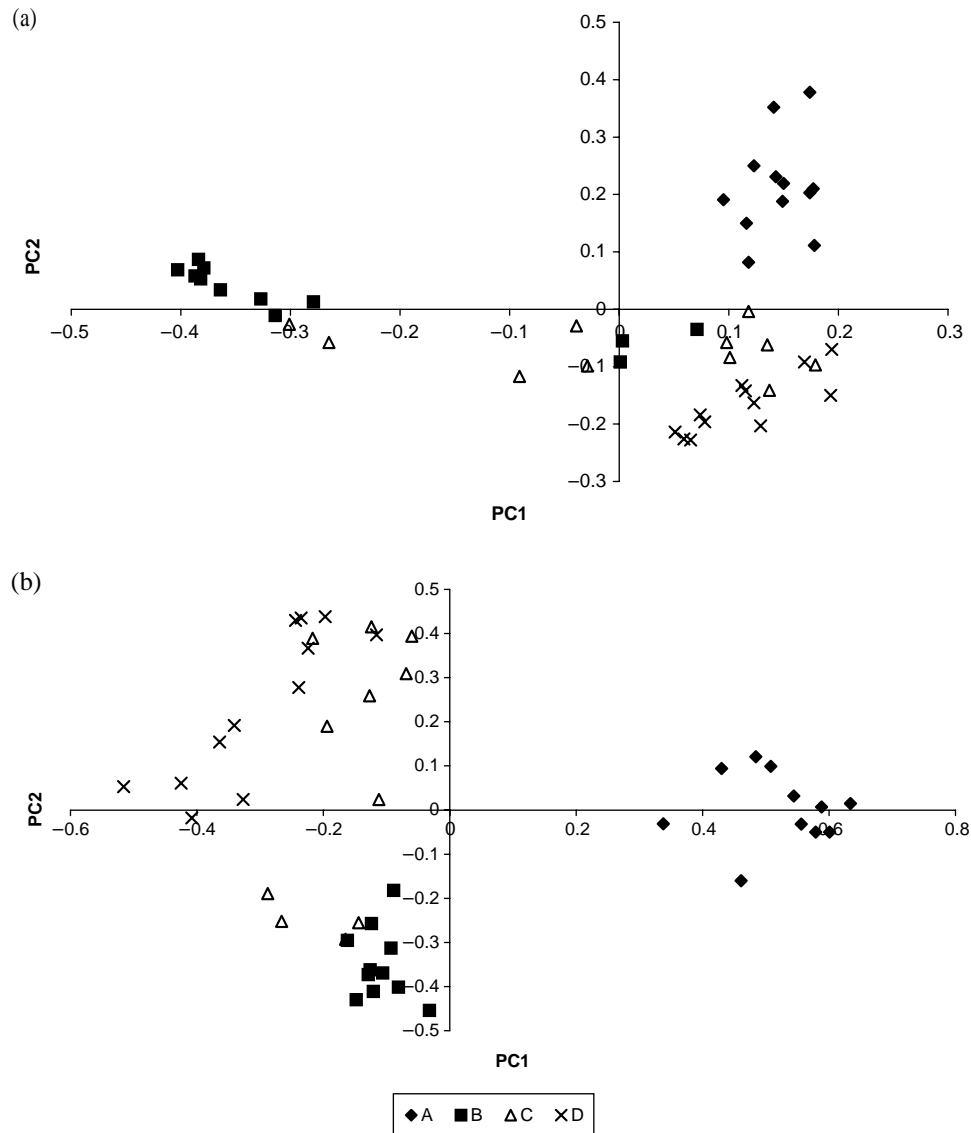
### Level 1: Distantly related species

The results of PCOA between species from separate series show that each primer combination produced discrimination between the two species along the first PC axis. In addition, combined primer pair data indicate that successively greater discrimination is obtained when two and three primer pairs are used, with data points being separated further along the first axis when additional primer pair

data is incorporated (Fig. 2a). Mantel test results for the intergroup study show strong association between primer pair matrices with  $P < 0.001$  in all six comparisons.

