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Infraspecific diversity of *Beauveria bassiana* from India based on DNA microsatellite markers

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Abstract

Beauveria bassiana is an insect-associated fungal species and it is economically important as an environment-friendly mycoinsecticide and a good source of biologically active secondary metabolites. In this study, we employed DNA microsatellite markers to investigate the infra-specific diversity of 102 isolates of *B. bassiana sensu stricto* from India. All the microsatellite markers were easily amplifiable for all the isolates and provided a good genotype resolution. The microsatellite analysis indicated the presence of highly polymorphic, randomly distributed populations of *B. bassiana* with variable host range and apparently no host-specificity. We, however, observed region-wise clustering of the populations within *B. bassiana sensu stricto* from India.

Keywords – allelic diversity – biocontrol – genetic diversity – host relationships – insect-pathogenic fungi – polymorphism

Introduction

Beauveria bassiana (Bals.–Criv.) Vuill. includes mainly fungal pathogens of phylogenetically diverse insect-hosts; however, some isolates survive and thrive as endophytes, epiphytes and saprobes (Ownley et al. 2010, Roy et al. 2010). This species, as delimited in traditional taxonomy, has been found to include multiple morphologically-similar phylogenetic species (Rehner et al. 2006). It is also known that *B. bassiana sensu lato* has a clonal genetic structure (Meyling et al. 2009) and the isolates belonging this taxon are believed to be generalist pathogens, apparently lacking host-specificity, probably the geographical distribution influencing their pathogenic behaviour (Bidochka et al. 2002, Wang et al. 2003, Rehner & Buckley 2005, Meyling et al. 2009). The studies related to the role of ecological factors such as habitat and hosts relationships on population structure of *B. bassiana* indicate at effect of habitat selection but varied relationships of associated hosts, highlighting the importance of ecological factors and geographical origin on *B. bassiana* isolates (Bidochka et al. 2002, Wang et al. 2003).

It is known that microsatellite regions can distinguish closely related microbial entities and reveal population genetic structure (Avise 2004, Enkerli & Widmer 2010) of a fungal species. Isolation and characterization of microsatellite markers have already been reported for a variety of

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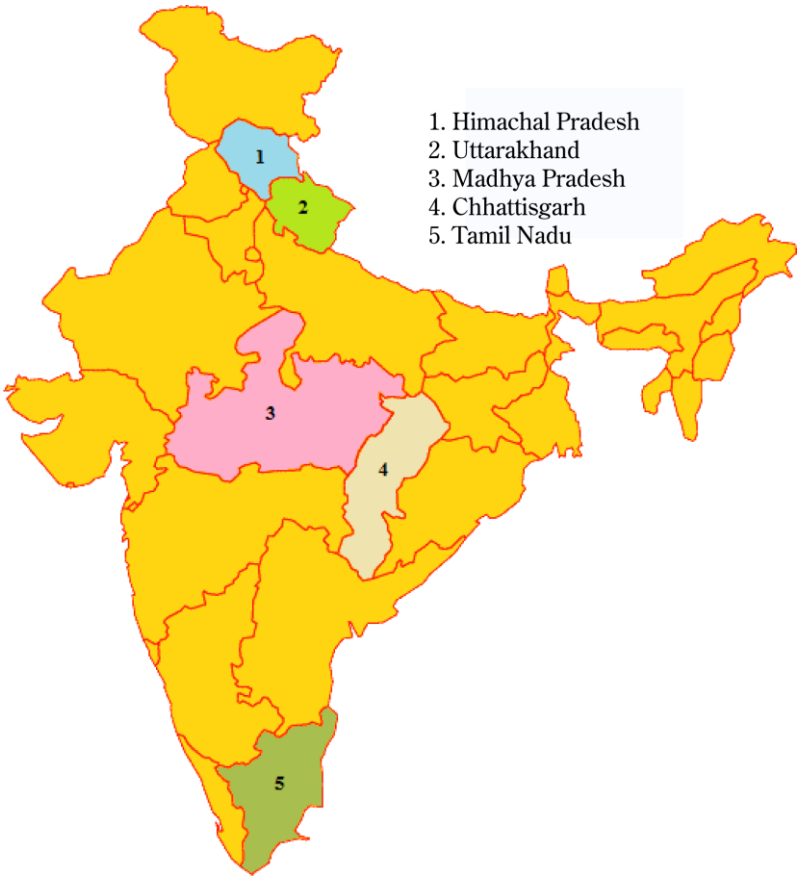

Fig. 1 – Site locations of *Beauveria bassiana* isolates included in the study (marked 1-5)

entomopathogenic fungi, including *B. bassiana sensu lato* (Rehner & Buckley 2003, Meyling et al. 2009), *B. brongniartii* (Enkerli et al. 2001), *Metarhizhium anisopliae* (Enkerli et al. 2005, Oulevey et al. 2009), *Paecilomyces fumosoroseus* (Dalleau-Clouet et al. 2005) *etc.* Regardless the extensive studies on mycoinsecticide potential, host specificity, virulence and pathology of *B. bassiana* from Indian region (Haraprasad et al. 2001, Devi et al. 2008, Challa et al. 2013), fundamental understanding related to its ecology, genetic diversity, population biology and species delimitation is limited from India (Devi et al. 2006).

