Molecular characterization of ladybird predators (Coleoptera: Coccinellidae) of aphid pests (Homoptera: Aphididae) in North East India

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Ladybird beetles are potential and promising biological control agents for the management of insect pests. These insects show variations in biological fitness in diverse habitats and subsequently in term of genotypes. We used cytochrome oxidase I (COI) gene sequences to study within-species genetic variation in four species of ladybird predators, viz. *Coccinella transversalis* (Fab.), *Cheilomenes sexmaculata* (Fab.), *Micraspis discolor* (Fab.) and *Anisolemnia dilatata* (Fab.) collected from different cultivated habitats of Tripura, North East India. Results of multiple sequence alignments of partial COI gene (553 bp) of mitochondrial origin showed 100% homology among different populations (within species) of three ladybird species. The molecular identity of *M. discolor* could not be established due to the absence of matching nucleotide sequence for this region of COI gene in the NCBI database. Three of the four populations of *Micraspis* species showed 100% homology in partial COI gene sequencing, but one representative population showed 52 nucleotide mutations, of which 1 mutation was found to result in the alteration of the codon from valine to isoleucine, and seemed to represent a different *Micraspis* species previously not known from NE India. This study shows that the three most common species of ladybird predators of aphid pests in NE India are fairly homogenous with respect to the COI gene, but species of *Micraspis* are genetically diverse and need further studies to address this issue.

Keywords: Aphid pests, genetic variation, ladybird beetles, molecular characterization.

SEVERAL species of aphids are important pests of crops and other economically important plants in Tripura and elsewhere in North East India. *Aphis gossypii* Glover, *Myzus persicae* (Sulzer), *Aphis craccivora* Koch and wax-producing horned aphids of bamboo are common among them[1,2]. Natural control agents like predators are considered important in the management of pest populations of these aphids. Ladybird predators are widely recognized as potential and promising natural control agents[3,4]. Biological fitness of natural populations of ladybird species varies in response to differences in biotic (quality of aphid preys and host plants) and abiotic (temperature and relative humidity, in particular) factors of different habitats[5,6]. Polyphagous ladybird predators that feed on different aphid prey species on different cultivated plants show differences in biological fitness and, therefore, are likely to differ in their genotypes[3,7]. The most potent genotypes of a predator species showing greater predation efficiency will be an important source for biocontrol agents. Unlike other insect species, limited molecular studies have been undertaken in the members of the subfamily Coccinellinae of the insect order Coleoptera[7,8]. Fu and Zhang[9] used partial COI gene and studied taxonomic relationship of 16 species across four subfamilies. The 5′ region of the COI gene of mitochondrial DNA (mtDNA) is considered to be informative and has also been widely used for detecting molecular genetic variation within and between species[10]. The COI gene has also been successfully used in the determination of host-associated differences in insect species[11]. In this study, we used COI gene of mtDNA for molecular characterization of multiple populations of four species of ladybird, viz. *Coccinella transversalis* (Fab.), *Cheilomenes sexmaculata* (Fab.), *Micraspis discolor* (Fab.) and *Anisolemnia dilatata* (Fab.) collected from different cultivated habitats of Tripura. The aim was to quantify the extent of genetic variation, if any, in different populations of ladybird species collected from different habitats, and to also establish evolutionary relationships among them.

Multiple specimens of the respective species of ladybird beetles were collected from different habitats (Table 1). All the samples were identified taxonomically using established taxonomic keys[12]. Representative specimens were preserved in 100% analytical-grade ethanol for molecular characterization work. DNA was extracted by Qiagen's DNeasy® Blood and Tissue Kit using the supplier's protocols. Qualitative and quantitative analysis of DNA was performed in Nano Drop at the ICAR Research Complex Facility, Umiam, Barapani, India. All the samples were subjected to PCR amplification using standard barcoding primers LepF1 and LeR1 (ref. 10) which target partial COI gene of mitochondrial genome, which indeed is considered as a standard barcoding region of this gene. Sequencing of all the samples was carried out commercially (M/S. Xcelris Pvt Ltd, Ahemdabad) by sending the post-PCR products (40 ml) under frozen conditions. The success of PCR amplification was tested on 1.5% agarose gel. Two adult specimens from each habitat of the four ladybird species were sequenced from both the ends (5′ and 3′). Sequence analysis was performed using the software Staden Package[13]. Molecular identity of the sequences was confirmed through BLASTn search in the NCBI portal (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was carried out in ClustalW[14]. All the analysed sequences were submitted to NCBI for accession

