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Source: Journal of Orthoptera Research, 10(2) : 203-207

Published By: Orthopterists' Society

URL: https://doi.org/10.1665/1082-6467(2001)010[0203:AFLPFT]2.0.CO;2

Amplified fragment length polymorphism for the analysis of genetic structure in grasshopper populations

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Abstract

A recently developed PCR-based assay, amplified fragment length polymorphism (AFLP), has been used for assessing phylogenetic relationships and the genetic structure of populations in many organisms including insects. Preliminary studies show that the procedure does not work well in acridid grasshoppers since it leads to very large numbers of indistinct bands, probably due to the large size of the genome. To overcome this drawback, we developed long primer sets with four selective nucleotides, and used these for the grasshoppers *Chorthippus brunneus* and *C. jacobsi* in northern Spain. This approach has allowed us to reduce the number of bands, so that we can compare homologous bands easily. Using the revised method, it appears that the degree of interspecific genetic differentiation of *C. brunneus* and *C. jacobsi* is relatively small.

Key words

Chorthippus biguttulus, species group, AFLP, genetic differentiation, +4 primers

Introduction

Recent advances in molecular phylogenetics using mitochondrial and nuclear DNA polymorphisms have contributed to providing a detailed picture of microevolutionary patterns in various organisms. Some fingerprinting techniques, such as restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD), have been used for the rapid screening of genetic diversity among lower-level taxa; however, no single technique is universally ideal because each available technique exhibits both strengths and weaknesses (Mueller & Wolfenbarger 1999).

A recently developed polymerase chain reaction (PCR)-based marker system, amplified fragment length polymorphisms (AFLPs), has repeatability and resolution superior or equal to those of other markers. Originally developed primarily for genetic mapping, it has also been used for detecting genetic differentiation at fine taxonomic levels (*e.g.*, Giannasi et al. 2001). Although there is difficulty in identifying homologous markers (alleles), rendering AFLPs less useful for studies that require precise assignment of allelic status (Mueller & Wolfenbarger 1999), the advantages nevertheless outweigh the disadvantages.

Some difficulties concerning data acquisition still exist with AFLPs; one of them is how to regulate the number of bands resolved by AFLPs to be suitable for reliable scoring of phenotypes from gels. Preliminary studies show that large numbers of indistinct bands can be observed in particular taxa so that it is very difficult to find homologous bands. This is presumably due to the large size of the genome. However, there are two ways to solve this problem. One of them is to use a restriction enzyme with a longer (*e.g.*, six-base) recognition site to reduce the number of restriction fragments (Hawthorne 2001). Another is to apply long primer sets to selective PCR (Young *et al.* 2001). We found that the standard AFLP procedure developed by Vos *et al.* (1995) leads to many unclear bands in acridid grasshoppers. Here, we evaluate the use of long primer sets to produce a reasonable number of clear AFLP bands from two members of the *biguttulus* species group of the genus *Chorthippus*, *C. brunneus* (Thunberg) and *C. jacobsi* (Harz), in northern Spain. These species form a hybrid zone with a complex spatial distribution of hybrid and parental phenotypes (Bridle *et al.* 2001, Bridle 1998, Bailey 2000). AFLPs may provide valuable markers for the study of this zone.

Materials and Methods

Population sampling and DNA extraction.— We collected nymphs and adults of *C. brunneus* and *C. jacobsi* from four sites in northern Spain (Fig. 1). All nymphs collected in the field were transferred into 30-cm high, 24-cm diameter larval rearing cages. They were reared to adult in a laboratory where the temperature was maintained at a minimum of 25°C by an overhead 60W lamp. The bottom of the cage was covered in fresh *Dactylis glomerata* grass, which was replaced every two or three days. All adults were preserved in 100% ethanol. Whole genomic DNA was prepared using the CTAB method (Winnepenninckx *et al.* 1993, Reineke *et al.* 1998).