We, therefore, aimed clarify if the above observation is applicable to the *B. bassiana sensu stricto* isolates from India. To achieve this, we employed microsatellite markers as they have been used in previous studies to reveal the infraspecific diversity in *B. bassiana sensu lato* (Rehner & Buckley 2003, Meyling et al. 2009).

Materials & methods

Fungal isolates and DNA isolation

A collection of 102 *B. bassiana* isolates (Table 1) were included in this study. The site locations of these isolates have been mapped in Fig. 1. Isolates were grown on potato dextrose agar (PDA, HiMedia) and incubated at 25 °C for 5–8 days. These were preserved as glycerol stocks prepared from fresh mycelia or harvested conidia in 10% glycerol and stored at -70 °C. The genomic DNA was extracted from fresh mycelia using ZR Fungal/ Bacterial DNA isolation kit (Catalogue number D6005, Zymo Research, USA). The quality and concentration of DNA extracted was assessed by 0.8% agarose gel electrophoresis and Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, USA).

Table 1 List of *B. bassiana* isolates under study and their general details

S. No.	Strain	Culture location	Host/Substrate	Host Order
1	MTCC 984	ATCC, USA	NA	NA
2	MTCC 3653	Jogendra Nagar, Mandi, HP	Oak tasar silkworm pupae	*Lepidoptera*
3	MTCC 4105	Maneri, Mandla, MP	*Hyblaea puera*	*Lepidoptera*
4	MTCC 4106	Gosalpur, Jabalpur, MP	Grasshopper	*Orthoptera*
5	MTCC 4107	Gosalpur, Jabalpur, MP	Grasshopper	*Orthoptera*
6	MTCC 4108	Gosalpur, Jabalpur, MP	Crop beetle	*Coleoptera*
7	MTCC 4109	Kanger, Bastar, Chhattisgarh	Black ant	*Hymenoptera*
8	MTCC 4110	Mandla, MP	*Eutectona machaeralis*	*Lepidoptera*
9	MTCC 4111	Bijadandi, Jabalpur, MP	small black ant	*Hymenoptera*
10	MTCC 4120	Pariyat, Jabalpur, MP	*Hyblaea puera*	*Lepidoptera*
11	MTCC 4121	Udaipur, Mandla, MP	*Pieris rapae*	*Lepidoptera*
12	MTCC 4122	Tikariya, Mandla, MP	Beetle	*Coleoptera*
13	MTCC 4492	Katni, MP	*Pieris rapae*	*Lepidoptera*
14	MTCC 4495	Katni, MP	*Hyblaea puera*	*Lepidoptera*
15	MTCC 4496	Katni, MP	Grasshopper	*Orthoptera*
16	MTCC 4497	Katni, MP	Crop beetle	*Coleoptera*
17	MTCC 4498	Balaghat, MP	*Eutectona machaeralis*	*Lepidoptera*
18	MTCC 4499	Balaghat, MP	Beetle	*Coleoptera*
19	MTCC 4500	Balaghat, MP	Black ant	*Hymenoptera*
20	MTCC 4501	Balaghat, MP	Grub	*Coleoptera*
21	MTCC 4503	Betul, MP	*Plusia orichalcea*	*Lepidoptera*
22	MTCC 4504	Betul, MP	NA	NA
23	MTCC 4505	Betul, MP	NA	NA
24	MTCC 4506	Betul, MP	Beetle	*Coleoptera*
25	MTCC 4507	Betul, MP	Beetle	*Coleoptera*
26	MTCC 4508	Betul, MP	Beetle	*Coleoptera*
27	MTCC 4509	Betul, MP	*Eutectona machaeralis*	*Lepidoptera*
28	MTCC 4510	Betul, MP	*Eutectona machaeralis*	*Lepidoptera*
29	MTCC 4511	Betul, MP	*Eutectona machaeralis*	*Lepidoptera*
30	MTCC 4512	Betul, MP	*Eutectona machaeralis*	*Lepidoptera*
31	MTCC 4513	Betul, MP	*Hyblaea puera*	*Lepidoptera*
32	MTCC 4514	Hoshangabad, MP	*Hyblaea puera*	*Lepidoptera*
33	MTCC 4515	Hoshangabad, MP	*Hyblaea puera*	*Lepidoptera*
34	MTCC 4516	Betul, MP	Beetle	*Coleoptera*
35	MTCC 4517	Betul, MP	*Hyblaea puera*	*Lepidoptera*
36	MTCC 4528	Chhindwara, MP	*Plusia orichalcea*	*Lepidoptera*
37	MTCC 4529	Betul, MP	*Plusia orichalcea*	*Lepidoptera*
38	MTCC 4530	Betul, MP	*Plusia orichalcea*	*Lepidoptera*
39	MTCC 4531	Betul, MP	*Plusia orichalcea*	*Lepidoptera*
40	MTCC 4532	Hoshangabad, MP	*Hyblaea puera*	*Lepidoptera*
41	MTCC 4533	Hoshangabad, MP	*Hyblaea puera*	*Lepidoptera*
42	MTCC 4534	Hoshangabad, MP	*Hyblaea puera*	*Lepidoptera*
43	MTCC 4535	Hoshangabad, MP	*Eutectona machaeralis*	*Lepidoptera*
44	MTCC 4536	Hoshangabad, MP	Black ant	*Hymenoptera*
45	MTCC 4537	Hoshangabad, MP	*Eutectona machaeralis*	*Lepidoptera*
46	MTCC 4538	Hoshangabad, MP	*Eutectona machaeralis*	*Lepidoptera*
47	MTCC 4539	Hoshangabad, MP	*Eutectona machaeralis*	*Lepidoptera*
48	MTCC 4540	Hoshangabad, MP	*Eutectona machaeralis*	*Lepidoptera*
49	MTCC 4542	Chhindwara, MP	*Eutectona machaeralis*	*Lepidoptera*