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Table 1. Records of samples of four species of ladybird predators, their habitats, aphid prey species and alternative food, dates of collection, NCBI accession numbers of nucleotide sequences and protein sequence lengths

Ladybird predator species	Habitat	Aphid prey species or alternative food	Date of collection	NCBI accession no.	Nucleotide sequence length (bp)	Protein sequence length (bp)
Anisolemnia dilatata	*Bambusa balcooa* 2	*Ceratovacuna silvestrii*, *Ceratovacuna indica*	18.11.14	KT693129	553	190
Anisolemnia dilatata	*Bambusa balcooa* 1	*Ceratovacuna silvestrii*, *Ceratovacuna indica*	20.09.14	KT693130	553	190
Chilomenes sexmaculata	*Ageratum conyzoides*	*Aphis gossypii*, *Aphis spiraecola*	09.09.14	KT693131	553	190
Chilomenes sexmaculata	*Cucurbita maxima*	*Aphis gossypii*	10.10.14	KT693132	553	190
Coccinella transversalis	*Brassica juncea*	*Lipaphis pseudobrassicae*	14.01.15	KT693133	553	190
Coccinella transversalis	*Chromolaena odorata*	*Aphis spiraecola*	15.01.15	KT693135	553	190
Micraspis sp.	*Oryza sativa*	Feed on pollens (alternative food)	10.10.14	KT693139	553	190
Micraspis sp.	*Melastoma melanensis*	*Aphis gossypii*	03.12.14	KT693140	553	190
Micraspis sp.	*Leucas aspera*	*Aphis gossypii*, *Aphis spiraecola*	03.12.14	KT693141	553	190
Micraspis sp.	*Cucurbita maxima*	*Aphis gossypii*	10.10.14	KT693142	553	190

bp, Base pairs.

Table 2. Per cent nucleotide identity matrix based on partial COI gene of mtDNA

	Micrs CM	Micrs OS	Micrs MM	Micrs LA	Csexm AC	Csexm CM	Ctrans CM	Adilat BB1	Adilat BB2
Micrs CM	100.00	100.00	100.00	90.78	82.46	82.46	84.09	82.82	83.00
Micrs OS	100.00	100.00	100.00	90.78	82.46	82.46	84.09	82.82	83.00
Micrs MM	100.00	100.00	100.00	90.78	82.46	82.46	84.09	82.82	83.00
Micrs LA	90.78	90.78	90.78	100.00	82.28	82.46	84.09	82.82	83.00
Csexm AC	82.46	82.46	82.46	82.46	100.00	99.82	82.64	84.45	84.63
Csexm CM	82.46	82.46	82.46	82.28	99.82	100.00	82.64	84.63	84.81
Ctrans CM	84.09	84.09	84.09	84.63	82.64	82.64	100.00	85.35	85.35
Adilat BB1	82.82	82.82	82.82	82.64	84.45	84.63	85.35	100.00	99.82
Adilat BB2	83.00	83.00	83.00	82.64	84.63	84.81	85.35	99.82	100.00

Micrs CM, *Micraspis* sp. from *Cucurbita maxima*; Micrs OS, *Micraspis* sp. from *Oryza sativa*; Micrs MM, *Micraspis* sp. from *Melastoma melanensis*; Micrs LA, *Micraspis* sp. from *Leucas aspera*; Csexm AC, *Cheilomenes sexmaculata* from *Ageratum conyzoides*; Csexm CM, *Cheilomenes sexmaculata* from *Cucurbita maxima*; Ctrans CM, *Coccinella transversalis* from *Cucurbita maxima*; Adilat BB1 and BB2, *Anisolemnia dilatata* from *Bambusa balcooa* at Amtali (BB1) and Ishanchandranagar (BB2) respectively.

numbers. Phylogenetic analyses were conducted in MEGA6 (ref. 15). The evolutionary history was inferred using the maximum likelihood (ML) method based on the Jukes–Cantor model[16].

