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Comparison of RAPD and PCR-RFLP markers for classification and taxonomic studies of insects

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ABSTRACT

Experiments were conducted to assess the efficiency of RAPD and PCR-RFLP marker systems as molecular tools for taxonomy and classification of insects. Three species of adult ants, *Camponotus maculatus, Monomorium pharoensis* and *Cataglyphis bicolor*, were used in this study. Total genomic DNA was extracted and used for PCR amplification of the ITS region. The purified PCR products were subjected to single digestion with *Eco*RI, *Msp*I, *Taq*I and *Sac*I enzymes and visualized on 2% agarose gel. In addition, 40 arbitrary primers were used to generate RAPD-PCR profiles. Data obtained from RFLP, RAPD and RFLP+RAPD were statistically and phylogenetically analyzed to determine the level of polymorphism in each case. Intraspecific, interspecific variation levels and phylogenetic relationships were investigated. Results were discussed, comparatively, and the final conclusion was: on the bases of ease, cost, technical labor, speed and amount of DNA needed, the **RAPD** marker system is preferentially used in taxonomic and classification studies. On the bases of degree of polymorphism, precision of genetic distance estimates and the statistical power of the test, **no differences** were observed between RAPD and PCR-RFLP tests.

Keywords: PCR-RFLP, RFLP, RAPD-PCR, RAPD, polymerase chain reaction.

INTRODUCTION

The past limitations associated with the pedigree data, morphological, physiological and cytological markers for assessing genetic diversity in many species have largely been circumvented by the development of DNA markers such as restriction fragment length polymorphisms (RFLPs; Botstein *et al.*, 1980) and random amplified polymorphic DNAs (RAPDs; Williams *et al.*, 1990). The detection and exploitation of naturally occurring DNA sequence polymorphisms are among the most significant developments in molecular biology. Polymorphic genetic markers have wide potential applications in animal and plant improvement programmes as a means for varietal and parentage identification, evaluation of polymorphic genetic loci affecting quantitative economic traits, and genetic mapping (Nagaraju *et al.*, 2001).

Although the discrimination power of RFLPs in diversity studies has been well documented (Smith *et al.*, 1990; Dudley *et al.*, 1991; Messmer *et al.*, 1993; Benchimol *et al.*, 2000), the limitations related to the technical complexity of performing RFLP analysis coupled with the widespread use of short-lived radioisotopes for detection have prompted researchers to look for alternative methods. The advent of the PCR has resulted in the development of a large number of molecular techniques, which offer an effective alternative to the hybridization methods of RFLP analysis. PCR-based approaches use only small quantities of DNA, avoid DNA blotting and use of radioactivity, and are amenable to automation. The RAPD method described by Williams *et al.* (1990) and Welsh & McClelland (1990)

generates PCR products by annealing to randomly distributed homologous target sites of the template DNA. This technique mostly generates dominant markers, although length polymorphisms caused by insertions/ deletions can also occur at low frequencies. Because of its relative simplicity, RAPD technology is being extensively used in genetic analysis of various plant and animal species. However, limitations in the applications of RAPDs such as the dominant nature of the markers, chance co-migration of bands at different loci, and requirement for stringent protocol standardization to ensure reproducibility have also been encountered (Black, 1993). As PCR technology finds increased use in genetic analysis, novel variations of this technique are emerging which promise precision, economy and speed (Wu *et al.*, 1994; Zietkiecwicz *et al.*, 1994; Vos *et al.*, 1995).

The aim of the present study was to evaluate RFLPs and RAPD-PCR assays as molecular marker systems for classification and taxonomic studies using representative formicid species of the order Hymenoptera. This evaluation was based on terms of cost, speed, amount of DNA needed, technical labor, degree of polymorphism, precision of genetic distance estimates and the statistical power of the test.

