

Genetic Relationship Among *Leptogenys Chinensis* and *Myrmicaria Brunnea* (Hymenoptera: Formicidae) by RAPD Molecular Markers

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ABSTRACT

Ants are important factor in ecosystem. They are greatly affected with environmental changes and human interference. Ants biomass do not look a lot like species richness and so their genetic information is valuable. *Myrmicaria brunnea*, harvester ant and *Leptogenys chinensis*, long legged ant used in study as representatives of Myrmicinae and Ponerinae respectively. RAPD technique is most common, reliable and cost-effective for study of genetic variability among different species. DNA marker was used for assessment of genetic integrity and variation among *Myrmicaria brunnea* and *Leptogenys chinensis*. Three RAPD markers, as P1, P2 and P3 generated 151 bands. *M. brunnea* and *L. chinensis* generated 66 and 85 bands respectively. Maximum polymorphic bands were generated by P2 primer (37) while, maximum monomorphic (17) bands were generated by primer P1. Study of monomorphic and polymorphic bands were carried out and statistical data generated were used to construct phylogenetic tree and fluorograms. *M. brunnea* and *L. chinensis* were found linked together at about 0.4 genetic distance.

Keywords: Ants, *Myrmicaria*, *Leptogenys*, RAPD, PCR, Hymenoptera.

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I. INTRODUCTION

In ecosystem, ants are of great significance due to their biomass and work for maintaining health and stability of the environment. Ants act as pollinators, seed dispersers and are important to nutrient cycling and ecosystem functioning. [7] [18]

Among 660 species from 87 genera of ants, *Myrmicaria* family has maximum diversity i.e. 45% of total Indian ants [18]. Ponerinae is largest subfamily of poneromorphs, 23 genera of ponerini found on all continents and major islands [4] [15] while, subfamily ponerinae with 14% diversity of species among which *Leptogenys* shows maximum diversity [8] still least studied. From a common evolutionary ancestral group poneromorphs suggested to be descended, diverged early on, near base of formicide tree [19].

In taxonomy, precise anatomical differences are used for classifying closely related species. In case of ants, it requires lots of taxonomic knowledge. Morphological data [6] and molecular data [3] [21] [27] [28], gives undisputable support for monophyletic origin of ants [18].

Environmental changes, vehicular vibration and additional manuring affects ant's population. Extinction is a demographic process that is likely to be influence of genetic factors [1]. Among social hymenopterans, though ants share common character like social behaviour and haploid male but differ in perennial and iteroparous colonies. Perennial species react more slowly to changing environment. Ants are omnipresent but their quantity (biomass) does not correspond to species richness. Wilson [29], Holldobler and Wilson [15] propose that more than one third of ant biodiversity still undiscovered and may go extinct before being discovered and studied [22].

Myrmicaria commonly known harvester ant and *Leptogenys* commonly known as long legged ants having rare ecological status [16]. For nesting *Myrmicaria* and *Leptogenys* prefers tree base and logs respectively. *Leptogenys chinensis* and *Myrmicaria brunnea* are major predators of *Antheraea mylitta* and affects tasar silk production [10]. *Leptogenys khammouanesis* newly identified species collected from cave shown characters like reduced eyes, light pigmentation, slender body, very elongated legs and antennae [20].

Recent years modern techniques in molecular biology such as polymerase chain reaction (PCR) and automated DNA sequencing greatly used in biology [14] for assessment of genetic variability. RAPD or Random Amplified Polymorphic DNAs technique is most common, reliable and cost-effective. DNA markers on PCR amplification detects small inverted repeats scattered throughout the genome helps in analysis of relatedness between genotype [12]. Diagnostic molecular characters can be generated with different primers, for taxonomic levels. In systematic relationships, constant fragments may be diagnostic for any one operational taxonomic units (OTU) and polymorphic for between OTUs [12].

Present study aims to examine genetic integrity and variation of *Leptogenys chinensis* and *Myrmicaria brunnae* assessed by RAPD markers using Fluorescent labelled primers.

II. MATERIAL AND METHOD

A. Sample collection

Leptogenys chinensis and *Myrmicaria brunnae* collected from Rashtrasant Tukdoji Maharaj, Nagpur University educational campus, Nagpur, Maharashtra state of India. The ants were collected by brush and transferred to collection tube containing 100% alcohol. The samples were coded *Leptogenys chinensis* (L1) as S1 and *Myrmicaria brunnae* (M2) as S2.

