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Identification of pumpkin beetle (*Aulacophora* spp.) through mtDNA-COI barcode approach in cucurbitaceous vegetable crops

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ABSTRACT

Cucurbitaceous vegetables are frequently attacked by various *Aulacophora* spp., making accurate species identification essential for effective pest management. However, traditional morphological methods have notable limitations in distinguishing these species. The present study was carried out during 2022 and 2023 at Bihar Agricultural University, Sabour, Bihar using molecular barcoding to provide a more reliable and precise identification approach. Genomic DNA was isolated from the mitochondria-rich legs and wings of 10 *Aulacophora* specimens collected from different agro-climatic zones of Bihar and the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using universal primers. The PCR-amplified COI gene fragments were sequenced, and the resulting barcode sequences were deposited in the NCBI GenBank database, where accession numbers were assigned to all 10 entries. BLAST analysis of these sequences confirmed the presence of two species i.e. *Aulacophora lewisii* and *Aulacophora indica* through comparison with reference sequences from multiple countries available in GenBank. A phylogenetic tree constructed at a 0.020 bar scale grouped the sequences into three distinct clusters. Haplotype analysis indicated that *A. lewisii* and *A. indica* diverged from a common ancestor through specific mutational events. Furthermore, several highly expressed codons were detected in both species, which may influence protein synthesis and hold potential for future functional studies.

Keywords: *Aulacophora* spp., Barcode approach, Cucurbitaceous vegetable, Haplotype analysis

The red pumpkin beetle has been found to be widely distributed throughout all zoogeographic regions of the world except the Neoarctic and Neotropical regions and all over the South-East Asia, Africa, Mediterranean region towards the west and Australia in the east (Khan 2018). Among the pests, the red pumpkin beetle is a major pest that can lower output and cause yield losses of between 30 and 100%, particularly in seedlings of practically all cucurbitaceous crops (Khan 2012). Several researchers have reported on the prevalence of the adult stage of the red pumpkin beetle (RPB) on several cucurbits (Nath 1964, Sarker *et al*. 2016). Additionally, it has been observed that by creating irregular holes on affected plant parts, this pest damages up to 35–75% of cotyledons at the seedling stage, roughly 70% of leaves, and 60% of flowers (Khan 2018). There aren't many reports in Bihar. A study has been initiated to determine the occurrence of the red pumpkin beetle at different growth stages of various cucurbitaceous vegetable crops in Bihar. Currently, some guidelines are available regarding the diversity of red pumpkin beetles across different cucurbitaceous crops (Kamal *et al.* 2014). However, identification based solely on morphological characteristics has several limitations. For instance, even experts find it difficult to accurately distinguish female and juvenile stages, which is critical for effective pest management. Apart from morphological analysis, DNA-based molecular markers are extensively employed to address systematic problems in other organisms, such as pest coleopterans (Das *et al.* 2020). Furthermore, for phylogenetic and haplotype investigations on insect pests, mitochondrial DNA (Mt-DNA) has been employed as a molecular marker (Simon *et al.* 1994). For species level identification, the widely used standard 658 bp COI segment has shown to be very informative and helpful (Bergsten *et al*. 2012). Any pest's barcoding can be difficult, but it's well worth the effort because it may represent multiple evolutionary stages. Thus, keeping these facts in mind, the present study was carried out to identify pumpkin beetle (*Aulacophora* spp.) through molecular barcode approach in cucurbitaceous vegetable crops. It is our aim that this study would provide guidance for future

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Table 1 List of *Aulacophora* spp. and their accession number used in the present study