S. No.	Strain	Culture location	Host/Substrate	Host Order
50	MTCC 4543	Chhindwara, MP	*Eutectona machaeralis*	*Lepidoptera*
51	MTCC 4544	Chhindwara, MP	*Eutectona machaeralis*	*Lepidoptera*
52	MTCC 4546	Chhindwara, MP	*Plusia orichalcea*	*Lepidoptera*
53	MTCC 4547	Chhindwara, MP	Fruit fly	*Diptera*
54	MTCC 4548	Chhindwara, MP	*Plusia orichalcea*	*Lepidoptera*
55	MTCC 4549	Mandla, Chhattisgarh	Black ant	*Hymenoptera*
56	MTCC 4550	Seoni, MP	*Eutectona machaeralis*	*Lepidoptera*
57	MTCC 4551	Seoni, MP	*Eutectona machaeralis*	*Lepidoptera*
58	MTCC 4552	Mandla, Chhattisgarh	Beetle	*Coleoptera*
59	MTCC 4553	Seoni, MP	*Eutectona machaeralis*	*Lepidoptera*
60	MTCC 4554	Seoni, MP	*Eutectona machaeralis*	*Lepidoptera*
61	MTCC 4557	Mandla, Chhattisgarh	*Eutectona machaeralis*	*Lepidoptera*
62	MTCC 4559	Mandla, Chhattisgarh	*Eutectona machaeralis*	*Lepidoptera*
63	MTCC 4560	Mandla, Chhattisgarh	*Eutectona machaeralis*	*Lepidoptera*
64	MTCC 4562	Mandla, Chhattisgarh	Fly	*Diptera*
65	MTCC 4563	Mandla, Chhattisgarh	unidentified insect	NA
66	MTCC 4564	Mandla, Chhattisgarh	Beetle	*Coleoptera*
67	MTCC 4565	Jabalpur, MP	*Plusia orichalcea*	*Lepidoptera*
68	MTCC 4566	Jabalpur, MP	*Plusia orichalcea*	*Lepidoptera*
69	MTCC 4567	Jabalpur, MP	*Plusia orichalcea*	*Lepidoptera*
70	MTCC 4568	Jabalpur, MP	*Plusia orichalcea*	*Lepidoptera*
71	MTCC 4569	Jabalpur, MP	*Plusia orichalcea*	*Lepidoptera*
72	MTCC 4571	Mandla, Chhattisgarh	*Eutectona machaeralis*	*Lepidoptera*
73	MTCC 4572	Mandla, Chhattisgarh	*Eutectona machaeralis*	*Lepidoptera*
74	MTCC 4575	Jabalpur, MP	*Eutectona machaeralis*	*Lepidoptera*
75	MTCC 4576	Jabalpur, MP	*Eutectona machaeralis*	*Lepidoptera*
76	MTCC 4577	Jabalpur, MP	*Eutectona machaeralis*	*Lepidoptera*
77	MTCC 4578	Jabalpur, MP	*Eutectona machaeralis*	*Lepidoptera*
78	MTCC 4579	Jabalpur, MP	*Eutectona machaeralis*	*Lepidoptera*
79	MTCC 4580	Jabalpur, MP	Insect	NA
80	MTCC 4581	Jabalpur, MP	*Plusia orichalcea*	*Lepidoptera*
81	MTCC 4582	Jabalpur, MP	*Plusia orichalcea*	*Lepidoptera*
82	MTCC 4599	Shahdol, MP	Beetle	*Coleoptera*
83	MTCC 4600	Shahdol, MP	*Plusia orichalcea*	*Lepidoptera*
84	MTCC 4605	Shahdol, MP	Beetle	*Coleoptera*
85	MTCC 6095	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
86	MTCC 6097	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
87	MTCC 6098	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
88	MTCC 6099	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
89	MTCC 6100	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
90	MTCC 6286	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
91	MTCC 6287	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
92	MTCC 6288	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
93	MTCC 6289	Mandla,MP	*Eutectona machaeralis*	*Lepidoptera*
94	MTCC 6291	Dantewada, Chhattisgarh	*Hyblaea puera*	*Lepidoptera*
95	MTCC 6297	Dantewada, Chhattisgarh	*Hyblaea puera*	*Lepidoptera*
96	MTCC 6298	Bastar, Chhattisgarh	*Eutectona machaeralis*	*Lepidoptera*
97	MTCC 6341	Mandla, MP	*Eutectona machaeralis*	*Lepidoptera*
98	MTCC 6685	Dindigul, Tamil Nadu	Soil	NA
99	MTCC 6779	Dindigul, Tamil Nadu	Field infected larva	NA
100	MTCC 7689	Dindigul, Tamil Nadu	Field infected larva	NA