MATERIALS AND METHODS

Insect material and DNA extraction

Three species of adult ants, *Camponotus maculatus, Monomorium pharoensis* and *Cataglyphis bicolor*, were used in the present study. All DNA extractions were performed using DNeasy tissue kit (QIAGEN, GmbH, Hilden, Germany) following the manufacturer's instructions. Individuals from each insect species were pooled to reach ≈0.1-0.5 g tissue weight and homogenized in a sterile 2 ml microcentrifuge tube and incubated in lysis buffer overnight at 60 ◦C. The lysate of the whole insect body was used to isolate total genomic DNA.

RAPD analysis

RAPD analyses were performed using 40 primers obtained from Operon Technologies Inc., Alameda, Calif. (kits A and B). Reactions were performed in 25 µl volumes containing 50 mM Tris, pH 8.5, 1 mM MgC12, 20 mM KC1, 500 mg/ ml BSA, 2.5 % Ficoll 400, 0.02 % Xylene cyanole, 1 unit *Taq* polymerase, 50 ng template DNA, 1 µM primer, and 0.2 mM dNTPs. A negative control, containing water instead of template, was included in each reaction set. The amplification program used was 5 min at 94°C (hot start), 1 min at 94°C, 2 min at 30°C and 2 min at 72°C for 40 cycles followed by one cycle of 72°C for 7 min. PCR amplification was carried out in a DNA thermal cycler (Model 380 A, Applied Biosystems, CA, USA). Following amplification, the reactions were visualized on 2 % agarose gel prepared in lx TAE buffer (Sambrook *et al.*, 1989) containing 0.1 µg/ ml of ethidium bromide and photographed using gel documentation system.

PCR-RFLP analysis

The DNA was suspended in 100 µl of TE buffer (pH 8.0) and used for PCR amplification of the ITS region. The universal primers ITSF and ITSR (Collins and Paskewitz, 1996) were used for the amplification of ITS using the PCR cycling parameters as described by Proft *et al.* (1999). The PCR individual bands of ITS region were visualized and eluted from the gel using GenClean Kit (Invitrogen Corporation, San Diego, CA, USA) as described by the manufacturer. The purified PCR products were subjected to single digestion with *Eco*RI, *Msp*I, *Taq*I and *Sac*I enzymes (Promega, Corp. Madison, WI, USA) overnight at 37 ◦C in a 20 µl reaction

volume. The digested fragments were resolved on a 2% agarose gel, visualized and photographed using gel documentation system.

Data analysis

In both RFLP and RAPD assays, banding patterns of the samples were scored for the presence (1) or for absence (0) of each amplified band. All RAPD assays were repeated thrice and only the reproducible bands were scored. For considering a marker as polymorphic, the absence of an amplified product in at least one sample was used as a criterion. For genetic distance analysis, data sets were fed into the clustering program of SPSS (Version 14.0) and similarity matrix was determined using Jaccard's coefficient. Next, distance matrix (distance = 1 - similarity) was calculated. Based on similarity matrices using the unweighted pair group method analysis, STATISTICA program for Windows, 1995 (StatSoft, Inc., USA) was used to generate UPGMA dendrogram (Norusis, 1994). The Chi-square test was used to analyze the data obtained and differences were considered statistically significant if $P< 0.05$.

RESULTS

Levels of polymorphism

Banding patterns:

Forty RAPD primers were used to amplify random sequences from the total DNA of the three ant species, *C. maculatus, M. pharoensis* and *C. bicolor*. All the primers gave clear banding patterns, as shown in Fig. (1).