No of samples	Species name	Country	NCBI-GeneBank accession number	Web link	Query ID	Query length (bp)
Sample 1	*Aulacophora* spp. 1	Sabour, Bihar, India	OR272361	https://www.ncbi.nlm.nih.gov/nuccore/OR272361	OR272361.1	652
Sample 2	*Aulacophora* spp. 2	Sabour, Bihar, India	OR268547	https://www.ncbi.nlm.nih.gov/nuccore/OR268547	OR268547.1	681
Sample 3	*Aulacophora* spp. 3	Purnia, Bihar, India	OR196699	https://www.ncbi.nlm.nih.gov/nuccore/OR196699	OR196699.1	626
Sample 4	*Aulacophora* spp. 4	Purnia, Bihar, India	OR196688	https://www.ncbi.nlm.nih.gov/nuccore/OR196688	OR196688.1	638
Sample 5	*Aulacophora* spp. 5	Sabour, Bihar, India	OR195796	https://www.ncbi.nlm.nih.gov/nuccore/OR195796	OR195796.1	673
Sample 6	*Aulacophora* spp. 6	Noorsarai, Bihar, India	OR301257	https://www.ncbi.nlm.nih.gov/nuccore/OR301257	OR301257.1	595
Sample 7	*Aulacophora* spp.7	Noorsarai, Bihar, India	OR497830	https://www.ncbi.nlm.nih.gov/nuccore/OR497830	OR497830.1	564
Sample 8	*Aulacophora* spp. 8	Noorsarai, Bihar, India	OR497831	https://www.ncbi.nlm.nih.gov/nuccore/OR497831	OR497831.1	563
Sample 9	*Aulacophora* spp. 9	Sabour, Bihar, India	OR497805	https://www.ncbi.nlm.nih.gov/nuccore/OR497805	OR497805.1	519
Sample 10	*Aulacophora* spp. 10	Sabour, Bihar, India96	OR497807	https://www.ncbi.nlm.nih.gov/nuccore/OR497807	OR497807.1	593

research and help researchers understand the effectiveness of barcoding pest species.

MATERIALS AND METHODS

Sample collection and extraction of genomic DNA: During the 2022 and 2023, collection of *Aulacophora* spp. was done from (Sabour) Bhagalpur, Noorsarai, and Purnia districts of Bihar using sweeping nets. Somatic parts, such as the wings and legs that were abundant in mitochondria, were carefully removed, washed with 70% ethanol, and then preserved for molecular analysis. In the *kharif* season of 2023, the genomic DNA of *Aulacophora* spp. was extracted from the leg and wing tissues at the Toxicology Laboratory of the Department of Entomology, Bihar Agricultural University, Sabour, Bihar, using the CTAB method, with certain modifications, as described by Doyle and Doyle (1990).

PCR amplification and gel documentation: A universal primer (LCO1490 (F): 5'-GTCAACAAATCATAAAGATATTGG-3' and HC02198 (R): 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') was used to facilitate the polymerase chain reaction. PCR amplification protocol was done according to the (Sahani *et al.* 2023). Gels made of 2% top vision agarose (Fermentas) were used to separate the results of amplification processes. Gels were stained using a solution of ethidium bromide. The size marker employed to ascertain the molecular size of the amplified products was a ProxiO 100 bp DNA ladder. In 0.5X TBE buffer, electrophoresis was carried out for 90 min at 100 V. The gels were documented using a gel documentation system (Alpha Innotech) following electrophoresis. After undergoing 1.5 hours of 2% agarose gel electrophoresis at 90 volts, the amplified product, which had an expected product size of about 710 bp (Fig. 1) was seen and recorded in a UV transilluminator. ProxiO 100 bp DNA ladder was used to assess the size of the amplified fragment.

DNA sequencing and bioinformatic analysis: Quality parameters were examined in the sequencing results that came from a sequencer (Barcode Biosciences, Bengaluru). Using BioEdit version 7.2.5 for Windows, a consensus area sequence was created after the undesirable sequences from the original paired-end data were removed (Hall 1999).

