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The sequence between mitochondrial nad1-nad2 genes are potential molecular markers of harpactorinae predators (Heteroptera: Reduviidae)

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ABSTRACT

Assassin bugs are terrestrial predators belonging to the family Reduviidae. Among the 24 subfamilies, harparctorinae is predominant and extensively investigated for biocontrol applications compared to the others subfamilies in Reduviidae. Being natural enemies of phytophagous insects, understanding the phylogeny of these predatory bugs can precise the selection of candidates to employ in insect pest management. In addition to morphological systematics, complete mitochondrial genome sequences provide great insights into the phylogeny for resolving evolutionary complexity. Complete mitochondrial genomes of four potential predatory harpactorinae and one outgroup triatominae were retrieved from NCBI GenBank database. Comparative analysis of the five mitogenomes and the nucleotide sequence between *nad1* and *nad2* genes were selected as the best option to distinguish. The nucleotide sequence between *nad1-nad2* are found to be biased towards A and T similar to their respective complete mitogenomes. Tajima's test of neutrality suggest that the evolutionary selection at *nad1-nad2* was parallel to the complete mitogenome and showed positive and significant ($p > 0.1$) with high nucleotide diversity. Unequal evolutionary rate at *nad1-nad2* between lineages observed in Tajima's relative rate test and proved the nucleotide sequences of *nad1-nad2* between species are highly variable. Comparing the phylogenetic trees generated using the complete mitogenomes and *nad1-nad2* genes uncovered the correlation between the trees and having identical branches with varying bootstrap values. Conventionally the highly conserved protein-coding *cox1* gene is used for molecular taxonomy whereas this study provides an additional and/or a possible alternative molecular marker for genetic comparative test (the nucleotide sequence between *nad1-nad2*) to understand the systematics and phylogeny of Reduviidae. The significant nucleotide diversity, high genetic distance and less genetic similarity of the sequence between *nad1-nad2* genes among the species studied, *Agriosphodrus dohrni, Rhynocoris fuscipes, Scipinia horrida,* and *Velinus nodipes* undoubtedly propose the possible utilization of *nad1-nad2* region as distinguishable molecular marker.

Keywords: Reduviidae, Biocontrol agent, Mitochondrial genome, Molecular markers, Phylogeny

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INTRODUCTION

Assassin bugs are terrestrial predatory heteropterans belonging to the family Reduviidae. There are about 7000 species including subspecies in 24 sub-familes. With the exception of hematophagous triatominae all the other

subfamilies are predatory. Harpactorinae is the largest subfamily consisting of morphologically diverse species about 2,800 in number across 300 genera (Ambrose, 1999; Weirauch *et al.*, 2014). They are generalist predators feeding on multiple phytophagous insects through different orders viz., Coleoptera, Hemiptera, Hymenoptera, Dictyoptera, Lepidoptera, Orthoptera and Arachnids. Being natural enemies of many insects, their diurnal behaviour and plant-dwelling habit, the harpactorinae assassin bugs are proved to be potential biocontrol agents and augmented successfully in control of many pests more than the other subfamilies of Reduviidae (Ambrose, 1995; Sahayaraj, 2007 and 2014; Schuh and Weirauch, 2020). For example, *Agriosphodrus dohrni* employed in the control of forest pest forest pest *Hyphantria cunea* (Du *et al*., 2018); *Rhynocoris fuscipes* against groundnut pests such as *Aphis craccivora, Spodoptera litura* and *Mylloceros sp*. (Sahayaraj *et al.,* 2002); *Scipinia* horrida on psyllid *Heteropsyila cubana* (Barrion *et al.,* 1987) and *Velinus nodipes* versus *Linaeideu aenea* (Takeno, 1998). However, research around the world for the utilization of assassin bugs as biocontrol agents is lag (Ambrose and Kumar, 2016). Exploration into any group of living organisms starts with proper identification and classification as it is fundamental to the study of life science (Quicke, 1993). Sole dependance on classical taxonomy is time consuming and is not valued by the modern scientific community. Application of molecular technology for systematic studies solve the evolutionary complexities in cryptic groups (Yi *et al*., 2022). Molecular systematics relies heavily on mitochondrial genomes due to their conserved nature across species. In addition to morphological systematics, complete mitochondrial genome sequences resolve evolutionary complexities (Kjer, 2016). Typical invertebrates including hemipteran mitogenomes feature 13 protein-coding genes, 22 transfer RNA genes and 2 ribosomal RNA subunit genes (Cameron, 2014i and 2014ii; Wang *et al.*, 2015). Conserved protein-coding genes such as *cox1* are used in molecular identification i.e., DNA barcoding (Jung, 2010). This study probes the nucleotide sequences between the protein-coding genes and their chances of being utilized as species specific molecular markers. Preliminary investigations with Triatominae suggest the highly variable nucleotide sequences between *nad1-nad2* (Selvaraj *et al*., 2022) were useful and that the phylogenetic analysis in this study can validate its significance.