B. Extraction of genomic DNA

Genomic DNA was isolated from both samples using Chromus Genomic DNA isolation kit (RKN80/81). Samples homogenized with 200µl C-TAB isolation buffer on cooling added 10µl of RNase A solution. Samples firstly incubated at 37°C for 15 minutes and then for 30 minutes at 65°C. 1ml of Chloroform: isoamylalcohol (24:1) mix added on cooling and centrifuged at 10000rpm for 10 minutes, in supernatant equal volume of Phenol: (Chloroform: Isoamyl alcohol) 1:1 added and centrifuged at 10000 rpm for 10 minutes. Precipitation solution (1:1 ratio) added in supernatant. White strands of genomic DNA separated were incubated at -20°C for 30 minutes.

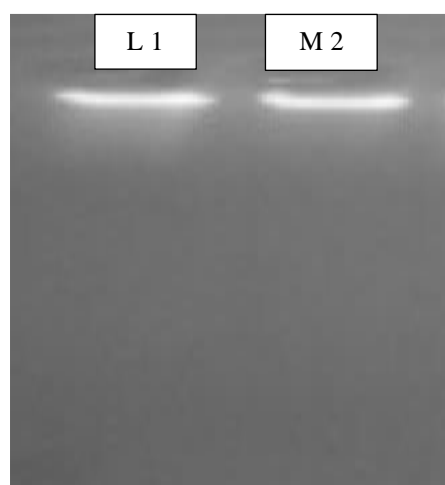


Fig. 1 Genomic DNA extracted from L1 *L. chinensis* and M2 *M. brunnae* loaded on 1% agarose gel.

C. RAPD primers screening

Genomic DNA was taken up for PCR amplification using 06 different randomly selected fluorescent labelled 20-mer RAPD primers (Chromous Biotech Pvt. Ltd., India). Primers that gave clear profiles were chosen for further study. Details of RAPD primers and their sequences are given in Table 1.

TABLE 1: Sequence, code and nucleotide length of primers used for RAPD analysis

S. N.	Primer sequence (5'→3')	Primer code	Nucleotide length
1	-CCCHGCAMCTGMTGCGCACHC-	P1	20-mers
2	-AGGHCTCGATAHCMGVY-	P2	17-mers
3	-MTGTAMGCTCCTGGGGATTCHC-	P3	22-mers

D. PCR Amplification

Reaction mixture (25 µl) containing 10X Chrom Taq Assay buffer (2.5 µl), 50ng of genomic DNA (0.5 µl), 100ng/µl RAPD primer (2.0 µl), 10mM dNTPs (1.0 µl), Chrom-Taq DNA polymerase (3U/ µl) (0.5 µl) and 18.5 µl of sterile distilled water incubated in ABI Thermal cycler:2720 DNA engine thermal cycler programmed for 40 cycles, denaturation at 94 °C for 5 minutes, annealing at 55°C for 1minute and 2 minutes for elongation at 72°C.

Amplification product were electrophoresed on 2% agarose gel in 1X TAE buffer mixed with 5 µl of staining dye at 100V and then photographed. Two molecular weight markers 500bp DNA ladder and 100bp DNA ladder were used for assessment.

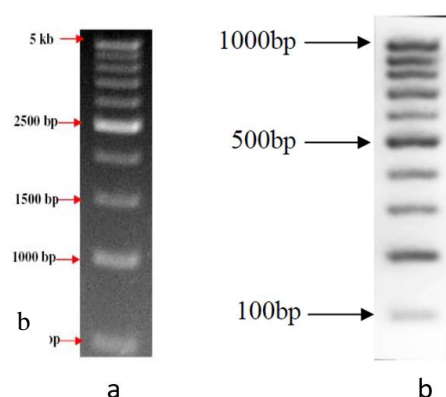


Fig. 2: (a) 500bp ladder contains 10 DNA fragments of size 500bp, 1000bp, 1500bp, 2000bp, 2500bp, 3000bp, 3500bp, 4000bp, 4500bp and 5000bp. (b) 100bp DNA ladder contains 10 DNA fragments of size 100bp, 200bp, 300bp, 400bp, 500bp(spiked), 600bp, 700bp, 800bp, 900bp and 1kb.