Phylogenetic tree construction and haplotype analysis: The end-to-end alignment done through clustalW and the dendrogram were made employing MEGA11 software

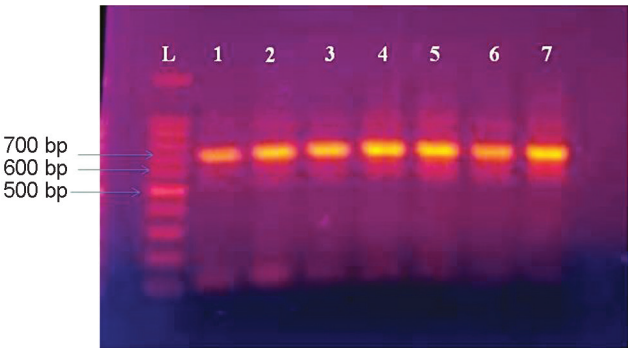

Fig. 1 Amplification of mitochondrial cytochrome c oxodase submit I (COI) gene of *Aulacaphora* spp. by universal primer pair.
All wells (1–7) showed single band between 500–700 bp which indicated confirmation of COI gene amplification. L = ProxiO 100 bp DNA ladder.

version 11.0.11 with neighbour-joining (NJ) with comprehensive gap finding resampled and the alignment of maximum composite likelihood with 1000 bootstrap replication (Tamura *et al*. 2021). Additionally, using the TCS network as a basis, Popart 1.7 software was used to generate the haplotype analysis (Clement *et al*. 2000).

Nucleotide composition and relative synonymous codon usage (RSCU) of COI gene sequences: The nucleotide composition of *Aulacophora* spp. was analysed manually. According to Shields and Sharp (1987), RSCU values are the ratio of the observed usage frequency of a single codon in a gene sample to the expected usage frequency in the synonymous codon family, assuming that all codons for a given amino acid are used equally. Consequential codons with RSCU values >1.0 were classified as plentiful because they exhibited positive codon usage bias, whereas those with RSCU values <1.0 were classified as less-abundant because they exhibited negative codon usage bias. Additionally, a codon was deemed to have no bias if its RSCU value was equal to 1.0, and to be inactive if its RSCU value was equal to zero.

Data availability: The datasets analysed in present study are available in the NCBI repository (https://www.ncbi. nlm.nih.gov/). The NCBI-GenBank accession number and web link of the public datasets evaluated in present are mentioned in Table 1.

RESULTS AND DISCUSSION

Taxonomic identification: Species of *Aulacophora* spp. from three different districts in two different zones of Bihar had been collected and intensively studied in the field of cucurbits, and the identification was done at Bihar Agricultural University, Sabour, Bhagalpur, Bihar. It was further verified by the concerned scientist working in the coleopteran group from ICAR-Indian Agricultural Research Institute, New Delhi. The following two species of *Aulacophora* were recognized based on morphological characters, i.e. *A. indica*, and *A. lewisii* from the different zones of Bihar. *Aulacophora indica* had reddish to yellowish colour elytra and yellowish-brown pygidium, and *Aulacophora lewisii* had dark, shiny black colour elytra. To confirm species identification, mtDNA-COI barcoding techniques were employed for additional verification.

Using universal primer pair namely LCO1490 (F) and HC02198 (R), amplification of COI gene was successfully achieved. Subsequently, the intended COI gene product produced by PCR was observed using a gel documentation system. All the entries (10 samples of *Aulacophora* spp.) in the present study yielded single band which indicated confirmation of COI gene amplification. While, there were no double bands among these 10 samples which indicated that sequences obtained were targeted only mitochondrial DNA and not nuclear DNA. Across all entries, the amplified products total sizes varied between 500 and 700 bp. For precise identification, the COI gene of *Aulacophora* spp. was sequenced using a molecular barcode technique.