S. No.	Strain	Culture location	Host/Substrate	Host Order
101	MTCC 7690	Dindigul, Tamil Nadu	Soil	NA
102	MTCC 9348	US Nagar, Uttarakhand	*Ideoscopus clypealis*	*Hemiptera*

[#]MTCC denotes to Microbial Type Culture collection; NA: Information not available; MP: Madhya Pradesh; HP: Himachal Pradesh

Microsatellite typing

A set of eight previously described microsatellite markers (Rehner & Buckeley 2003) were used (Table 2) for the microsatellite typing. Touchdown polymerase chain reaction (PCR) was performed to amplify the microsatellite-containing-loci from genomic DNA. The PCR reactions were carried out in an Eppendorf Mastercycler with following cycling parameters: initial denaturation at 95 ˚C for 2 minutes followed by 10 cycles of denaturation at 94 ˚C for 30 seconds, annealing at 66 ˚C for 30 seconds and further reduction in annealing temperature by 1 ˚C at each succeeding cycle, and extension at 72 ˚C for 1 minute. Then, 36 cycles of 30 seconds at 56 ˚C and 1 minute at 72 ˚C and a final extension at 72 ˚C for 30 minutes was done at the end of the PCR amplification and the reaction was held at 4 ˚C until further processed. The resulting microsatellite amplicons were resolved on 4% Tris-Borate-EDTA agarose gels stained with ethidium bromide (0.5 µg/ ml) and visualized under UV light.

Microsatellite data analysis

Identity version 1.0 (Wagner & Sefc 1999) was used for microsatellite data analysis and generating allele frequency and null allele frequency. The presence and absence of different alleles over each locus was scored as binary data comprising of 1 and 0, respectively. A binary matrix was prepared containing allele information for cluster analysis. The microsatellite binary matrix was imported into TREECON (Van de peer & De Wachter 1994) for further analysis. An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram was constructed following Nei and Li index (Nei & Li 1979) with 1000 bootstrap replicates (Felsenstein 1985). Simpson's index of diversity (D) was used to calculate the discriminatory power of microsatellite markers to ensure whether any two randomly selected isolates belong to same genotype for a given (combination of) marker(s). A 'D' value of '1' designates all isolates to be different whereas a 'D' value of 0 designates all isolates to be identical.

Results

All microsatellite markers used in this study were found to be polymorphic, displaying up to 55 (Ba02 locus) allelic frequencies. The estimated frequency of null alleles was in the range of ~ 0.01–0.04 for all the markers, except Ba01 locus which depicted high frequency of null allele, i.e., 0.46 (Table 2). Also, the Ba01 locus was found to be least polymorphic, with the amplification of 15 alleles (Table 2). This marker, therefore, was found to be least informative in the present population structure analysis. The discriminatory powers for individual markers ranged from 0.9901 to 0.9905 (Table 3). The 'D' value for combined panel of all markers was found to be 0.8563 (Table 3). This represents unrelated and polymorphic alleles among the *B. bassiana* isolates from this study.

Table 2 Microsatellite data of 102 *Beauveria bassiana* isolates using 8 Loci (Identity1.0)

Locus	Repeat motif	No. of alleles, size Range (bp)	Estimated frequency of null alleles (%)
Ba01	$(CA)_{14}$	15 (78-115)	46
Ba02	$(CA)_{20}$	55 (93-178)	1
Ba03	$(CA)_{26}$	50 (112-162)	1
Ba05	$(GAT)_{15}$	43 (88-178)	3
Ba06	$(GTT)_{10}$	43 (92-117)	2
Ba08	$(AGG)_{10}$	47 (168-250)	2
Ba12	$(CTT)_{7}$	43 (135-224)	4
Ba13	$(AAG)_{9}$	44 (135-213)	3

Table 3 Discriminatory power (D) of individual markers and the complete set of 8 markers

Marker	D	Complete Set	D
Ba01	0.9903	Complete marker set	0.8563
Ba02	0.9901		
Ba03	0.9903		
Ba05	0.9904		
Ba06	0.9905		
Ba08	0.9904		
Ba12	0.9904		
Ba13	0.9904		

The unrooted UPGMA tree based on microsatellite data for all the populations is presented in Fig. 2. The tree separated the populations into five clusters (Cluster I to Cluster V). However, the UPGMA dendrogram based on the combined microsatellite data revealed no apparent correlation among the genotype of the isolates, host-substrate and geographical location (Fig. 2). It represented high allelic diversity among *B. bassiana* isolates from this study.