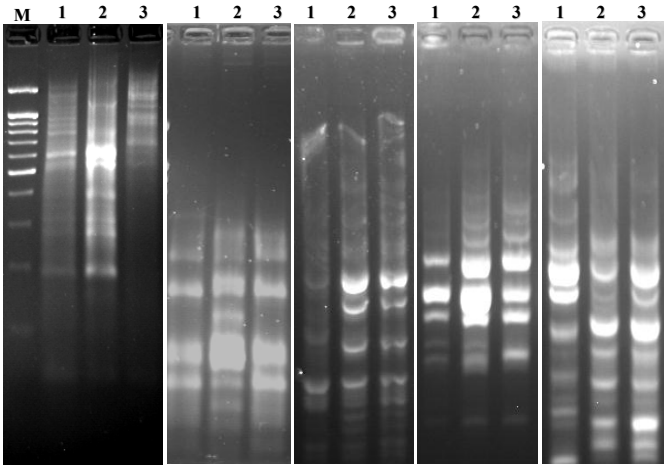

Fig. 1: Representative 2% agarose gels of RAPD-PCR patterns generated from **three** ant species, *C. maculatus, M. pharoensis* and *C. bicolor* using forty arbitrary primers OPA and OPB. Lane M: DNA marker 100 bp Ladder, lane 1: *C. maculatus*, lane 2: *C. bicolor* and lane 3: *M. pharoensis*.

An average of 7.5 bands per primer were obtained for one amplification and a total of 100 bands per species were compared among the 3 species. In RFLP assay, an average of 5 bands were observed for each reaction (Fig. 2) and 7 bands per species were obtained with four restriction enzymes in 36 RFLP reactions. RAPD analysis revealed that the highest average percentage of common (monomorphic) bands (78.1%) was observed within *C. maculatus*. Meanwhile, the highest average percentage of polymorphic bands (45.5%) was observed within *M. pharoensis* (Table 1).

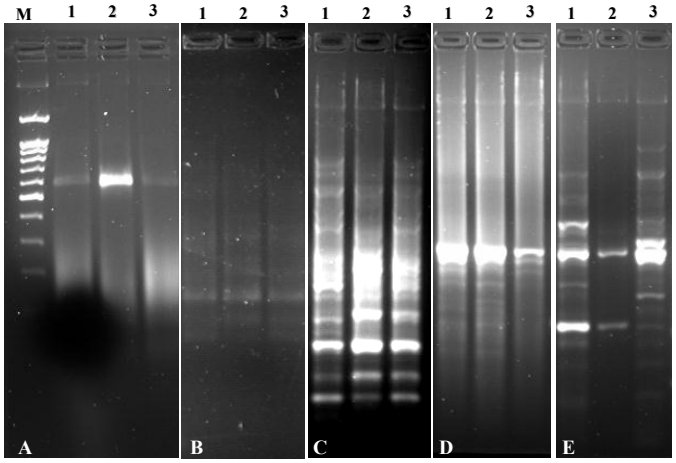

Fig. 2: Representative 2% agarose gels of PCR-RFLP patterns generated from three ant species, *C. maculatus, M. pharoensis* and *C. bicolor* using forty arbitrary primers. Lane M: DNA marker 100 bp Ladder, lane 1: *C. maculatus*, lane 2: *C. bicolor* and lane 3: *M. pharoensis*. A: PCR of ITS region, B: *Eco*RI, C: *Sac*I, D: *Taq*I and E: *Msp*I.

Table 1: Average percentages of common and polymorphic bands within each species as revealed by RAPD and RFLP analyses.

Ant species	Total no. of tested bands	RAPD molecular marker		RFLP molecular marker	
		% Monomorphic	% Polymorphic	% Monomorphic	% Polymorphic
C. maculatus	556	200/256 78.1	56/256 21.9	164/300 54.7	136/300 45.3
M. pharoensis	741	216/396 54.5	180/396 45.5	250/345 72.5	95/345 27.5
C. bicolor	563	187/248 75.4	61/248 24.6	172/315 54.6	143/315 45.4

On the other hand, RFLP assay clarified that the highest average percentage of monomorphic bands (72.5%) was observed within *M. pharoensis*. Meanwhile, the highest average percentage of polymorphic bands (45.4%) was observed within *C. bicolor* (Table1). Furthermore, RAPD analysis revealed that the highest average percentage of monomorphic bands (90.7%) was observed between *M. pharoensis* and *C. bicolor*. Meanwhile, the highest average percentage of monomorphic bands resolved by RFLP (94.6%) was observed between *C. maculatus* and *C. bicolor* (Table 2). On the other hand, the highest average percentages of polymorphic bands resolved by RAPD and RFLP (18.4 and 15.8%) were observed between *M. pharoensis* and *C. maculatus* (Table 2).