Sequence results and BLAST analysis of all 10 sequences for Aulacophora *spp.*: For identification of *Aulacophora* spp. through molecular barcoding approach, Mt-COI barcode region from 10 samples of *Aulacophora* spp. were sequenced. After getting sequencing results, it was submitted to NCBI-GenBank and received NCBI-GenBank accession number of all 10 samples of *Aulacophora* spp. (Table 1). The query length ranged from 519–681 bp. Further, BLAST (Basic local alignment search tool) analysis was used to compare sequence identity between the obtained sequences of all 10 samples of *Aulacophora* spp. and existing GenBank library sequences of different countries from NCBI GenBank. BLAST analysis of sample 1 for *Aulacophora* spp. showed 100%, 99.69% and 99.69% homology with the sequences in GenBank submitted from China, Bangladesh and Meghalaya, respectively. Hence, it confirmed that the sample 1 is *Aulacophora lewisii.* BLAST analysis of sample 2 for *Aulacophora* spp. showed 99.37%, 99.09% and 99.05% homology with the sequences in GenBank submitted from China, Bangladesh and Meghalaya respectively. Hence, it confirmed that the sample 2 is *Aulacophora lewisii*. BLAST analysis of sample 3 for *Aulacophora* spp. showed 86.82%, 86.82% and 86.68% homology with the sequences in GenBank submitted from Bangladesh, China and Meghalaya, respectively. Hence, it confirmed that the sample 3 is *Aulacophora lewisii*. BLAST analysis of sample 4 for *Aulacophora* spp. showed 99.53%, 99.22% and 99.21% homology with the sequences in GenBank submitted from China, Bangladesh and Meghalaya, respectively. Hence, it confirmed that the sample 4 is *Aulacophora lewisii.* BLAST analysis of sample 5 for *Aulacophora* spp. showed 98.64%, 98.30% and 98.28% homology with the sequences in GenBank submitted from China, Bangladesh and Meghalaya, respectively. Hence, it confirmed that the sample 5 is *Aulacophora lewisii*. BLAST analysis of sample 6 for *Aulacophora* spp. showed 99.50%, 99.33%, 99.16%, 99.16%, 99.16% and 99.16%, homology with the sequences in GenBank submitted from China, Kerala, Meghalaya, Bangladesh, Vietnam and Tamil Nadu, respectively. Hence, it confirmed that the sample 6 is *Aulacophora lewisii.* BLAST analysis of sample 7 for *Aulacophora* spp. showed 100.00%, 99.45%, and 99.27% homology with the sequences in GenBank submitted from Bangladesh, Pakistan, and Australia, respectively. Hence, it confirmed that the sample 7 is *Aulacophora indica.* BLAST analysis of sample 8 for *Aulacophora* spp. showed 100.00%, 99.45%, and 99.45% homology with the sequences in GenBank submitted from Bangladesh, Pakistan, and Australia, respectively. Hence, it confirmed that the sample 8 is *Aulacophora indica.* BLAST analysis of sample 9 for *Aulacophora* spp. showed 100.00%, 99.40%, and 99.40% homology with the sequences in GenBank submitted from Bangladesh, Pakistan, and Australia, respectively. Hence, it confirmed that the sample 9 is *Aulacophora indica.* BLAST analysis of sample 10 for *Aulacophora* spp. showed 100.00%, 99.48%, and 99.48% homology with the sequences in GenBank submitted from Bangladesh, Pakistan, and Australia, respectively. Hence, it confirmed that the sample 10 is *Aulacophora indica*.

Phylogenetic tree analysis: To confirm the origin and evolution, molecular phylogenetic tree was drawn using the Neighbour-Joining method (Fig. 2). This analysis involved 20 nucleotide sequences in which 10 nucleotide sequences of *Aulacophora* spp. were compared with 10 sequences from different parts of countries like China, Bangladesh, Pakistan, Australia, Vietnam and India (Kerala, Tamil Nadu and Meghalaya). At 0.020 bar scale, this tree was clustered into three major groups (I, II, III) which were further divided into sub-groups. Group I contained only *Aulacophora lewisii* and group II contained only *Aulacophora indica.* Group III also contained *Aulacophora lewisii* but this group was slightly different from both group I and group II. Further, in group I, *Aulacophora lewisii* having accession number MH198035 from Meghalaya, India showed 95% similarity with another *Aulacophora lewisii* having accession number OM757838 from Tamil Nadu, India. Similarly, *Aulacophora lewisii* having accession number OR196688 from Purnia, Bihar, India showed 57% similarity with another *Aulacophora lewisii* having accession number OR301257 from Noorsarai, Bihar, India. While in group II, *Aulacophora indica* having accession number KY846455 from Pakistan showed 83% similarity with another *Aulacophora indica* having accession number MW093414 from Australia. Additionally, *Aulacophora indica* having accession number OR497831 from Noorsarai, Bihar, India showed 47% similarity with another *Aulacophora indica* having accession number OR497805 from Sabour, Bihar, India.