The AFLP technique (Vos *et al.* 1995) consists of two consecutive PCRs (preselective and selective amplifications, see Fig. 2), which amplify the DNA fragments generated by a double-restriction digestion and subsequent ligation of specific nucleotide adapters. In the selective PCR step, amplification was performed with the addition of cytosine as the first selective nucleotide on both the *Eco*RI and *Mse*I linkers (Fig. 2). To compare the resolution of different

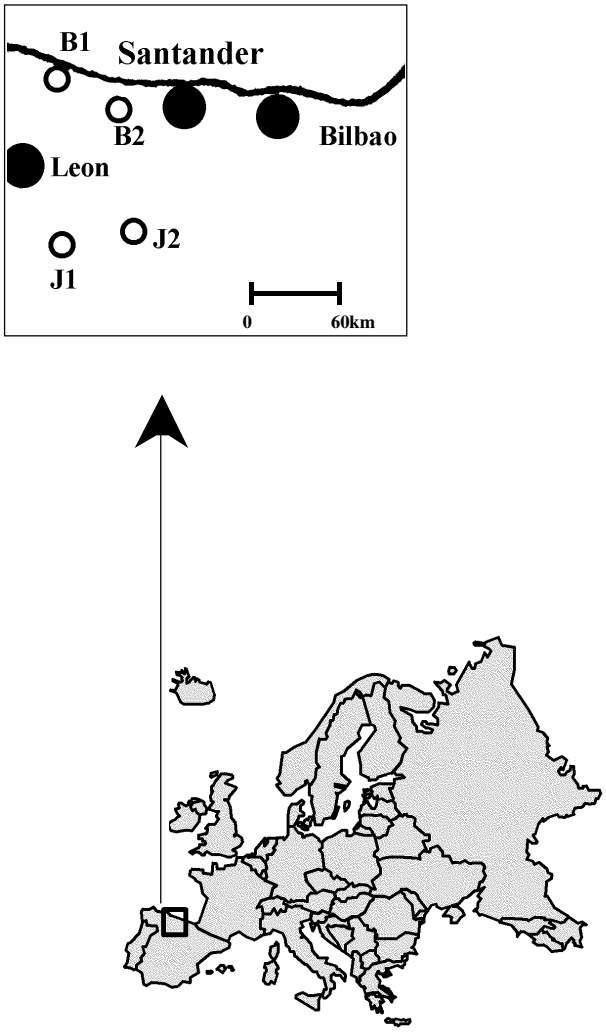

Fig. 1. Map showing the collection sites of two *Chorthippus* species. Samples were collected from the following populations (number of males and females): (B1) *C. brunneus* population 1 (29); (B2) *C. brunneus* population 2 (23); (J1) *C. jacobsi* population 1 (22); (J2) *C. jacobsi* population 2 (25).

lengths of primers, we used +3+3 (*Eco*RI-CTC, *Mse*I-CAA), +4+3 (*Eco*RI-CTCC, *Mse*I-CAA), and +4+4 (*Eco*RI-CTCC, *Mse*I-CAAT) primer combinations to generate the AFLP markers in selective amplifications. Since +4+4 primer combinations provide the most definite bands (see Fig. 3), we used several combinations of +4+4 primer pairs for the phylogenetic analysis described below. *Eco*RI primers were labelled using γ^{33}P ATP.

PCR products resulting from selective amplifications were separated on a 6% acrylamide gel using a sequencing electrophoresis apparatus (Gibco BLR) run for 3 h at 55W. Bands were detected using autoradiography with exposure times ranging from 48 to 72 h depending on the strength of the radioactive signal. Because of the number of specimens used in this study each primer pair combination was run on a separate gel, thus avoiding the potential problems of scoring bands across gels.

Data analysis.— The presence or absence of each AFLP marker band was scored by hand from the autoradiograph and coded as a binary character. For all analyses, data from the five primer combinations (Table 1) were combined to form a raw data matrix consisting of all bands. Based on the raw data matrix, we calculated allele frequencies (assuming Hardy-Weinberg equilibrium since the 'presence' allele is dominant to the 'absence' allele) and hence Nei's (1972) genetic distance between each pair of samples. A dendrogram was then constructed using the Neighbor-Joining (NJ) method (Saitou & Nei 1987). Calculation of the genetic distance and NJ tree was conducted using NTSYS-pc (Rohlf 1994).