DNA was extracted successfully from all the specimens. Standard barcoding primers LepF1 and LepR1 successfully amplified the standard barcoding region of COI gene of mitochondrial genome for all the specimens. Good-quality sequences were obtained for all the specimens; no insertions or deletions (INDELs) were detected among the different species of ladybird, irrespective of their habitats and geographic locations. Single nucleotide polymorphisms (SNPs) were not detected in different populations of three ladybird species, namely *C. transversalis*, *C. sexmaculata* and *A. dilatata*; these showed 100% nucleotide homology with each other, i.e. within species variation (Table 2). The representative sequences characterized at molecular levels have been submitted to NCBI vide accession numbers KT693129 to KT693142 (Table 1). In case of *M. discolor*, though its morphological identity was established taxonomically[12,17], no molecular matching data were found for the four representative populations of this species in NCBI (as on February 2016). Therefore, we restrict ourselves to designate these as *Micraspis* species. Three of the four representative populations of *Micraspis* sp. that were collected from *Cucurbita maxima*, *Oryza sativa* and *Melastoma melanensis* respectively, were identical in their nucleotide sequences, but the one collected from *Leucas aspera* host plant had 52 nucleotide mutations (SNPs) (Figure 1). Of these, 51 mutations appeared to be synonymous and only one mutation was found to result in the alteration of a codon for the amino acid from valine to isoleucine within the aliphatic group of amino acids (Figure 2). This finding clearly indicates that the population collected from host *L. aspera* was indeed different from the three other populations of *Micraspis* species collected in this study and should be recognized as a separate species.