MATERIALS AND METHODS

Complete mitochondrial genomes of biocontrol agents from the subfamily Harpactorinae (Heteroptera: Reduviidae) catalogued in the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore) were downloaded. The selected species with their GenBank accession number are A-*Agrisphodrus dohrni* (NC015842), B-*Rhynocoris fuscipes* (MZ440304), C-*Scipinia horrida* (NC037744), D-*Velinus nodipes* (NC037738) and outgroup E-*Rhodnius prolixus* (NC050328). Circular genome map was created using CGview (https://proksee.ca/). Dataset FASTA files were generated for analysis using Unipro UGENE software v44.0 (http://ugene.net/). Multiple sequence alignment was performed using MAFFT 7 online server (https://mafft.cbrc.jp/). Tajima's test for neutrality and relative rate was computed in MEGA 11 (Tamura *et al.,* 2021) with codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). Trees were constructed using neighbourhood-joining method, Jukes-Cantor substitution model and 200 Bootstrap resampling. The constructed trees were processed in ITOL web server (https://itol.embl.de/). The results were presented as tables and graphs using Microsoft Office Excel 16.

RESULTS

Nucleotide composition

The nucleotide sequence between *nad1-nad2* features five transfer RNAs (Leucine-1, Valine, Isoleucine, Methionine and Glutamine), 2 ribosomal RNA subunits and a control region (Figure 1).

Table 1. Nucleotide composition of the selected complete mitogenome sequence and the sequence between *nad1-nad2* of harpactorinae biocontrol agents.

Species	Length (bp)		A%		T(U)%		G%		C%	
	Complete	*nad1-nad2*	Complete	*nad1-nad2*	Complete	*nad1-nad2*	Complete	*nad1-nad2*	Complete	*nad1-nad2*
Agriosphodrus dohrni	16470	4039	38.99	39.47	33.22	33.97	12.19	10.47	15.60	16.09
Rhynocoris fuscipes	15542	3234	40.94	40.29	31.08	32.53	11.50	10.14	16.48	17.04
Scipinia horrida	15660	3376	40.78	39.90	32.48	34.06	11.35	10.28	15.38	15.76
Velinus nodipes	15904	3458	41.13	41.82	32.11	32.94	11.50	9.86	15.26	15.38
Average	15894	3527	40.46	40.37	32.22	33.38	11.63	10.19	15.68	16.07
*Rhodnius prolixus**	15789	3524	41.03	40.87	28.57	30.77	11.05	9.82	19.35	18.54

(* Outgroup)