The phylogenetic tree was drawn using the Neighbour-Joining method to confirm the evolutionary relationships of *Aulacophora* spp. (Fig. 2). The percentage of replicate trees in which the associated *Aulacophora* spp. clustered together in the bootstrap test (1000 replicates) were shown next to the branches. This analysis involved 20 nucleotide sequences. There were a total of 656 positions in the final dataset. The optimal tree with the sum of branch length (SBL) was 0.31561831.

Relative synonymous codon usage of two identified species: In *A. lewisii*, the synonymous codons such as AAA, AAT, ACA, AGA, AGT, ATA, ATT, CAA, CAT, CCA, CCT, CGA, CTA, GAA, GAT, GCA, GGA, GTA, GTT, TAT, TCA, TCC, TCT, TGA, TGT, TTA, and TTT showed RSCU value >1.0 and hence, indicated strong codons while in *A. indica*, the synonymous codons such as AAC, ACC, ACT, ATA, ATC, CAA, CCC, CTG, GAG, GAT, GCG, GCT, GGG, GTA, GTC, GTT, TAT, TCA, TCC, TGC,

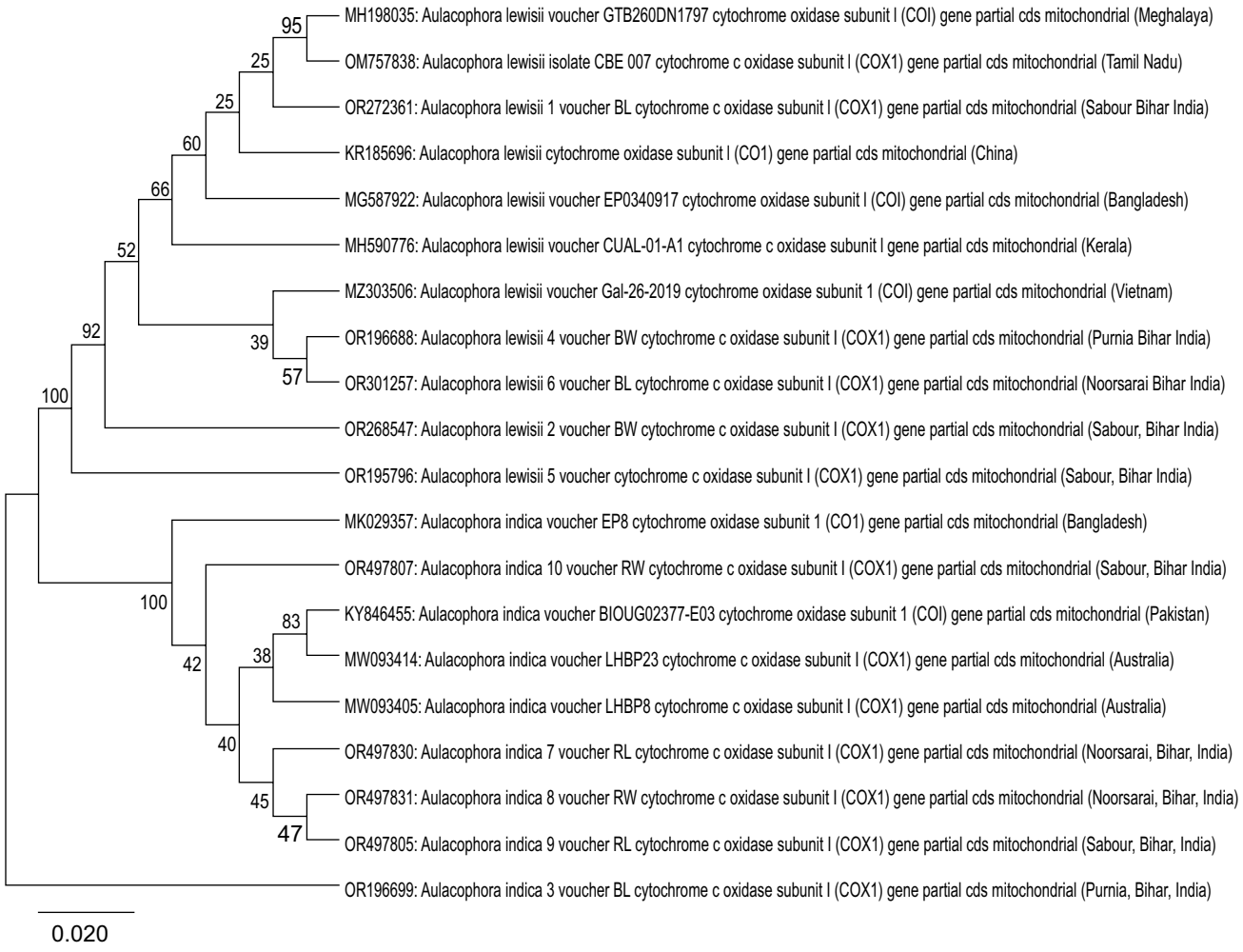

Fig. 2 Neighbour-joining phylogenetic tree of *Aulacophora* spp. detected in various samples of Bihar.

TTA, TTG and TTT showed RSCU value >1.0 and hence, indicated strong codons (Table 2). These strong codons can be used in translation process. Additionally, in *A. lewisii*, the synonymous codons such as ACC, ACG, ACT, ATG, CAC, CCC, CTT, GAC, GCC, GCG, GCT, GGC, GGG, GGT, TTC and TTG