E. Statistical Analysis

Analysis of the molecular data for *L. chinensis* and *M. brunnae* scored on the basis of clear genomic DNA bands 1 for presence of a band and 0 for absence of band. If amplified product was absent in any one of the samples used for analysis, counted as polymorphic, and considered monomorphic if the amplified product was present in both samples.

The binary data from the fluorescent labelled RAPD primers by gene mapper (ABI Genetic Analyser; 3500xL) produced the RAPD profiles, data was subjected to Neighbor-

joining cluster analysis using Free Tree and Tree View software for phylogenetic analysis.

Coefficient of simple coincidence (S_{ij}), in which the genetic similarity between the i^{th} and j^{th} individual calculated by:

$$S_{ij} = a + d / a + b + c + d$$

Where,

a = number of bands present in both sample (1,1)

b = number of bands present in first sample and absent in second sample (1,0)

c = number of bands in absent first sample and present in second sample (0,1)

d = absence of bands in both samples (0,0)

for coefficient of similarity we considered double absence i.e d. absence of bands as evidence of homology (Skorch et al. 1992, Waldschmidt et al. 2002). The genetic similarity converted to genetic distance using expression:

$$D_{ij} = 1 - S_{ij}$$

Similarity index was used to calculate the genetic distance values and to construct the phylogenetic tree/ dendrogram.

III. RESULTS:

A. RAPD profiles

Isolated DNA from *L. chinensis* and *M. brunnae* were used to produce RAPD-based DNA profiles. Three random primers were applied for RAPD amplification.

TABLE 2: Bands obtained in sample 1 (*L. chinensis*) and sample 2 (*M. brunnae*) with three random primers

Sr.No.	Primer code	Bands Obtain	
		Sample 1	Sample 2
1	P1	34	34
2	P2	26	37
3	P3	6	14

Total bands generated by sample 1 (*L. chinensis*) were 66 and sample 2 (*M. brunnae*) were 85. The number of bands generated by individual primers shown in Table 2, Fig. 3.

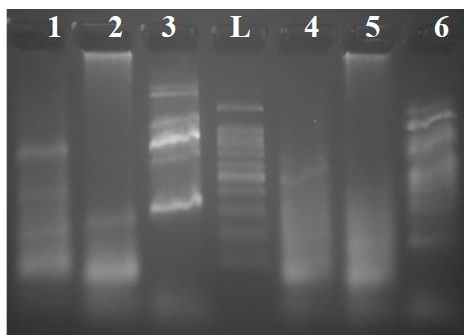


Fig. 3: Banding patterns of RAPD fragments of sample 1 (*L. chinensis*) and sample 2 (*M. brunnae*) with primer P1, P2, P3. Lane 1 is sample 1 v/s P1, Lane 2 is sample 1 v/s P2, Lane 3 is sample 1 v/s P3, Lane L is a marker 100bp ladder, Lane 4 is sample 2 v/s P1, Lane 5 is sample 2 v/s P2, Lane 6 is sample 2 v/s P3.

B. Level of Polymorphism

1. Banding pattern:

RAPD profiles obtained through amplification of genomic DNA of *L. chinensis* and *M. brunnae* showed different primers generated monomorphic and polymorphic bands. Compare to *L. chinensis* and *M. brunnae* primers generated 151 fragment bands. Percentage of polymorphic bands and monomorphic bands produced by 3 primers shown in Table 3 and 4. Furthermore, polymorphic bands produced by RAPD analysis in *L. chinensis* and *M. brunnae* with percentage of polymorphic bands using 3 primers shown in Table 5 and 6. The results showed different primers produced different fragment pattern i.e. different number and different DNA length (Fig.6-11).

TABLE 3: Polymorphic bands between *L. chinensis* and *M. brunnae* by RAPD markers.

Sr. No.	Primer	Number of scorable bands	Molecular size (bp)	Number of Polymorphic bands	% Polymorphic band
1	P1	51	50-326	34	66.66
2	P2	40	50-326	37	92.5
3	P3	18	52-500	16	88.88

TABLE 4: Monomorphic bands between *L. chinensis* and *M. brunnae* by RAPD markers.

Sr. No.	Primer	Number of scorable bands	Molecular size (bp)	Number of Monomorphic bands	% Monomorphic band
1	P1	51	52-161	17	33.33
2	P2	40	51-302	13	25.49
3	P3	18	267-271	02	11.11

TABLE 5: Polymorphic bands in *L. chinensis*.