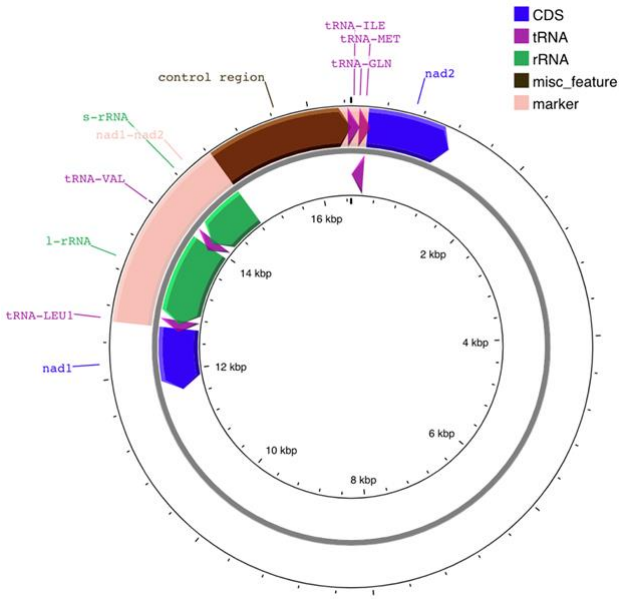
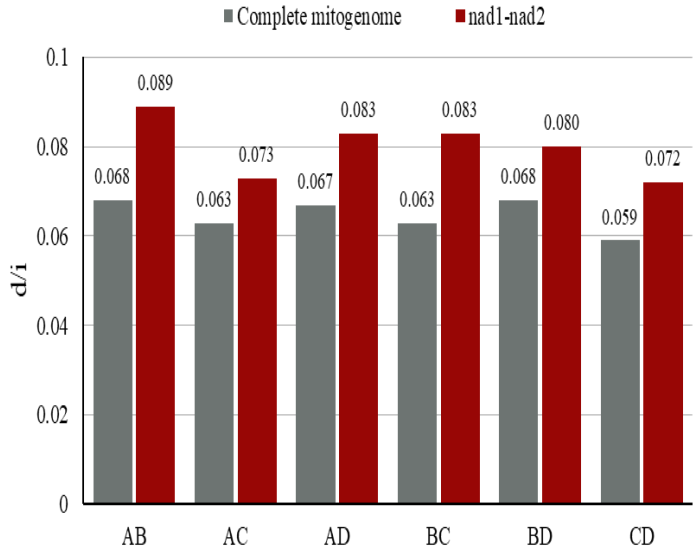


Figure 1. Representative circular map of the harpactorinae mitogenome showing the features present between the genes *nad1-nad2*.

Agriosphodrus dohrni has the largest (16,470 bp) mitgenome among the reported mitogenome of harpactorinae. The length of the complete mitogenome sequences of the harpactorinae biocontrol agents taken for the study vary from 15,542 bp (*Rhynocoris fuscipes*) to 16,670 bp (*Agriosphodrus dohrni*) with an average 15,894 bp. Whereas, the length of the sequence between the genes *nad1-nad2* range from 3,324 bp (*Rhynocoris fuscipes*) to 4,039 bp (*Agriosphodrus dohrni*). Base composition of complete mitogenome revealed that the percentage of adenosine (A 40.46 %) is predominant over the other nucleotides (T 32.22T% > C 15.68% > G 11.63%). Similar characteristics on the composition of nucleotides in the sequence between *nad1-nad2* was observed (A 40.37% > T 33.38% > C 16.07% > G 10.19%) (Table 1). The difference among nucleotide composition of the selected four species of harpactorinae on the complete mitogenome and *nad1-nad2* revealed their genetic conservation.

Test of neutrality for evolutionary selection

Test of neutrality analysis of *nad1-nad2* genes of 5 nucleotide sequences (4 Harpactorinae and 1 Triatominae as outgroup) showed positive and significant ($p > 0.1$) (Table 2). The level of significance on nucleotide diversity and the Tajima test statistic showed that neutrality analysis of *nad1-nad2* genes (D = 0.925) were similar in significance compared to complete mitogenome (D = 0.931). There were a total of 14,975 (complete mitogenome) and 3,001 (*nad1-nad2*) positions in the final dataset with segregating sites



(S)

Figure 2. Tajima's relative rate test - Divergent sites(d)/Identical sites(i) where species A-D are Harpactorinae tested in combination with outgroup E - *Rhodnius prolixus**

6,049 and 1,303 for the complete mitogenome and *nad1-nad2*, respectively. High nucleotide diversity was observed for the (π) of 0.217 and 0.234 for complete mitogenome (π = 0.217) and *nad1-nad2* (π = 0.234) with slight higher diversity in *nad1-nad2*. The high nucleotide diversity is a clue of distance genetic relationship of species studied in harpactorinae.

Table 2. Tajima's test of neutrality for the selected biocontrol agents mitogenome complete sequence and the sequence between *nad1-nad2* (*Rhodnius prolixus* - Outgroup).

	Complete mitogenome	***nad1-nad2***
m	5	5
n	14975	3001
S	6049	1303
p_s	0.404	0.434
Θ	0.194	0.208
π	0.217	0.234
D	0.931*	0.925*

m = number of analysed sequences, *n* = total number of sites and *S* = Number of segregating sites. $p_s = S/n$, $\Theta = p_s/a_1$, π = nucleotide diversity and *D* is the Tajima test statistic. * Significant (p > 0.1).