Table 1. The number of marker bands obtained for each of five kinds of four-base pair primer combinations.

Primer pair	*Eco*RI	*Mse*I	Number of markers
1	CAGG	CCTG	23
2	CAGG	CAAG	47
3	CTCC	CAAT	16
4	CTCC	CGAG	28
5	CAGA	CCTG	40

Results and Discussion

The number of bands resolved by different primer combinations was reduced as the selective primers became longer (Fig. 3). Among the three primer combinations, the +4+4 primer combinations were the most suitable for reliable scoring of homologous bands because each band was clearly distinct. We applied the +4+4 primer combinations to other species of acridid grasshoppers, and here too they provided a reasonable number of bands (Tatsuta & Butlin, unpublished data). It is likely that the large number of bands resolved by three-base primer combinations suggested by Vos *et al.* (1995) is due to a large size of the genome of Acridid grasshoppers, a result similar to other animal species (*e.g.* Young *et al.* 2001). Thus +4+4 primer combinations rather than +3+3 or +4+3 ones, would be the best solution for obtaining distinct bands suitable for data analysis when the shorter length primers give many ambiguous bands. A total of 159 markers was scored and 94% of them were polymorphic.

The NJ tree demonstrates that there is a low level of genetic differentiation among populations and between *C. brunneus* and *C. jacobsi* (Fig. 4). Genetic distances between intraspecific populations were as large as interspecific dis-