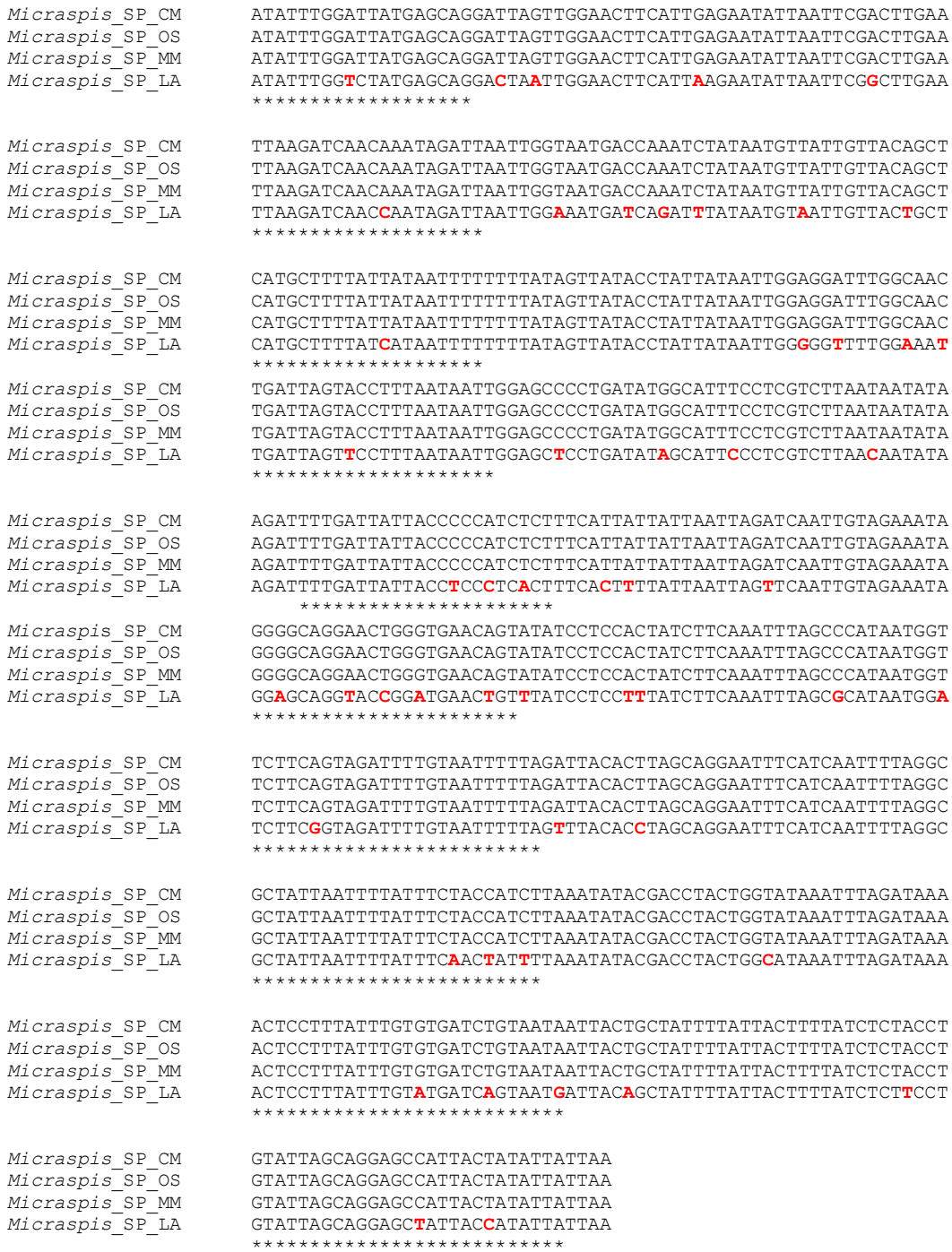

Figure 1. Multiple sequence alignments of partial COI gene (553 bp) from four representative populations of *Micraspis* species (abbreviations CM, OS, MM, LA are the same as in Table 2).

As expected, *C. transversalis*, *C. sexmaculata* and *A. dilatata* formed a separated clade in the ML tree supported by 100% bootstrap values (Figure 3). *Micraspis* sp. collected from *L. aspera* separated from the other three populations of the same genus within the clade (Figure 3). The evolutionary divergence determined for *Micraspis* sp. collected from *L. aspera* against three populations of the same genus was 0.105 (Table 3), which is considered to be the genetic distance between two separate species. These results suggest that *Micraspis* sp. collected from *L. aspera* stands out as a different species. Results show that three of the four species of ladybird predators of aphid pests are fairly homogenous with respect to COI gene that is considered to be functionally active in cells. This may also imply that these ladybird predators show homogeneity in their reproductive and

Table 3. Estimate of evolutionary divergence[16] based on partial COI gene in four species of ladybird beetles

Ladybird species	Csexm CM	Csexm AC	Adiala BB	Adiala AC	Ctrans CO	Ctrans BJ	Micrs LA	Micrs MM	Micrs OS	Micrs CM
Csexm CM										
Csexm AC	0.002									
Adiala BB	0.190	0.193								
Adiala AC	0.193	0.196	0.002							
Ctrans CO	0.226	0.226	0.182	0.182						
Ctrans BJ	0.226	0.226	0.182	0.182	0.000					
Micrs LA	0.232	0.229	0.226	0.226	0.193	0.193				
Micrs MM	0.229	0.229	0.220	0.223	0.202	0.202	0.105			
Micrs OS	0.229	0.229	0.220	0.223	0.202	0.202	0.105	0.000		
Micrs CM	0.229	0.229	0.220	0.223	0.202	0.202	0.105	0.000	0.000	

Abbreviations are the same as in Table 2.

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Micraspis_SP_CM      MFGLWAGLVGTSLSMLIRLELSSTNSLIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGN
Micraspis_SP_OS      MFGLWAGLVGTSLSMLIRLELSSTNSLIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGN
Micraspis_SP_MM      MFGLWAGLVGTSLSMLIRLELSSTNSLIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGN
Micraspis_SP_LA      MFGLWAGLIGTSLSMLIRLELSSTNSLIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGN
                     ********:*************************************************