Relative rate test for evolution

Tajima's relative rate test was computed to test the equality of evolutionary rate between sequences AB, AC, AD, BC, BD and CD with outgroup sequence E (2 harpactorinae taxa with 1 outgroup triatominae). Results of the computation presented in the Figure 2 shows the Divergent sites(d)/Identical sites(i) form the relative rate test varies from 0.059 (CD) to 0.068 (AB and BD) for the complete mitogenome and 0.072 (CD) to 0.089

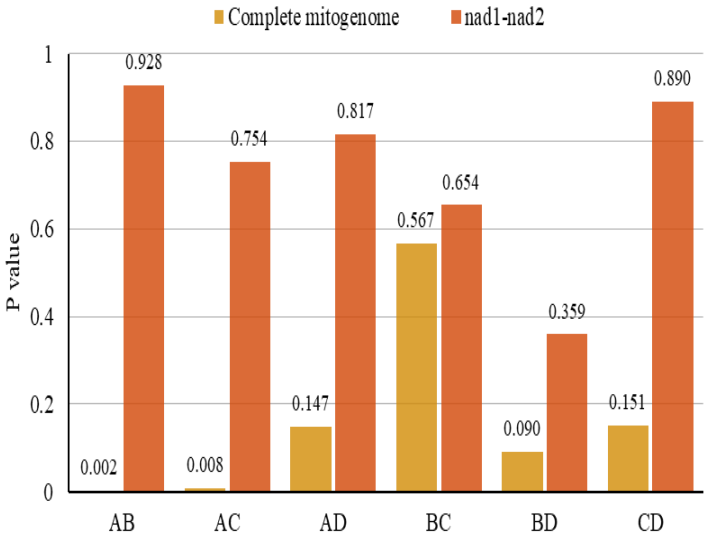


Figure 3. Tajima's relative rate test - P value, where species A-D are Harpactorinae tested in combination with outgroup E - *Rhodnius prolixus**. P<0.05 rejects null hypothesis for equality of evolutionary rate (AB) for the *nad1-nad2*.

The observed p value < 0.05 rejects null hypothesis for equality of evolutionary rate (Figure 3) at AB (0.002) and AC (0.008) for the complete mitogenome (Figure 3).

Phylogenetic tree

Similar trees were obtained using *nad1-nad2* (Figure 4 - left) and complete mitogenome (Figure 4 - right). Length of the branch from the outgroup is identical (0.16). Bootstrap estimates are low at 63 and 31 at the *nad1-nad2* corresponding to the high estimates at 100. *Agriosphodrus dohrni* is closely related to *Velinus nodipes*. However, the genetic distance between *Agriosphodrus dohrni* and outgroup Rhodnius prolixus was relatively far. *Rhynocoris fuscipes* is more similar to the

outgroup *Rhodnius prolixus.* Outline of phylogenetic trees using *nad1-nad2* region and mitogenome of *Agriosphodrus dohrni, Velinus nodipes, Scipinia horrida, Rhynocoris fuscipes* and outgroup *Rhodnius prolixus* revealed that the tested predators showed high genetic distance and less genetic similarity among the tested harpactorinae (Figure 4).

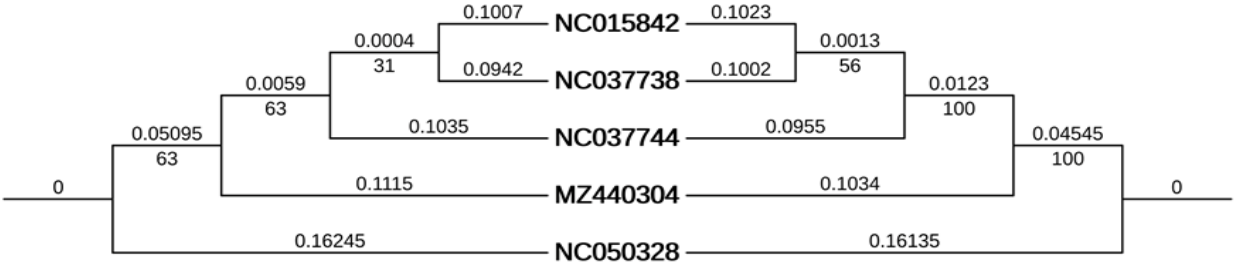


Figure 4. Phylogenetic tree constructed using nucleotide sequence between *nad1-nad2* (left) and complete mitogenome (right) with neighbourhood-joining method, Jukes-Cantor substitution model (200 bootstraps replications). NC015842: *Agrisphodrus dohrni;* NC037738: *Velinus nodipes*; NC037744: *Scipinia horrida*; MZ440304: *Rhynocoris fuscipes* and outgroup NC050328: *Rhodnius prolixus.*