### Level 2: Closely related species

The clear species discrimination along the first principal axis demonstrated at level 1 was repeated in the PCA results for the more closely related species at level 2.



**Fig. 3** Principal coordinate scatter plots of AFLP data from three primer pairs showing the distribution of *Anolis oculatus* using (a) all polymorphic bands and (b) a subset of selected bands (20%) generated from the secondary analysis. Symbols represent sample sites of conspecific populations across putative transition zone shown.

Discrimination also increased with the number of primer pairs used (Fig. 2b). Again, Mantel test results showed association between all primer pair matrices.

### Level 3: Marked population differentiation

In comparison with the interspecies studies, the population-level principal coordinate analysis of *A. oculatus* shows considerably less discrimination, even when using three primer pairs (Fig. 3a). The plot shows limited separation by transect locality spread along both axes, which account for 26.3% of the total variation (Table 2), however, the orientation in character space of the first principal

coordinate axis does not correspond to that of the transect in geographical space.

Subsequently, the identification of a subset of population-informative markers was undertaken (see Secondary data analysis in Materials and methods). This technique resulted in greater population differentiation along the transect, with localities being distributed in geographical order along the first principal coordinate axis (Fig. 3b). This axis described 26% of the total variation compared with 16% using all markers (Table 2). Furthermore, AFLP population change was highly congruent with changes in microsatellite, mtDNA and morphological variation as can be seen in a plot of character change

**Table 2** Cumulative eigenvalue percentage on the first three principal coordinate axes for *Anolis oculatus* and *A. roquet*. A greater percentage of total variation is incorporated into the three axes using principal coordinate analysis based on selected bands

| Principal coordinate analysis       | % variation on axis 1 | % variation on axes 1–2 | % variation on axes 1–3 |
|-------------------------------------|-----------------------|-------------------------|-------------------------|
| <i>A. oculatus</i> (all bands)      | 16.6                  | 26.3                    | 32.8                    |
| <i>A. oculatus</i> (selected bands) | 26.1                  | 45.1                    | 52.2                    |
| <i>A. roquet</i> (all bands)        | 9.1                   | 13.2                    | 17.9                    |
| <i>A. roquet</i> (selected bands)   | 13.1                  | 21.3                    | 28.8                    |

against transect distance, which shows a rapid transition occurring in all markers over a small geographical range (Fig. 4).

#### Level 4: Subtle population differentiation

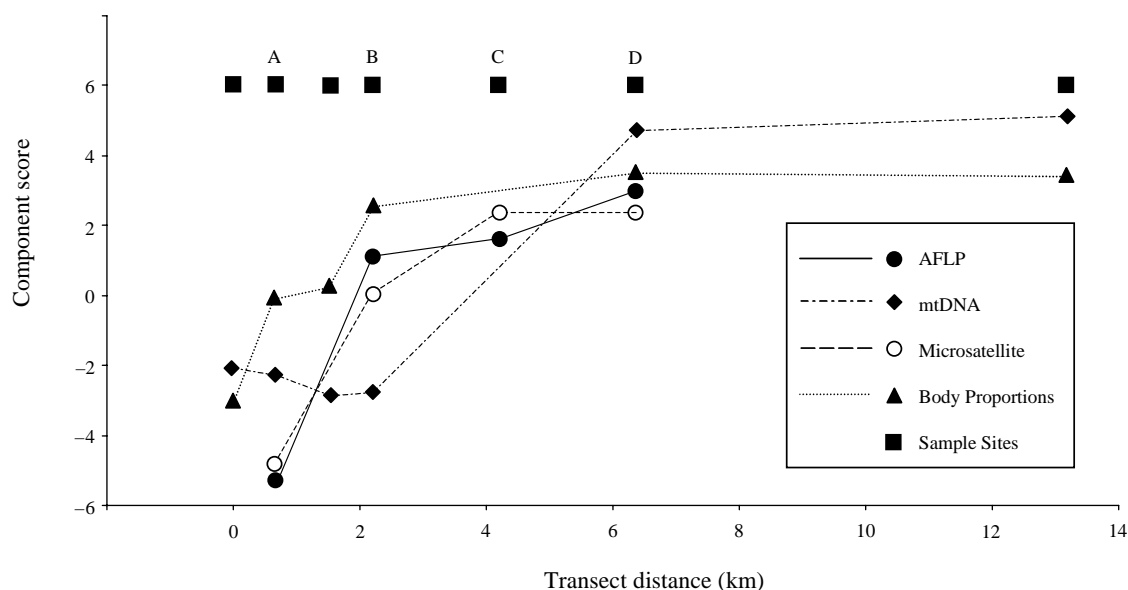
At the finest taxonomic level under investigation, population differentiation was negligible. PCOA results for all available AFLP bands show almost no discrimination between *A. roquet* sampling localities (Fig. 5a) with just 13.2% of variation described by the first two axes (Table 2).