Table (2): Average percentages of common and polymorphic bands between species as revealed by RAPD and RFLP analyses.

Ant species	Total no. of tested bands	RAPD molecular marker		RFLP molecular marker	
		% Monomorphic	% Polymorphic	% Monomorphic	% Polymorphic
M. pharoensis and *C. maculatus*	1297	532/652 81.6	120/652 18.4	543/645 84.2	102/645 15.8
M. pharoensis and *C. bicolor*	1304	584/644 90.7	60/644 9.3	562/660 85.2	98/660 14.8
C. maculatus and *C. bicolor*	1119	447/504 88.7	57/504 11.3	582/615 94.6	33/615 5.4
M. pharoensis, C. maculatus and *C. bicolor*	1860	603/900 67.0	297/900 33.0	586/960 61.0	374/960 39.0

Intraspecific variation (within each species):

The average percentages of monomorphic bands within each species are shown in Table (1). Among all species, the average percentages of monomorphic bands were 67.0% and 61.0% for RAPD and RFLP analyses, respectively (Table 2). These overall similarity values indicated the detectability of polymorphism with the two kinds of molecular marker systems. The intraspecific variation within the three ant species, *C. maculatus, M. pharoensis* and *C. bicolor*, was shown in Table (1).

Interspecific variation (between species):

The average percentages of monomorphic bands between species are shown in Table (2). Between all species, the average percentages of monomorphic bands were 33.0% and 39.0% for RAPD and RFLP analyses, respectively (Table 2). The average percentages of polymorphic bands were higher between *M. pharoensis* and *C. maculatus* (18.4% and 15.8% for RAPD and RFLP, respectively) than between *M. pharoensis* and *C. bicolor* (9.3% and 14.8% for RAPD and RFLP, respectively) and between *C. maculatus* and *C. bicolor* (11.3% and 5.4% for RAPD and RFLP, respectively) (Table 2). These overall dissimilarity values indicated the detectability of polymorphism with the two kinds of molecular marker systems. The interspecific variation between the three ant species, *C. maculatus, M. pharoensis* and *C. bicolor*, was shown in Table (2).

Phylogenetic analyses:

Phylogenetic analyses have been performed on the RAPD results and RFLP results as well as for the data of RAPD+RFLP (Fig. 3). RAPD, RFLP and RAPD+RFLP data.

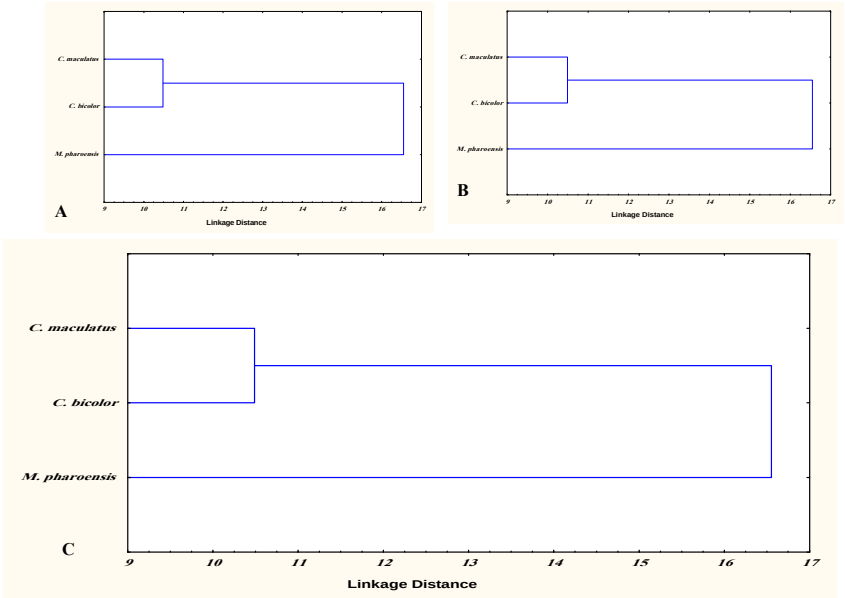

Fig. 3: Dendrograms constructed on the basis of similarity index among three ant species, *C. maculatus, M. pharoensis* and *C. bicolor* using data of RAPD, PCR-RFLP and RAPD+PCR-RFLP. A: RAPD, B: PCR-RFLP and C: RAPD+PCR-RFLP.