Micraspis_SP_CM      WLVPLMIGAPDMAFPRLNNMSFWLLPPSLSLLLISSIVEMGAGTGWTVYPPLSSNLAHNG
Micraspis_SP_OS      WLVPLMIGAPDMAFPRLNNMSFWLLPPSLSLLLISSIVEMGAGTGWTVYPPLSSNLAHNG
Micraspis_SP_MM      WLVPLMIGAPDMAFPRLNNMSFWLLPPSLSLLLISSIVEMGAGTGWTVYPPLSSNLAHNG
Micraspis_SP_LA      WLVPLMIGAPDMAFPRLNNMSFWLLPPSLSLLLISSIVEMGAGTGWTVYPPLSSNLAHNG
                     ************************************************************

Micraspis_SP_CM      SSVDFVIFSLHLAGISSILGAINFISTILNMRPTGMNLDKTPLFVWSVMITAILLLLSLP
Micraspis_SP_OS      SSVDFVIFSLHLAGISSILGAINFISTILNMRPTGMNLDKTPLFVWSVMITAILLLLSLP
Micraspis_SP_MM      SSVDFVIFSLHLAGISSILGAINFISTILNMRPTGMNLDKTPLFVWSVMITAILLLLSLP
Micraspis_SP_LA      SSVDFVIFSLHLAGISSILGAINFISTILNMRPTGMNLDKTPLFVWSVMITAILLLLSLP
                     ************************************************************

Micraspis_SP_CM      VLAGAITMLL
Micraspis_SP_OS      VLAGAITMLL
Micraspis_SP_MM      VLAGAITMLL
Micraspis_SP_LA      VLAGAITMLL
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Figure 2. Multiple sequence alignment of partial protein (190 amino acids) sequence of COI gene for four representative populations of *Micraspis* species (abbreviations CM, OS, MM, LA are the same as in Table 2).

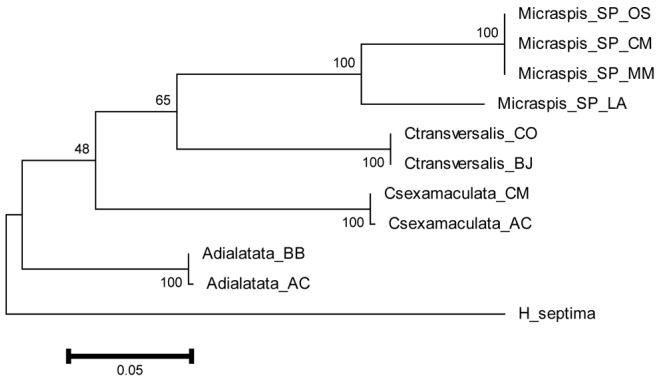

Figure 3. Molecular phylogenetic analysis by maximum likelihood method: the percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences.

feeding efficiency in the environment of Tripura. That is, diverse populations of three species of ladybird predators, *C. transversalis*, *C. sexmaculata* and *A. dilatata* found in Tripura, mutually interbreed and consist of randomized individuals with equal genetic and biological potency. However, *Micraspis* species collected from *L. aspera*, and those from three other habitats are not *M. discolor* and are mutually exclusive. These should belong to possibly two different species, which are to be identified with more sample size.