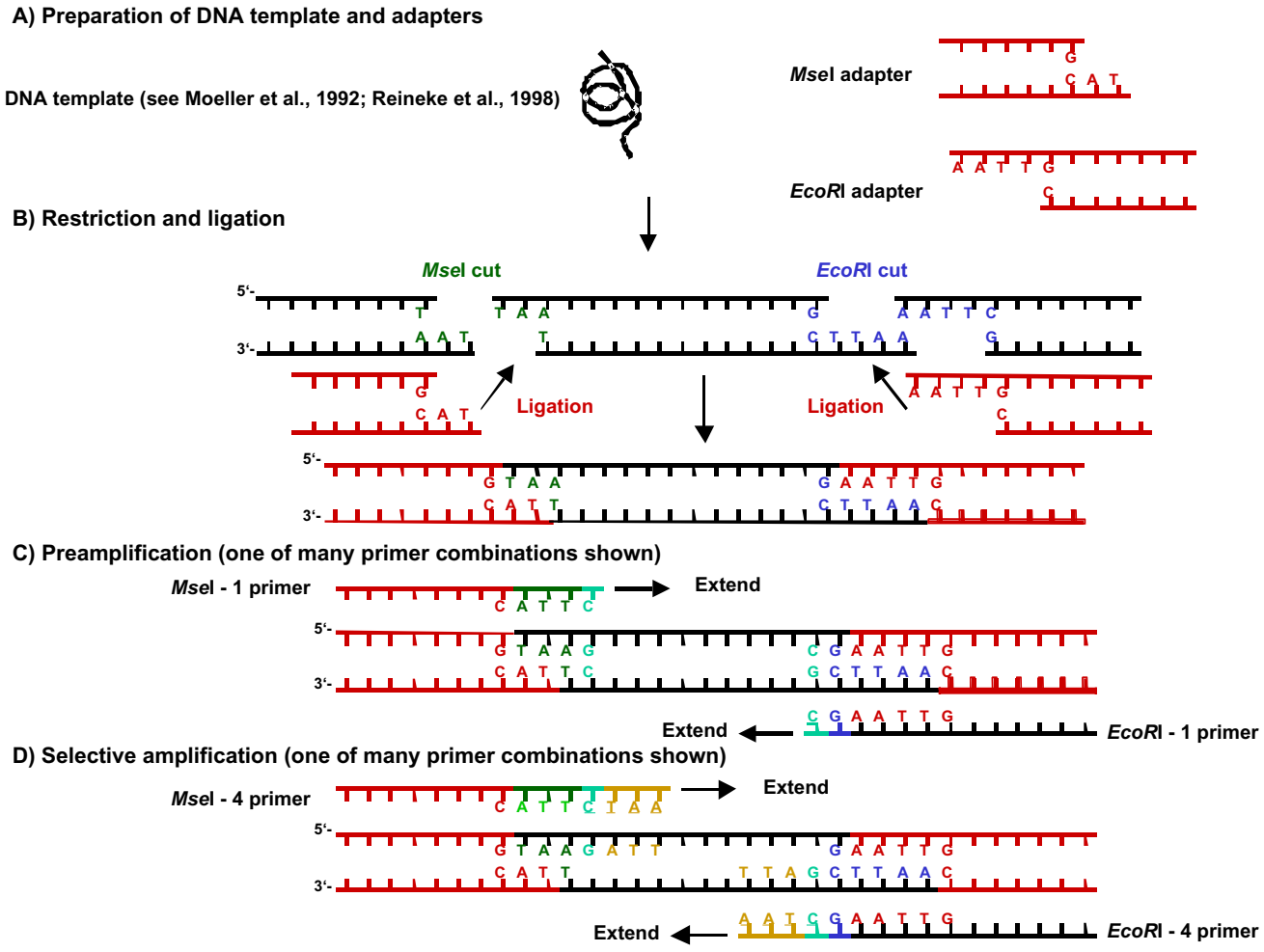

Fig. 2. Flowchart of AFLP method using four-base primer combinations (after Mueller & Wolfenbarger 1999). Details are given in text.

tances, suggesting that the degree of genetic differentiation between species is small relative to the variation within species as has been observed for other markers (Mason *et al.* 1995). Further analysis using additional populations is needed to understand genetic differentiation and diversification of the *C. biguttulus* species group, but the AFLP technique provides a powerful tool for this purpose.

Acknowledgements

We cordially thank Jon R. Bridle and Ayumi Tatsuta for their field assistance. Thanks are also due to Craig Wilding for his kind guidance for AFLP analysis, and three anonymous reviewers for useful comments on earlier versions of this paper. This study was supported in part by The Japan Society for the Promotion of Science (JSPS) Research Fellowship for Japanese Young Scientists (No. 08608, H. T.) and a British Council Grant for JSPS Fellows (H. T.).

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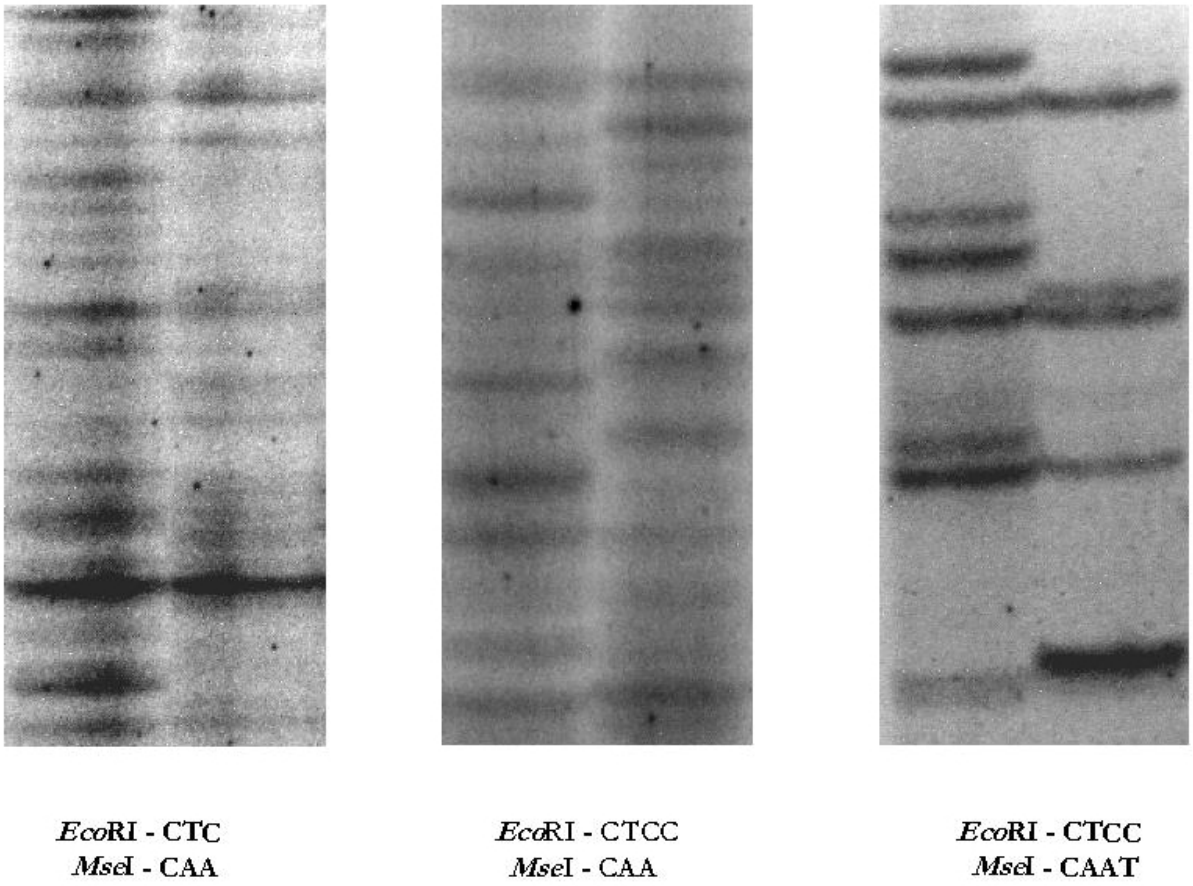