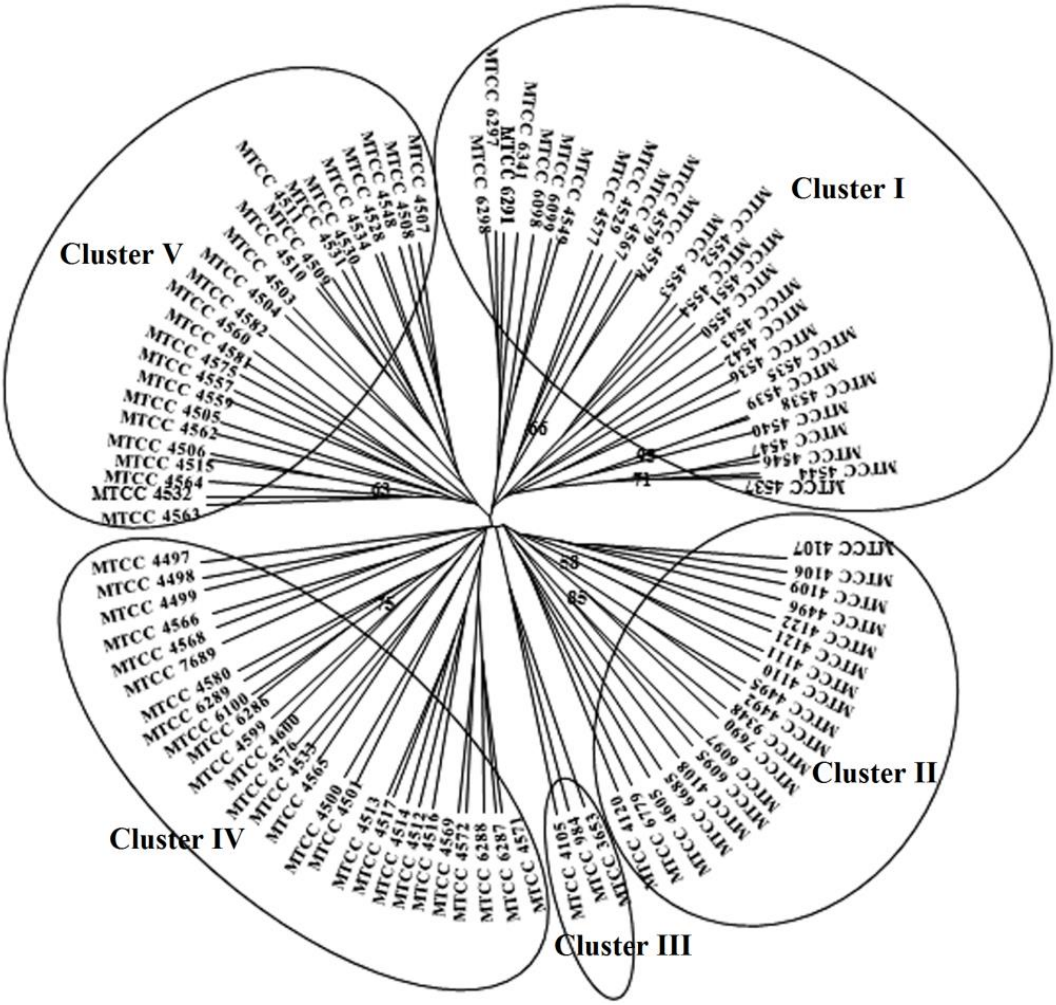

Fig. 2 – UPGMA Cluster analysis. Split tree prepared using TREECON software representing the genetic correlations of all combined 102 *B. bassiana* isolates from India. Bootstrap values above 50% are highlighted