Following the identification of a subset of population-informative markers, some differentiation by transect locality became apparent with population groups distributed along the first axis (Fig. 5b). The change in mean locality component score with transect distance closely parallels a clinal change in morphology across the same localities (Fig. 6). This clearly indicates that informative AFLP markers are capable detecting genotypic change at a fine taxonomic level, although the effect is small compared with the higher levels investigated here.

#### Discussion

A recombining molecular marker that can simultaneously discriminate between species and populations is a valuable asset in molecular ecological and conservation research. Until recently it has been necessary to rely on relatively lengthy and expensive techniques such as restriction fragment length polymorphism (RFLP) analysis of nuclear DNA (nrDNA). AFLPs have now provided an alternative approach to such studies. The work presented here examines the usefulness of AFLP over graduated taxonomic levels within the *Anolis* genus. Its ability to separate species and the potential application to population level studies, combined with the comparative cost in terms of both time and money, suggest that AFLP has enormous potential in these areas.

The clear discrimination produced in this study between both *Anolis oculatus* and *A. roquet* (level 1), and *A. luciae* and *A. extremus* (level 2), supports the use of AFLP in



**Fig. 4** Transect multiple marker component data for *Anolis oculatus* along the west coast of Dominica. Rapid variation in component score occurs between sites A and D in all markers. (Diagram adapted from Malhotra & Thorpe 1994; microsatellite data from Stenson 2000.)