1. Blackman, R. L. and Eastop, V. F., *Aphids on the World's Crops: An Identification and Information Guide*, John Wiley, UK, 1984.
2. Ganguli, R. N. and Agarwala, B. K., Aphid association of agricultural crops in Tripura, North East India. *Indian Agric*., 1985, **29**(4), 281–287.
3. Hodek, I. and Evans, E. W., Food relationships. In *Ecology and Behaviour of the Ladybird Beetles* (*Coccinellidae*) (eds Hodek, I., van Emden, H. F. and Honek, A.), Blackwell Publishing Ltd, London, 2012, pp. 141–274.
4. Agarwala, B. K., Bardhanroy, P., Yasuda, H. and Takizawa, T., Prey consumption and oviposition of the aphidophagous predator *Menochilus sexmaculatus* (Coleoptera: Coccinellidae) in relation to prey density and adult size. *Environ. Entomol*., 2001, **30**(6), 1182–1187.
5. Dixon, A. F. G., *Insect Predator – Prey Dynamics, Ladybird Beetles and Biological Control*, Cambridge University Press, Cambridge, UK, 2000.
6. Agarwala, B. K., Das, S. and Senchowdhuri, M., Biology and food relation of *Micraspis discolor* (F.), an aphidophagous coccinellid in India. *J. Aphidol.*, 1988, **2**(1–2), 7–17.
7. Margo, A., Lecompte, E., Magne, F., Hemptinne, J.-L. and Crouau-Roy, B., Phylogeny of ladybirds (Coleoptera: Coccinellidae): are the subfamilies monophyletic? *Mol. Phylogenet. Evol*., 2010, **54**, 833–848.
8. Aruggoda, A. G. B., Shunxiabg, R. and Baoli, Q., Molecular phylogeny of ladybird beetles (Coccinellidae: Coleoptera) inferred from mitochondrial 16S rDNA sequences. *Trop. Agric. Res.*, 2010, **21**, 209–217.
9. Fu, J. and Zhang, Y. C., Sequence analysis of mtDNA – COI gene and molecular phylogeny on twenty seven species of coccinellids (Coleoptera: Coccinellidae). *Entomotaxonomia*, 2006, **28**, 179–185.
10. Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H. and Hallwachs, W., Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 14812–14817.
11. Behere, G. T., Tay, W. T., Russell, D. A., Heckel, D. G., Appleton, B. R., Kranthi, K. R. and Batterham, P., Mitochondrial DNA analysis of field populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae) and of its relationship to *H. zea*. *BMC Evol. Biol*., 2007, **7**, 117.
12. Majerus, M. E. N., *Ladybirds*, New Naturalist Series No. 81, Harper Collins, London, UK, 1994.
13. Staden, R., Beal, K. F. and Bonfield, J. K., The Staden Package. *Methods Mol. Biol.*, 2000, **132**, 115–130.
14. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G., The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 1997, **24**, 4876–4882.
15. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol*., 2013, **30**, 2725–2729.
16. Jukes, T. H. and Cantor, C. R., Evolution of protein molecules. In *Mammalian Protein Metabolism* (ed. Munro, H. N.), Academic Press, New York, USA, 1969, pp. 21–132.
17. Poorani, J., An annotated checklist of the Coccinellidae (Coleoptera) (excluding Epilachninae) of the Indian region. *Orient. Insects*, 2002, **36**, 307–383.

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Evaluating a survey landscape for tiger abundance in the confluence of the Western and Eastern Ghats

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Due to the current depleting trends in tiger population, range countries have committed to double tiger numbers by the year 2022. However, some areas, including source sites, across the range countries lack scientifically estimated tiger numbers both at the larger landscape and at the protected area level. Here we report a population of tigers, from Biligiri Rangaswamy Temple Tiger Reserve (BRTTR), using camera trap based capture-mark recapture in a spatially explicit likelihood and Bayesian analyses that yielded an estimate of ~55 tigers with a density of about 6.8 tigers/100 km^2. BRTTR nestled in a larger tiger landscape, perhaps contributes dispersing individuals to the adjoining forests, calling for integrated monitoring and management efforts for the entire landscape. This data set could help in designing long-term, landscape level plans and outcomes.

Keywords: Biligiri Rangaswamy Temple Tiger Reserve, camera trapping, capture–recapture method, tiger.

CURRENTLY, tigers (*Panthera tigris*) survive in a mere 7% of their former range with less than 3,500 individuals estimated to be living in the wild[1,2]. Within the 13 range countries, India is a key site for long-term survival of tigers in the wild[3]. However, Cambodia recently announced the local extinction of its tiger species, thus depicting depleting populations. Project Tiger, initiated in 1972, was perhaps the pioneering collaborative programme between government and non-government organizations towards the protection of tigers. Since then, in India and the world over, substantial financial investments and resources have been spent on conservation of the species during the past five and half decades.

Sizeable funding has also been invested on research and monitoring activities[4,5]. In recent years the Indian federal government has taken initiatives to estimate tiger numbers on a nation-wide scale[6–8], which in itself is a laudable effort. In a country that is large and complex in so many different ways, attempting to collate data on such a spatial scale is exemplary.

Walston *et al*.[9] argue source sites as key areas for long-term conservation of the species. However, lack of

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