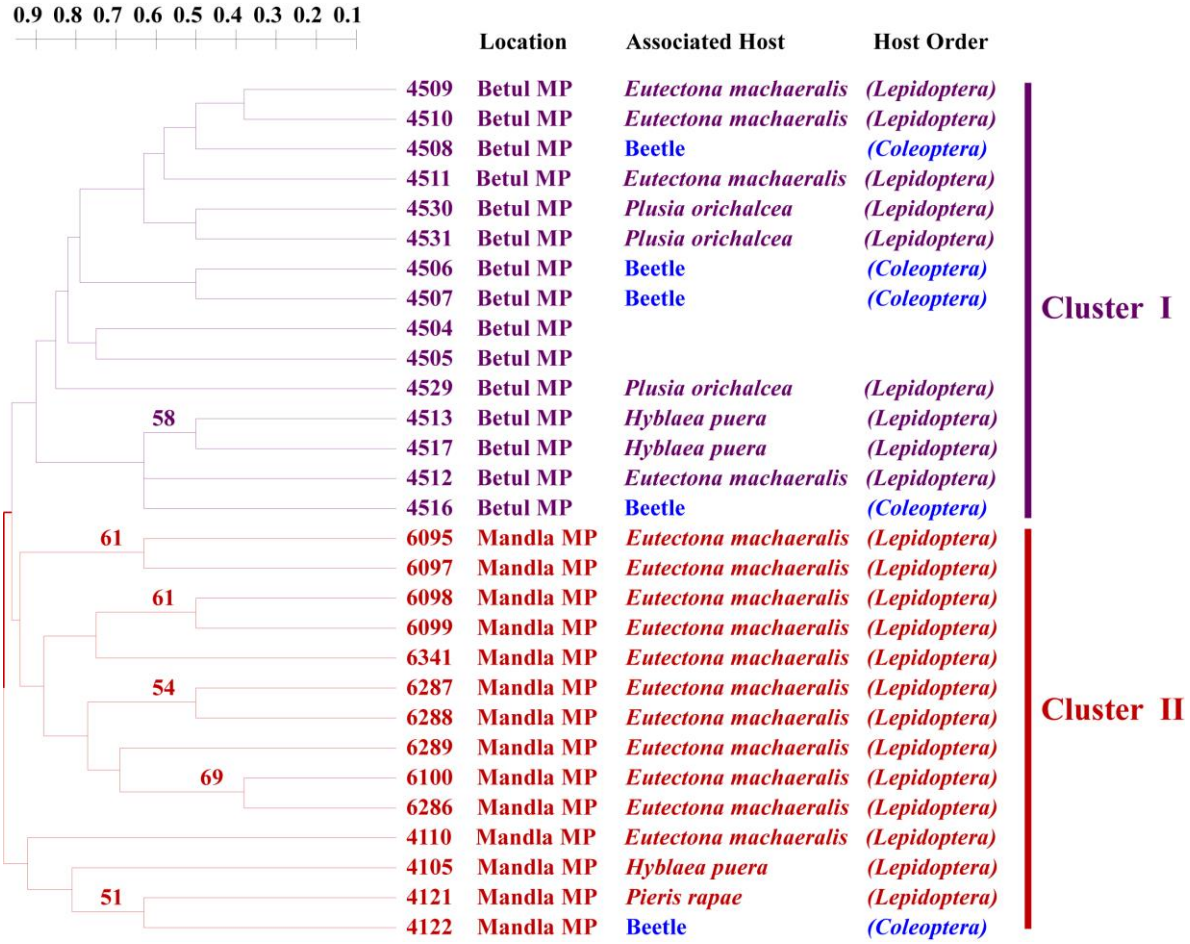

Fig. 3 – UPGMA Cluster analysis representing region-wise genetic correlations of Betul and Mandla (from Madhya Pradesh population site) origins of *B. bassiana* isolates from India. Bootstrap values above 50% are highlighted.

We then looked into reduced datasets with locality related origin (Betul vs. Mandla) or host related association (*Eutectona machaeralis* vs. *Plusia orichalcea*) (Figs. 3, 4). UPGMA cluster analysis was performed for these reduced datasets as already described for the combined microsatellite data. Two of the branches obtained in unrooted UPGMA dendrogram of Betul vs. Mandla subpopulations were identified as a distinct genetic cluster and designated as cluster I and II (Fig. 3). These two branches were significantly clustered on the basis of their location Betul and Mandla in the present dataset. However, there were no regular arrangements of favored host (*Lepidoptera* or *Coleoptera*) in each of the two clusters I and II. Similarly, in the host based reduced dataset, no grouping was found. A mixture of two hosts was observed in each cluster (Fig. 4). So, any evidence for host-wise selection was lacking from the *B. bassiana* isolates in this study from India.

Discussion

The microsatellite markers have revealed very high genetic diversity and polymorphic population structure in *B. bassiana sensu stricto* from India. This result is comparable to the earlier studies employing microsatellite loci for depiction of genetic diversity of *B. bassiana* (Fernandes et al. 2006, Takatsuka 2007, Meyling et al. 2009, 2012, Castrillo et al. 2010, Yao et al. 2012). The microsatellite markers provided a good genotype resolution, indicating that all selected isolates belong to *B. bassiana sensu stricto* (Meyling et al. 2012). The relationships of *B. bassiana* genotypes with its associated host were proposed to be accidental co-occurrence of such host associated population structure for *B. bassiana*, which were supported to be primarily habitat associated rather than host associated (Bidochka et al. 2002).