Amplification with 40 random primers generated 603 monomorphic bands and 297 polymorphic bands in a total of 900-banded RAPD patterns. Whenever, RFLP

generated 586 monomorphic band and 374 polymorphic bands in a total of 960-banded RFLP patterns. A total of 900 (RAPD), 960 (RFLP) and 1860 (RAPD+RFLP) distinct bands obtained were used for cluster analysis. The three UPGMA dendrograms revealed that 80% similarity cut-off value gave the same two major clusters. *C. maculatus* and *C. bicolor* were clustered in the same genotype while the *M. pharoensis* was clustered in a separate genotype (Fig. 3). *Chi* square and Fisher's tests revealed significant differences between both *M. pharoensis* and *C. maculatus* and between *M. pharoensis* and *C. bicolor*. However no significant difference between *C. bicolor* and *C. maculatus* was observed in cases of RFLP and RAPD+RFLP data.

DISCUSSION

Compared to standard RFLP analysis, PCR-RFLP has many advantages. However, amplification of specific regions requires knowledge of the flanking sequences to obtain the primers needed for the reaction (Saiki *et al*. 1988). RAPD-PCR technique does not require knowledge of specific sequences, but rather uses random 10 base-pair primers. This method has become very useful for population genetic and taxonomic studies (Williams *et al*. 1990; Welsh and McClelland, 1990).

As reported in this study, after a screen of 40 random primers, 18.4, 9.3, 11.3 and 33.0% polymorphic DNA markers were obtained between *M. pharoensis* and *C. maculates*, *M. pharoensis* and *C. bicolor*, *C. maculates* and *C. bicolor* and between *M. pharoensis*, *C. maculates* and *C. bicolor*, respectively. Comparable levels of genetic polymorphism were obtained using RFLP marker system (15.8, 14.8, 5.4 and 39.0% between *M. pharoensis* and *C. maculates*, *M. pharoensis* and *C. bicolor*, *C. maculates* and *C. bicolor* and between *M. pharoensis*, *C. maculates* and *C. bicolor*, respectively). Interestingly, the phylogenetic and statistical analyses of the data obtained from RAPD, RFLP and RAPD+RFLP marker systems resulted in clustering *C. maculatus* and *C. bicolor* in one major genotype and *M. pharoensis* in a separate genotype. These results suggested that *C. maculatus* and *C. bicolor* have a common ancestor and are genetically closer to each other than to *M. pharoensis*.

These results clarified that the same results (in terms of degree of polymorphism, precision of genetic distance estimates and the statistical power of the test) were obtained using either the data of RAPD or RFLP marker systems. El-Alfy *et al*. (2009) assessed genetic variation among nile tilapiine fishes by RAPD markers. Alam *et al*. (2006) used PCR-RFLP successfully to identify four members of *Anopheles annularis* group of mosquitoes. Nagaraju *et al.* (2001) compared four marker systems (RFLP, RAPD, ISSR-PCR and SSRs) in genetic analysis of the silkworm, *Bombyx mori*. They concluded that ISSR-PCR is best suited for generation of the volume of information required for performance of such a task. Chapini *et al.* (1999) investigated the genetic relationship between four *Anagrus* hymenopteran species using RAPD markers and found that they represent four genetically distinct species. Aljanabi *et al*. (1998) indicated the potential of RAPD markers to demonstrate the genetic variability of stink bug egg parasitoids. Wilkerson *et al.* (1993) reported that RAPD analysis will prove to be a powerful and technically accessible tool in elucidating the systematics of uncharacterized dipteran species complexes from natural populations. They also discussed some problematic characteristics of RAPD banding patterns and approaches to overcome these problems were suggested.