showed RSCU value <1.0 and thus, indicated weak codons while in *A. indica*, the synonymous codons such as ATG, ATT, CAG, CCA, CTA, CTC, CTT, GAA, TAC and TTC showed RSCU value <1.0 and thus, indicated weak codons. Further, in *A. lewisii*, the synonymous codon such as CGT showed RSCU value =1.0 and hence, indicated no codon bias while in *A. indica*, the synonymous codons such as AAA, AAG, CAC, CAT, CCG, CCT and GGT showed RSCU value =1.0 and hence, indicated no codon bias. Moreover, in *A. lewisii*, the synonymous codons such as AAC, AAG, AGC, AGG, ATC, CAG, CCG, CGC, CGG, TAA, CTC, CTG, TAG, TGG, TGC, TCG, TAC, GTG, GTC and GAG showed RSCU value =0.0 and hence, indicated inactive codons while in *A. indica*, the synonymous codons such as AAT, ACA, ACG, AGA, AGC, AGG, AGT, CGA, CGC, CGG, CGT, TAA, GCA, GCC, GGA, GGC, GTG, TCG, TCT, TGA, TGG, TGT and TAG showed RSCU value =0.0 and hence, indicated inactive codons.

Haplotype analysis: Haplotypes analysis of mitochondrial *COI* gene of *Aulacophora* spp. was constructed by Popart 1.7 software based on TCS network. *A. indica* (OR497830) was separated by 63 mutational steps from the common ancestor. However, *A. lewisii* (OR196699) was separated

Table 2 List of codon, amino acid and relative synonymous codon usage (RSCU) of two identified species namely *Aulacophora lewisii* and *Aulacophora indica*

