International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 06-12-2021, Accepted: 22-12-2021, Published: 07-01-2022
Volume 7, Issue 1, 2022, Page No. 4-6

Molecular barcoding and phylogeny reconstruction analysis of *Belionota prasina* (Thunberg, 1789) (Coleoptera: Buprestidae)

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Abstract
This is the first COI barcoding of *Belionota prasina* (Thunberg, 1789) from south India. Previously, only one COI sequence of *B. prasina* available from India, which was reported from North India. In this study, we sequenced and reconstructed the phylogeny of *B. prasina* and focused on the sequence divergence. Currently, seven sequences of COI are available in NCBI GenBank. In a maximum-likelihood phylogeny analysis, our species was found to be closely related to Indian *B. prasina* species. Nevertheless, interesting findings were observed in K2P divergence analysis, with our sequence showing 0.731% of divergence from the previously deposited sequence. The genetic variability of CO1 among *B. prasina* collected from various geographical areas is described in this study.

Keywords: *Belionota prasina*, COI barcoding, phylogeny analysis, genetic divergence

Introduction
In the modern biological taxonomic study, DNA barcoding can mitigate the challenges of accurate identification has become a global research effort since being proposed by Hebert *et al*. (2003) as a standardized approach for identifying unknown specimens species-level (Pentinsaari *et al*. 2019) [12]. In recent years, mitochondrial DNA-based species identification methods have become a viable alternative to traditional morphology-based methods (Hebert *et al*., 2003; Ashfaq & Hebert, 2016) [2]. Even though a subsequent critical evaluation of the results using classical morphology by a taxonomic expert remains desirable, sequencing short mtDNA fragments with conservative primers has been repeatedly demonstrated to be a rapid, reliable, and cost-effective alternative. In addition, DNA-based methods enable the identification of immature stages, which may be the only available representatives of a species when economically significant damage is discovered for the first time. They are also quick and don't require any expertise (Ahrens *et al*., 2007) [1]. The barcode region of mitochondrial cytochrome c oxidase subunit I, a gene commonly used in phylogenetic and taxonomic studies of organisms (Derocles, 2012) [3], was used in this study. DNA barcodes have much potential as a Coleoptera identification tool and taxonomic tool for beetle taxonomists (Pentinsaari *et al*., 2014) [13]. Except for the *Agrilus* Curtis, 1825 genus, the other buprestid genus is the least studied group (Kelnarova *et al*., 2014) [7]. Buprestid taxonomy study in India is minimal. Although few sequences are available in NCBI GenBank, there are no proper publications related to molecular barcoding and phylogenetic analysis of buprestid species from India. This study focused on the molecular barcoding and phylogenetic analysis of one of the cashew buprestid pest species: *Belionota prasina* (Thunberg, 1789). *B. prasina* is a polyphagous insect pest widely distributed in India, China, Taiwan, Thailand, Malaysia, Indonesia, Borneo, and Northern Australia (see Ramasamy *et al*. 2018) [14]. This study examines the genetic divergence of *B. prasina* collected from different geographical regions.

Materials and Method
Belionota prasina (Thunberg, 1789) was collected using light traps from the Botanical Garden, University of Calicut, Kerala. Following collection, samples were placed in a vial containing 75% alcohol.

Isolation and sequencing of DNA
Genomic DNA was extracted from the thoracic leg using Nucleospin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. The extracted DNA was subjected to PCR amplification. PCR was performed in a reaction mixture containing 6.25µL master mix, 1.25µL forward and reverse primer, 1µL extracted DNA sample, and 3.25µL water. The total volume of the reaction mixture is 13 µL. For performing PCR, we used CO1 universal primer, LCO1490 (5'GGTCAACAAATCATAAAGATAT TGG 3') and HCO2198 (5' TAAACTTCAGG GTGACC AAAAAATCA 3') (Folmer *et al*. 1994). The thermal profiles of CO1 amplification were 5 min at 950 C, 40 cycles of 10 sec at 94oC, 1 min at 52oC, and 45 sec at 72oC, followed by a final extension of 10 min at 72oC. The purified PCR products were sequenced at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala, India, using the dideoxy chain termination method (Sanger, 1975). The forward and reverse strands were aligned using Clsutal W in MEGA X to ensure the sequences were clear without any mismatches.

Phylogeny analysis
The Sequences were checked in the BLAST tool to find similar sequences in the NCBI database. Similar sequences were retrieved from the NCBI database and used for phylogeny construction. All similar CO1 sequences of *B. prasina* with our sequence were aligned in MEGA X, MUSCLE alignment method (Kumar *et al*. 2018).
The evolutionary history was inferred by using the

Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993) [15]. The bootstrap consensus tree inferred from 100 replicates (Felsenstein 1985) [4] is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985) [4]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein 1985) [4]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. This analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 389 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*. 2018) [11].