Sr. No.	Primer	Total Number of bands	Molecular size (bp)	Number of polymorphic bands	% Monomorphic band
1	P1	34	53 – 325	17	50
2	P2	26	54 – 220	13	50
3	P3	06	80 – 281	04	66.66

TABLE 6: Polymorphic bands in *M. brunnae*.

Sr. No.	Primer	Total Number of bands	Molecular size (bp)	Number of polymorphic bands	% Monomorphic band
1	P1	34	59 – 326	17	50
2	P2	37	50 – 326	24	64.86
3	P3	14	53 – 500	12	85.71

2. Distance matrix table and Phylogenetic analysis:

Data from RAPD primers pooled together and genetic distance matrix was plotted using FreeTree and TreeView software Table 7, further subjected to Neighbor joining cluster analysis software and phylogenetic tree was constructed to evaluate the genetic diversity between *L. chinensis* and *M. brunnae*. The percent of polymorphism and genetic relationship among the both samples were analysed by developing phylogenetic tree Fig.4. Dendrogram cluster for *L. chinensis* and *M. brunnae* shown 2 clusters (Fig. 5) two samples were linked together with 100bootstrap.

TABLE 7: Distance matrix of *L. chinensis* and *M. brunnae* showing the genetic variation revealed by fluorescent-labelled RAPD markers using Nei's genetic diversity.

	<i>L. chinensis</i>	<i>M. brunnae</i>
<i>L. chinensis</i>		0.57616
<i>M. brunnae</i>	0.57616	

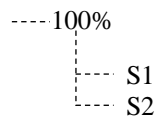


Fig. 4: Reference tree



Fig. 5: Dendrogram showing the clustering pattern between *L. chinensis* and *M. brunnae* revealed by FRAPD markers.

3. Fluorograms:

RAPD profiles of *L. chinensis* and *M. brunnae* with primer 1, 2 and 3 displayed as fluorograms. Single peak corresponds to single band on the gel.

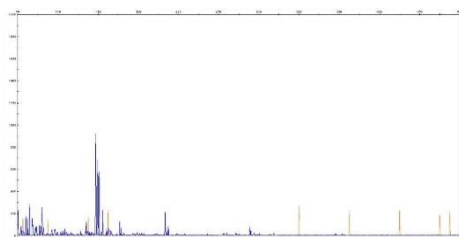


Fig. 6: Fluorogram of RAPD pattern of *L. chinensis* produced on the automated DNA sequencer (ABI genetic Analyser; 3500xL). Primer 1 was used for amplification reaction

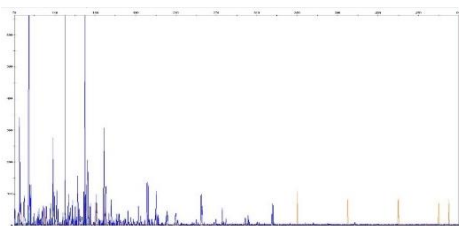


Fig. 7: Fluorogram of RAPD pattern of *M. brunnae* produced on the automated DNA sequencer (ABI genetic Analyser; 3500xL). Primer 1 was used for amplification reaction

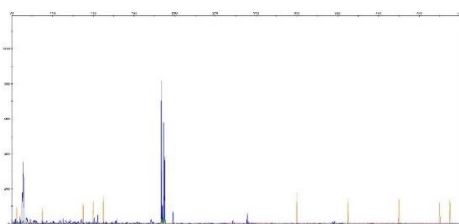


Fig. 8: Fluorogram of RAPD pattern of *L. chinensis* produced on the automated DNA sequencer (ABI genetic Analyser; 3500xL). Primer 2 was used for amplification reaction.

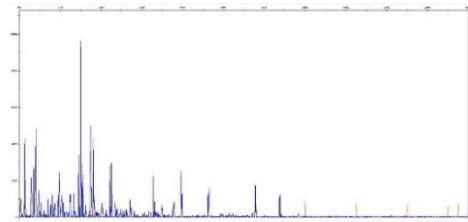


Fig. 9: Fluorogram of RAPD pattern of *M. brunnae* produced on the automated DNA sequencer (ABI genetic Analyser; 3500xL). Primer 2 was used for amplification reaction.