In this study, RAPD analysis proved to be as informative as PCR-RFLP analysis. The frequency of diagnostic primers observed in this study is quite high in comparison to studies of closely related species. There were no accepted methods for estimating genetic divergence from RAPD data and this may be considerable technical difficulty. However, the genetic similarity suggested by RAPD survey of *C. maculatus* and *C. bicolor* species is seemingly at odds with their extreme morphological dissimilarity. While RAPD analysis is clearly an approach of merit for such studies, some characteristics of RAPD reactions were encountered. First, RAPD band patterns must be empirically determined to be reproducible before their use as markers is justified. This could be made after repeated reactions to confirm the absence or presence of the band. Second, preferential amplification during RAPD reactions has little effect on the use of RAPD markers in diagnostic studies, but could be of serious importance in studies requiring independent markers, such as hybrid zone analysis, pedigree analysis or relatedness estimates based on band sharing. Finally, the inference that bands of similar size in different individuals are truly homologous should be made cautiously and rejected in cases of complex banding patterns or the bands that differ in intensity or reproducibility. Band homology can only be definitely determined by further investigation, such as Southern blotting or sequencing. To avoid complicated interpretation, the primers used should have some characteristics, such as strong diagnostic bands and simple patterns. Primers producing banding patterns that are in any way suspect should be passed over in favour of screening additional primers for markers with optimal characteristics.

Conclusively, on the bases of ease, cost, technical labor, speed and amount of DNA needed, the RAPD marker system is preferentially used in taxonomic and classification studies. On the bases of degree of polymorphism, precision of genetic distance estimates and the statistical power of the test, no differences were observed between RAPD and PCR-RFLP tests.

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ARABIC SUMMARY

مقارنة بين استخدام معلمات تفاعل البلمرة العشوائى المتسلسل و معلمات تفاعل الرفلاب التباينى فى الدراسات التقسيمية و تصنيف الحشرات

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أجريت التجارب لتقييم كفاءة معلمات تفاعل البلمرة العشوائى المتسلسل و معلمات تفاعل الرفلاب التباينى كأدوات جزيئية للتصنيف و تقسيم الحشرات. واستخدمت ثلاثة أنواع من النمل البالغ لهذه الدراسة، وهى: *كاتاجليفيس بيكولور* ، *مونوموريام فارونيسيس و كامبونوتاس ماكيولاتاس* . تم استخلاص الحمض النووي الجينومي من الحشرات ، ليستخدم بعد ذلك فى تفاعل الرفلاب التباينى مع أربعة من إنزيمات القطع و هى: *EcoRI، MspI ، Taq1 , SacI* و تم فصل الناتج على ٢ ٪ هلام أجاروز. وبالإضافة إلى ذلك ، تم استخدام ٤٠ بادىء عشوائى لتوليد معلمات تفاعل البلمرة العشوائى المتسلسل. بعد الحصول على البيانات من تفاعل البلمرة العشوائى المتسلسل و تفاعل الرفلاب التباينى وتحليلها إحصائيا و عمل الشجرة التصنيفية للعينات لتحديد مستوى التباين في كل حالة. وقد تمت دراسة مستويات التباين فى النوع الواحد ، ومستويات التباين فيما بين الأنواع وعلاقات النشوء والتطور. ونوقشت النتائج ، مع االدراسات السابقة ، وكان الاستنتاج النهائي كالآتى: على أسس سهولة العمل والتكلفة والعمالة والسرعة والكمية المطلوبة من الحمض النووي ، فإن معلمات تفاعل البلمرة العشوائى المتسلسل تعتبر الأفضل في الدراسات التصنيفية والتقسيمية. وعلى أسس مستوى التباين ، ودقة تقدير المسافة الجينية ، والقوة الإحصائية للاختبار ، لم يلاحظ أي اختلافات بين كفاءة معلمات تفاعل البلمرة العشوائى المتسلسل و معلمات تفاعل الرفلاب التباينى.