Test of the homogeneity of substitution patterns between sequences

The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences (Disparity Index test). A Monte Carlo test (500 replicates) was used to estimate the *P*-values (Kumar and Gadagkar 2001) [10], shown below the diagonal. *P*-values smaller than 0.05 are considered significant (marked with yellow highlights). The estimates of the disparity index per site are shown for each sequence pair above the diagonal. This analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 389 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*. 2018) [11].

Estimates of evolutionary divergence between sequences

The number of base substitutions per site from between sequences is shown. Standard error estimates (s) are shown above the diagonal and were obtained by a bootstrap procedure (100 replicates). Analyses were conducted using the Kimura 2-parameter model (Kimura 1980) [9]. This analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each pair (pairwise deletion option). There were a total of 1458 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*. 2018) [11].

Results

A total of seven COI sequences of *B. prasina is* available in the NCBI database (length above 500 bp). Currently, only one deposition of *B. prasina* is available from India and which showed around 85.11% of identity with our sequence (NCBI Accession no: OM033623) and covered 97% of the query length. On the other hand, six of the seven COI sequences were deposited from the USA, and they showed 85-86% of identity and covered 97% of the query area (Table 1).

In the Maximum-likelihood phylogeny analysis, our sequence was found to be very similar to *B. prasina* collected from India (NCBI accession no: KX009514.1) (Fig. 1).

In the homogeneity of substitution patterns between sequences analysis, four sequences showed a significantly ($p<0.05$) disparity index (MN380472.1, MN380464.1, MN380463.1, our sequences) compared to other sequences depositions (Table 2). The K2P divergence between sequences was calculated. Compared to previously Deposited *B. prasina* species, our sequences showed a significant increase in K2P divergence (Table 3). Our species had the highest divergence (0.731) from a previously deposited Indian *B. prasina* (NCBI accession no: KX009514.1), which was collected from north India.

Table 1: *B. prasina* COI sequence retrieved from NCBI GenBank.

Organism	NCBI Accession No.	Query cover (in percentage)	Per. Ident (in percentage)
Belionota prasina	KY357832.1	97	86.35 (USA)
Belionota prasina	MN380472.1	97	85.11 (USA)
Belionota prasina	MN380467.1	97	85.11 (USA)
Belionota prasina	MN380465.1	97	85.11 (USA)
Belionota prasina	MN380464.1	97	85.11 (USA)
Belionota prasina	MN380463.1	97	85.11 (USA)
Belionota prasina	KX009514.1	97	85.11 (India)

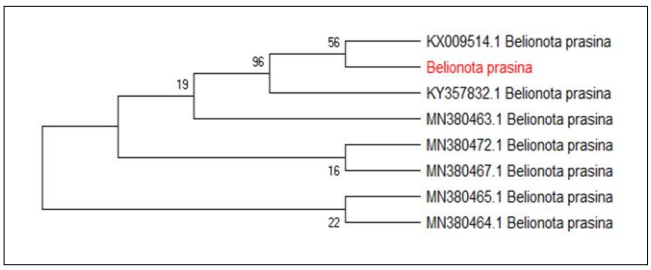

Fig 1: Maximum-likelihood phylogeny analysis of *B. prasina.* Our specimen collected from south India. Highlighted as red color (NCBI accession no: OM033623)

Table 2: Test of the Homogeneity of Substitution Patterns between Sequences. *P*-values smaller than 0.05 are considered significant (marked with yellow highlights), below diagonal. The estimates of the disparity index per site are shown for each sequence pair above the diagonal.

***B. prasina* (KY357832.1)**		**0.015**	**0.015**	**0.015**	**0.015**	**0.015**	**0.000**	**1.684**
B. prasina (MN380472.1)	0.040		0.000	0.000	0.000	0.000	0.000	1.481
B. prasina (MN380467.1)	0.058	1.000			0.000	0.000	0.000	1.481
B. prasina (MN380465.1)	0.050	1.000	1.000			0.000	0.000	1.481
B. prasina (MN380464.1)	0.048	1.000	1.000	1.000		0.000	0.000	1.481
B. prasina (MN380463.1)	0.036	1.000	1.000	1.000	1.000		0.000	1.481
B. prasina (KX009514.1)	1.000	1.000	1.000	1.000	1.000	1.00		1.383
B. prasina	0.010	0.004	0.006	0.010	0.002	0.012	0.006	

Table 3: Estimates of Evolutionary Divergence between Sequences. K2P divergence score represented in below diagonal and Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (100 replicates).