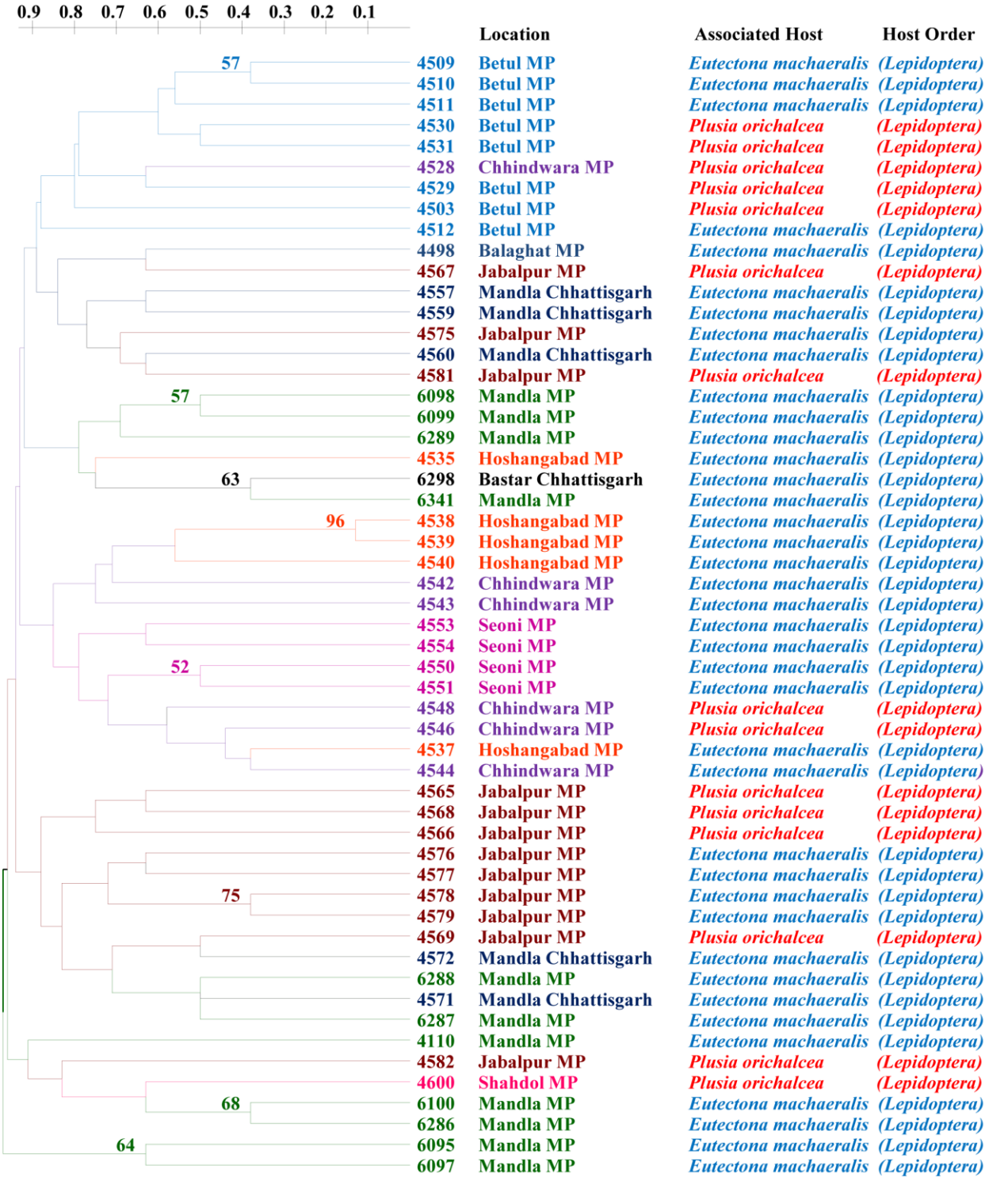

Fig. 4 – UPGMA Cluster analysis representing host-wise genetic correlations of *Eutectona machaeralis* and *Plusia orichalcea* hosts of *B. bassiana* isolates from India. Bootstrap values above 50% are highlighted

We further checked for more reduced datasets based on their geographical locations. Figs. 5 and 6 represent region-wise genetic correlations of Madhya Pradesh and Chhattisgarh reduced datasets, and of Tamil Nadu (Southern-India) with other parts of India (MP, HP and Chhattisgarh). We could clearly observe the region-wise clustering for all these reduced datasets, which signifies the greater role of geographical locations (ecological factors) rather than host-pathogen interactions for *B. bassiana sensu stricto* isolates from India.