DISCUSSION

The NCBI GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore) provides <80 Reduviidae complete mitochondrial genomes and only 48 are archived as reference genomes (https://www.ncbi.nlm.nih.gov/refseq).

Considering the biocontrol potential, the complete mitogenomes of four harpactorinae predators viz., *Agriosphodrus dohrni* (Yao *et al.,* 1995), *Rhynocoris fuscipes, Scipinia horrida* (Ambrose, 1999 and 2007) and *Velinus nodipes* (Takeno, 1998) were selected for the study. Selvaraj *et al*. (2022) reported the variable nucleotide sequences in complete mitogenomes of Triatominae and found the sequences between the genes *cytB-nad1* and *nad1-nad2* to be highly variable across ten species. This study is the next step in validating the pattern within harpactorinae biocontrol agents. The sequence length between the *nad1-nad2* especially is highly variable ranging from 3,324 to 4,039 bp directly proportionate to the length of the complete mitogenome. The nucleotide sequence between *cytB-nad1* encodes (5'-3') a non-coding RNA tRNA-Serine along with a few base pairs. Whereas the nucleotide sequence between *nad1-nad2* encodes complement (3'-5') tRNA-Leucine, 16s rRNA, tRNA-Valine, 12s rRNA and from 5'-3', a control region (miscellaneous feature), tRNA-Isoleucine, complement (3'-5') tRNA-Glutamine and 5'-3' tRNA-Methionine (Cameron, 2014i & ii; Zhao *et al.,* 2015; Chen *et* al., 2018; Zhao *et al*., 2020; Sun *et al.,* 2021; Yi *et al*., 2022). The base composition is heavily based towards A and T which is common in the mitochondrial genomes of hemipterans, similar richness on A and T was observed in the tested mitogenome (Wang *et al*., 2015).

Phylogenetic analysis using the Tajima's test of neutrality revealed similarity in the statistic values. This shows that the changes occurring in the complete mitogenome is reflected at the nucleotide sequence between the genes *nad1-nad2*. In the relative rate test, the Divergent sites(d)/Identical sites(i) where species A-D are Harpactorinae tested in combination with outgroup E - *Rhodnius prolixus* shows that rate of evolution at *nad1-nad2* is higher than the complete mitogenome i.e, it can be used as a molecular marker to validate the interspecific variations. Further, the P values observed in the complete mitogenome of AB and AC with outgroup E are significant (<0.05) showing equality in the evolutionary rate in contradiction to the values at *nad1-nad2* where the P values are >0.05. Due to this phenomenon the nucleotide sequence between genes *nad1-nad2* are unique to individual species (Nei and Kumar, 2000; Selvaraj *et al*., 2022)

The phylogenetic tree constructed from the whole mitogenome coverage is similar to the one constructed using *nad1-nad2.* Therefore, the nucleotide sequence between *nad1-nad2* can be considered to be a species-specific marker. Consequently, morphological systematics is compulsory to validate the species (Trautwien, 2012) and the phylogenetic trees are an observable estimate of a long period of genome evolution which is owed to change with the calculation algorithm (Bickel, 2022). However, the molecular assembly can be influenced by the reference genome used to assemble the sequenced contigs. Hence, it is essential to validate the reports of this study across the subfamilies, genera and species for adoption of this variable sequence between *nad1-nad2* for phylogenetic systematics.

In conclusion, the positive and significant nucleotide diversity on *nad1-nad2* genes and complete mitogenome revealed the high distance genetic and less genetic similarity among the species studied in Harpactorinae, *Agriosphodrus dohrni, Velinus nodipes, Scipinia horrida,* and *Rhynocoris fuscipes,* which clearly suggest the possible utilization of *nad1-nad2* genes as molecular marker to distinguish the species of Harpactorinae.

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