***B. prasina* (KY357832.1)**		**0.005**	**0.005**	**0.005**	**0.005**	**0.005**	**0.07**	**0.065**
B. prasina (MN380472.1)	0.014		0.000	0.000	0.000	0.000	0.07	0.068
B. prasina (MN380467.1)	0.016	0.000		0.000	0.000	0.000	0.07	0.067
B. prasina (MN380465.1)	0.014	0.000	0.000		0.000	0.000	0.07	0.068
B. prasina (MN380464.1)	0.014	0.000	0.000	0.000		0.000	0.07	0.068
B. prasina (MN380463.1)	0.014	0.000	0.000	0.000	0.000		0.07	0.067
B. prasina (KX009514.1)	0.034	0.028	0.028	0.028	0.028	0.028		0.073
B. prasina	0.731	0.720	0.720	0.720	0.720	0.717	0.782	

Discussion

The barcode region of mitochondrial cytochrome c oxidase subunit I, a gene commonly used in phylogenetic and taxonomic studies of organisms (Derocles, 2012) [3], was used in this study. This is the first COI sequence of *B. prasina* collected from south India and deposited from India. The first *B. prasina* COI sequences were deposited from North India. In the NCBI, only seven COI sequences *B. prasina* deposition available; all previous depositions showed significant divergence from our sequence.

Kim *et al*. (2014) [8] performed a phylogenetic analysis to determine whether *Chrysochroa coreana* is new. First, COI was amplified and sequenced in a partial region (532 bp). After that, the sequences were aligned and analyzed. Finally, they calculated genetic distances between them using the Kimura-2-parameter method. They concluded that *C. fulgidissima* from Korea was closely related to one from Taiwan, with only a small genetic distance between them (0.083), and also reported that Individuals from Korea (0.192) and Taiwan (0.183) were distantly related to the Japanese individual. Supporting the findings of Kim *et al*. (2014) [8], our sequences had the highest level of divergence compared to previously deposited sequences. However, the reason for the highest divergence is unknown; more haplotype gene mapping and haplotype network analysis are needed to understand the genomic divergence of *B. prasina.*

Acknowledgments

We thank the Department of Zoology, University of Calicut, for providing the infrastructural facility. The present study was financially supported by UGC-SAP (F.3-6/2012 (SAP-II) dated 10.10.2012).

References

1. Ahrens D, Monaghan MT, Vogler AP. DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). *Molecular Phylogenetics &* Evolution,2007:44:436-449.doi:10.1016/j.ympev.2007.02.024.
2. Ashfaq M, Hebert PDN. DNA barcodes for biosurveilance: regulated and economically important arthropod plant pests. Genome,2016:59:933-945.
3. Derocles SAP *et al*. Identification of molecular marker for DNA barcoding in the Aphidiinae (Hymenoptera: Braconidae). Mol. Ecol. Resour,2012:12:197-208.
4. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution,1985:39:783-791.
5. Herbert PDN, Ratnasingham S, Dewaard JR. Barcoding animal life: cytochrome oxidase subunit a divergences among closely related species. Proc. Royal Soc. B: Biol. Sci,2003:270:96-99.
6. Herbert PDN, Ratnasingham S, deWaard JR. Barcoding animal life: cytochrome c oxidase subunite 1 divergence among closely related species. Proc. Roy. Soc. Lon. B. Biol. Sci,2003:270:96-99.
7. Kelnarova I, Jendek E, Grebennikov VV, Bocak L. First molecular phylogeny of Agrilus (Coleoptera: Buprestidae), the largest genus on Earth, with DNA barcode database for forestry pest diagnostics. Bulletin of Entomological Research, 2014, 1-12.
8. Kim SK, Hwang UW, Kwon O. Three different genetic lineages of the jewel beetle Chrysochroa fulgidissima (Buprestidae; Chrysochroinae) inferred from mitochondrial COI gene. J. Ecol. Env,2014:37(1):35-39.
9. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution,1980:16:111-120.
10. Kumar S, Gadagkar SR. Disparity Index: A simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. Genetics,2001:158:1321-1327.
11. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution,2018:35:1547-1549.
12. Pentinsaari M *et al*. DNA barcodes reveal 63 overlooked species of Canadian beetles (Insecta, Coleoptera). Zookeys, 2019, 894:53.
13. Pentinsaari M, Herbert PDN, Muntanen M. Barcoding beetles: A regional survey of 182 species reveals high identification success and usually deep intraspecific divergences. Plos One,2014:9(9):e108651.
14. Ramasamy M. A scientific note on occurrence and infestation of jewel beetle Belionota prasina (Coleoptera: Buprestidae) on Cashew (Anacardium occidentale). Natl. Acad, 2018. Sci. Lett. .https://doi.org/ 10.1007/s40009-018-0706-2
15. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution,1993:10:512-526.