Fig. 3. Sections of AFLP autoradiographs based on different combinations of selective primers.

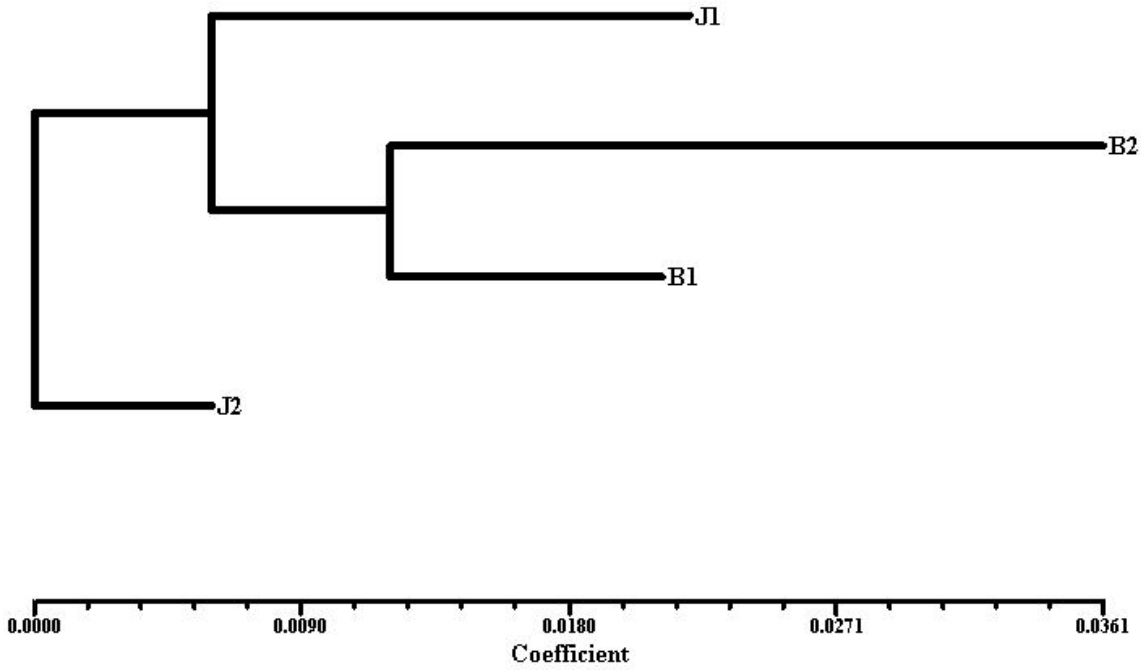

Fig. 4. Neighbor-joining tree based on pairwise Nei's genetic distance.