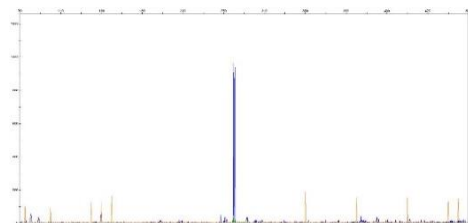


Fig. 10: Fluorogram of RAPD pattern of *L. chinensis* produced on the automated DNA sequencer (ABI genetic Analyser; 3500xL). Primer 3 was used for amplification reaction.

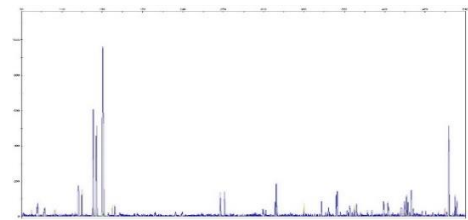


Fig. 11: Fluorogram of RAPD pattern of *M. brunnae* produced on the automated DNA sequencer (ABI genetic Analyser; 3500xL). Primer 3 was used for amplification reaction.

IV. DISCUSSION

L. chinensis and *M. brunnae* belongs to subfamily Ponerinae and Myrmicinae under the family Formicidae with unique synapomorphies [5] [19]. The present observation with molecular data support monophyly of this family with similarity index 0.6. Both samples formed strong clade with 100 bootstraps. It is suggested that the 32 monomorphic bands generated in both samples may be further used for identifying monophyly of other members of subfamilies.

During the present study *Leptogenys chinensis* and *Myrmecaria brunnae* used as representatives of two subfamily Ponerinae and Myrmicinae shown 57.61% polymorphism provides additional support for branching of poneromorphs from Formicoid [5] [26] with 0.4 genetic distance. 34 polymorphic bands generated i.e. 51.51% by *L. chinensis* and 53 polymorphic bands i.e. 62.35% generated by *M. brunnae* could prove to be potential genetic information for identifying differences and similarities among the attribute like genus and within the species using similar RAPD markers.

According to Seppa [22] among well-known ant species only 0.5% genetic information is available and so using representative species more genetic study should be carried out also added invasive ants are potential threat to the native species and suggested for study of non-neutral genes as many ants have extensive dispersal polymorphism. During the present study more polymorphism was observed with P1 primer followed by P2 primer in *L. chinensis* and *M. brunnae*,

could prove to be useful in identifying non-neutral genes in particularly those fragments ranging between 53bp to 326bp.

Anbalagan *et al.* [2] used RAPD to study genetic diversity within blackfly from different elevation of Palni hills. Hasegawa in 1995 suggested that if RAPD markers are to be used to demonstrate parent-offspring relationship, sperms from queen spermathecal should be incorporated to increase accuracy. Hasan *et al.* in 2009 [13] studied genetic variability among individuals of *A. capillaris* using ten primers to amplify DNA.

Earlier, RAPD molecular markers were also used to find out genetic relationship among the nests of carpenter ant located in some area by Matta *et al.* [17] and concluded low genetic differentiation between ant workers from different nests as well as workers within same nests.

Genetic relationship among Lycaenidae butterflies was also carried out using RAPD marker by Tiple *et al.* in 2009 [24]. Galal (2009) [9] used RAPD and PCR-RFLP markers and concluded that depending upon ease, cost, technical labour, speed and amount of DNA needed, the RAPD marker system should be preferred as they found no difference in RAPD and PCR-RFLP tests. Present study also supports the use of RAPD markers for taxonomic study and polymorphism. During the present study 3 RAPD markers used could be prove helpful for monitoring effects of environmental and chemical changes in soil due to excessive use of manures, fertilizers and other factors ultimately affecting ant's genera. With RAPD's genetic information it will be possible to monitor variation in genes of adopted ant species to changing environment. Monomorphic bands could be further investigated for presence in other related hymenopterans and will help to conclude for their common ancestry.

V. CONCLUSION

Dendrogram cluster for *L. chinensis* and *M. brunnea* shown 2 clusters two samples were linked together with 100bootstrap. *L. chinensis* and *M. brunnea* used as representatives of two subfamily Ponerinae and Myrmicinae shown 57.61% polymorphism and 0.57 genetic variation. The 32 monomorphic bands generated in both *L. chinensis* and *M. brunnea* by three primers may be further used for identifying monophyly of other members of subfamilies.

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