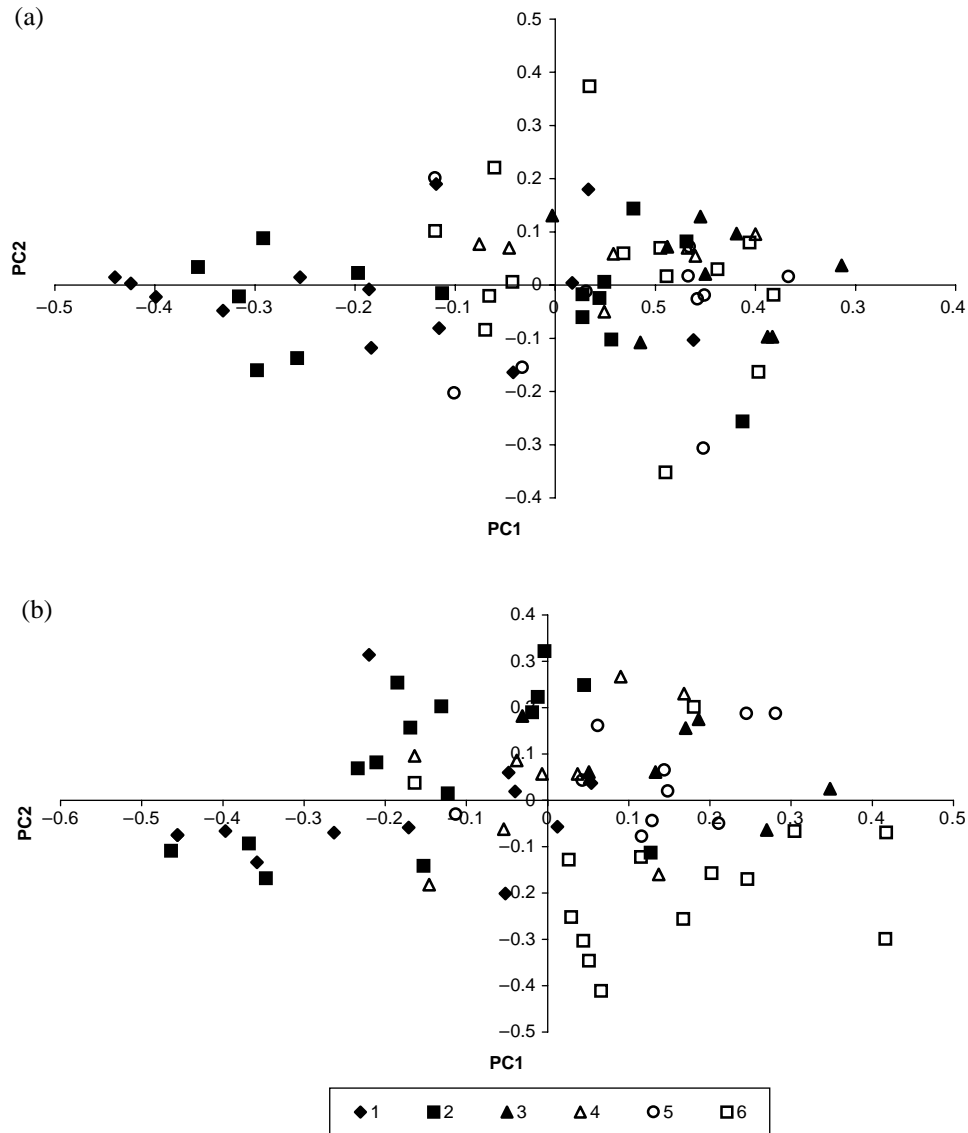
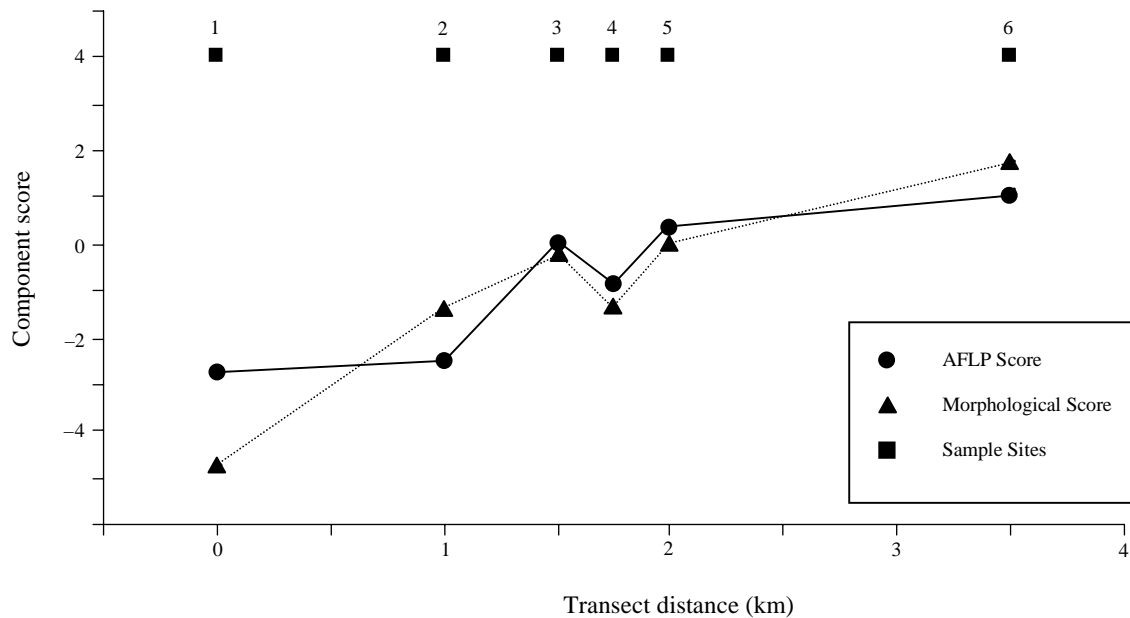