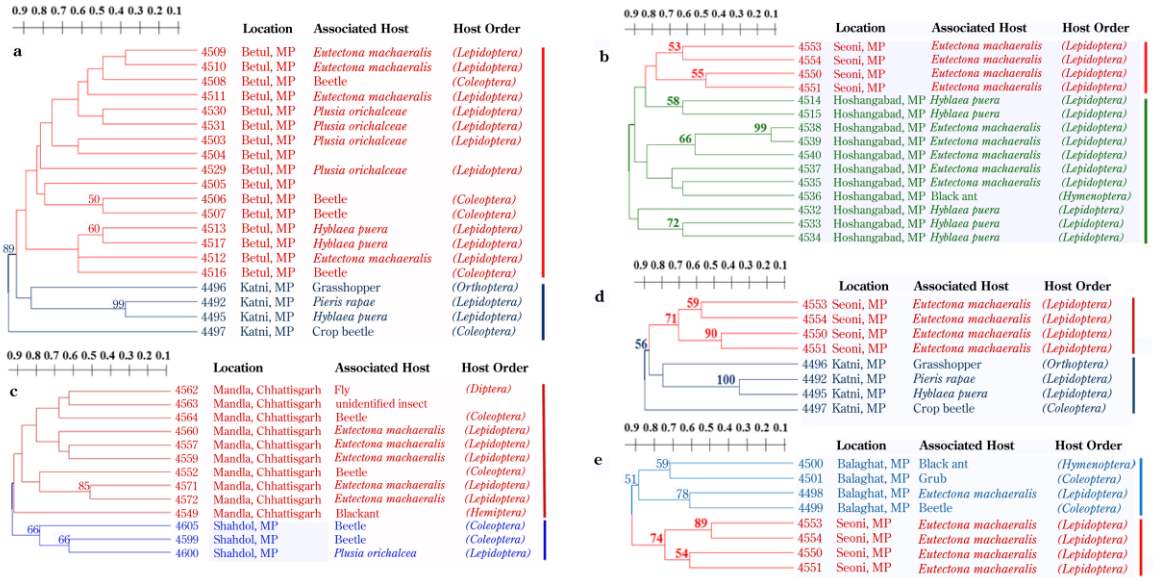

Fig. 5 – Region-wise genetic correlations of MP and Chhattisgarh reduced datasets. Bootstrap values above 50% are highlighted

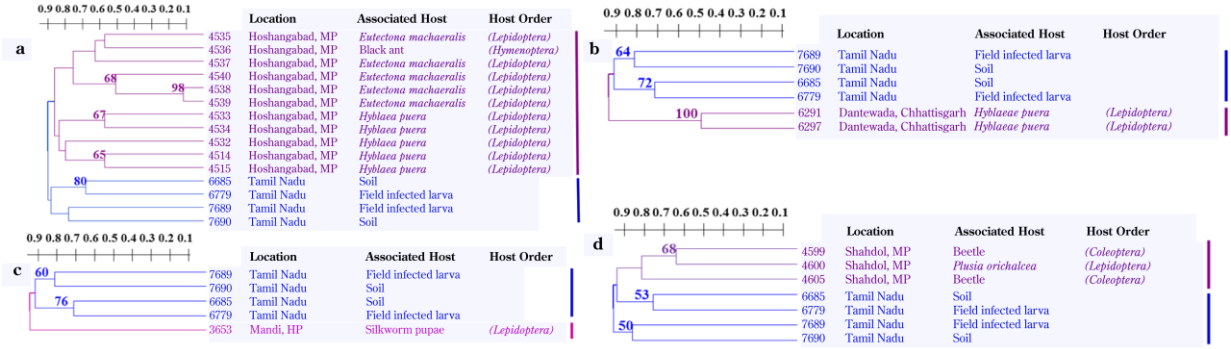

Fig. 6 – Region-wise genetic correlations of Tamil Nadu (Southern-India) with other parts of India (MP, HP and Chhattisgarh). Bootstrap values above 50% are highlighted

The genetic groupings obtained with reduced dataset based on its original locality (MP, HP, Chhattisgarh and Tamil Nadu) and host related associations (*Eutectona machaeralis* vs. *Plusia orichalcea*) supported the previous findings that *B. bassiana* isolates exhibit higher association with their habitat and geographical location rather than host species (Bidochka et al. 2002, Wang et al. 2003, Rehner & Buckley 2005, Devi et al. 2008, Ghikas et al. 2010). Bidochka et al. (2002) had analyzed the population structure of *B. bassiana* from the Canadian Arctic (forested and agricultural habitats) region and associated them with distinct genetic groups (three different habitats), but no any clear associations with host could be ascertained by them. Similarly, Wang et al. (2003) presented the genetic relatedness of *B. bassiana* isolates with geographical locality from China, Japan and USA regions. Nevertheless, in the present study we have not discussed the role of habitat type and the prevalent climatic factors like temperature, light, rainfall, salinity, *etc.*, that should be further explored in future studies from the Indian populations.

The observed genetic diversity, except for the locus Ba01, predicts the neutral allele theory of molecular evolution with the projected evolution in the adaptive direction (Duret 2008). It signifies the random processes in interpreting genotypic diversity or structural stability in evolution with limited role of allelic variation or polymorphic nature that highlights the role of habitat where the proximity in the habitat has been related to the acquisition of new hosts (Duret 2008, Kepler et al. 2012). Similar to the finding by Meyling et al. (2009), the present study indicates no specific host target for *B. bassiana* rather a versatile host range for a particular geographical location.

The geography-centric population structure of *B. bassiana sensu stricto* from India, as revealed from this study, could have useful implications in future biocontrol research on entomopathogenic fungi from India.

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