Fig. 5 Principal coordinate plots of amplified fragment length polymorphism data from three primer pairs showing the distribution of *Anolis roquet* using (a) all polymorphic bands and (b) a subset of selected bands (20%) generated from the secondary analysis. Symbols represent sample sites (populations) shown in Fig. 1.

species-level discrimination of *Anolis* taxa. The finding that any individual primer pair was sufficient to separate species using only the first principal coordinate axis, together with the significant correlation between matrices suggests that at this level, the choice of primer pair is not critical. The high percentage of fragment polymorphism in comparison with the within-island results, indicates that a large proportion of the AFLP markers were species specific, explaining the strength of species separation. The increase in discriminatory ability with the number of primer pairs scored is concordant with a recent study of cichlid fishes (Albertson *et al.* 1999), in which dendrographic bootstrap values and resolved nodes increased with primer pair number.

Within species (levels 3 and 4), lower levels of polymorphism reduced the number of informative markers and the remaining polymorphic markers displayed a high ratio of within-population to between-population variability. Therefore, although the number of informative markers could be increased by using more primer pairs, the ability to assign individuals to a population without prior identification of population markers remains limited. Previous AFLP studies of vertebrate populations have encountered similar problems. De Knijff *et al.* (2001), found that only 23% of AFLP polymorphisms were due to between-taxon differences in the herring gull assemblage, whereas low polymorphism levels have been found in catfish by Liu



**Fig. 6** The parallel between changes in amplified fragment length polymorphism and morphological marker mean component scores for *Anolis roquet* populations along the transect in southern Martinique.

*et al.* (1998, 1999), who tested 64 primer combinations to generate sufficient bands from which to isolate specific markers. It seems likely that such widespread screening and marker development would yield similar results in *Anolis* species, however, this would negate many of the advantages that result from the use of AFLP described here.

The application of a selection procedure to identify informative markers without examination of individual bands allows individuals to be clustered more accurately into geographical populations while maintaining experimental objectivity. The technique maximizes differentiation at the ends of the transect, but does not predefine the nature (gradual or stepped) or position of the transition between the two extremes. The use of an artificial marker to compare with bands produced from transect extremes enables markers describing variability along that transect to be selected. Some sort of character selection is standard in morphometric analysis in order to improve the signal-to-noise ratio (e.g. Douglas *et al.* 1998; Brede *et al.* 2000), but is not generally carried out in AFLP analysis.

This selection technique was most successful when applied to *A. oculatus* data (level 3), increasing the percentage variation described on the first two principal coordinate axes (from 26.3 to 45.1%) and improving population discrimination along the transect. The plot of mean population coordinate scores against geographical distance (Fig. 4), demonstrates the close correlation in variation between AFLP, mtDNA, microsatellite and morphological data further supporting AFLP as an alternative population marker.

As expected, the usefulness of AFLP was found to be most restricted at level 4, between morphologically distinct

populations of *A. roquet* in southern Martinique. Even so, the identification of parallel clinal change between population means in AFLP and morphological data demonstrates the ability of the selected marker analysis to promote population discrimination. However, individuals were not clearly separated by locality under PCOA, and the use of AFLP as a precise population discriminator at very fine taxonomic levels is not fully supported by this study.

The application of the AFLP technique to different taxonomic levels investigated in this study promotes its use as a species specific nuclear marker well suited to the needs of researchers in molecular ecology. However, although the selective screening of AFLP markers described herein can increase discriminatory ability, the usefulness of AFLP at the population level is more ambiguous, possibly necessitating the use of a large number of primer pairs and individual screening of several thousand markers, elevating the research investment required to a level comparable with that of other locus specific techniques.

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This study forms part of Rob Ogden's PhD thesis on the genetic evolution of Caribbean island lizards. The work is part of a programme of research, directed by Roger S Thorpe, on the factors influencing population differentiation and speciation in island lizards and the role of nuclear markers in evolutionary and systematic studies.

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