S. no.	Aulacophora lewisii						Aulacophora indica					
	Codon	Amino acid	RSCU	Codon	Amino acid	RSCU	Codon	Amino acid	RSCU	Codon	Amino acid	RSCU
1.	AAA	Lysine	2.000	CTT	Leucine	0.692	AAA	Lysine	1.000	CTT	Leucine	0.720
2.	AAC	Asparagine	0.000	GAA	Glutamic acid	2.000	AAC	Asparagine	2.000	GAA	Glutamic acid	0.933
3.	AAG	Lysine	0.000	GAC	Aspartic acid	0.857	AAG	Lysine	1.000	GAC	Aspartic acid	0.000
4.	AAT	Asparagine	2.000	GAG	Glutamic acid	0.000	AAT	Asparagine	0.000	GAG	Glutamic acid	1.067
5.	ACA	Threonine	2.400	GAT	Aspartic acid	1.143	ACA	Threonine	0.000	GAT	Aspartic acid	2.000
6.	ACC	Threonine	0.400	GCA	Alanine	2.286	ACC	Threonine	2.000	GCA	Alanine	0.000
7.	ACG	Threonine	0.400	GCC	Alanine	0.571	ACG	Threonine	0.000	GCC	Alanine	0.000
8.	ACT	Threonine	0.800	GCG	Alanine	0.286	ACT	Threonine	2.000	GCG	Alanine	2.000
9.	AGA	Serine	1.714	GCT	Alanine	0.857	AGA	Serine	0.000	GCT	Alanine	2.000
10.	AGC	Serine	0.000	GGA	Glycine	2.444	AGC	Serine	0.000	GGA	Glycine	0.000
11.	AGG	Serine	0.000	GGC	Glycine	0.444	AGG	Serine	0.000	GGC	Glycine	0.000
12.	AGT	Serine	1.143	GGG	Glycine	0.222	AGT	Serine	0.000	GGG	Glycine	3.000
13.	ATA	Methionine	1.333	GGT	Glycine	0.889	ATA	Methionine	1.200	GGT	Glycine	1.000
14.	ATC	Isoleucine	0.000	GTA	Valine	1.231	ATC	Isoleucine	1.111	GTA	Valine	1.333
15.	ATG	Methionine	0.667	GTC	Valine	0.000	ATG	Methionine	0.800	GTC	Valine	1.333
16.	ATT	Isoleucine	2.000	GTG	Valine	0.000	ATT	Isoleucine	0.889	GTG	Valine	0.000
17.	CAA	Glutamine	2.000	GTT	Valine	2.769	CAA	Glutamine	1.400	GTT	Valine	1.333
18.	CAC	Histidine	0.400	TAC	Tyrosine	0.000	CAC	Histidine	1.000	TAC	Tyrosine	0.556
19.	CAG	Glutamine	0.000	TAT	Tyrosine	2.000	CAG	Glutamine	0.600	TAT	Tyrosine	1.444
20.	CAT	Histidine	1.600	TCA	Serine	2.286	CAT	Histidine	1.000	TCA	Serine	6.400
21.	CCA	Proline	1.231	TCC	Serine	1.714	CCA	Proline	0.500	TCC	Serine	1.600
22.	CCC	Proline	0.615	TCG	Serine	0.000	CCC	Proline	1.500	TCG	Serine	0.000
23.	CCG	Proline	0.000	TCT	Serine	1.143	CCG	Proline	1.000	TCT	Serine	0.000
24.	CCT	Proline	2.154	TGA	Tryptophan	2.000	CCT	Proline	1.000	TGA	Tryptophan	0.000
25.	CGA	Arginine	3.000	TGC	Cysteine	0.000	CGA	Arginine	0.000	TGC	Cysteine	2.000
26.	CGC	Arginine	0.000	TGG	Tryptophan	0.000	CGC	Arginine	0.000	TGG	Tryptophan	0.000
27.	CGG	Arginine	0.000	TGT	Cysteine	2.000	CGG	Arginine	0.000	TGT	Cysteine	0.000
28.	CGT	Arginine	1.000	TTA	Leucine	3.923	CGT	Arginine	0.000	TTA	Leucine	1.320
29.	CTA	Leucine	1.154	TTC	Phenylalanine	0.400	CTA	Leucine	0.240	TTC	Phenylalanine	0.133
30.	TAA	Glutamine	0.000	TAG	Glutamine	0.000	TAA	Glutamine	0.000	TAG	Glutamine	0.000
31.	CTC	Leucine	0.000	TTG	Leucine	0.231	CTC	Leucine	0.840	TTG	Leucine	1.440
32.	CTG	Leucine	0.000	TTT	Phenylalanine	1.600	CTG	Leucine	1.440	TTT	Phenylalanine	1.867

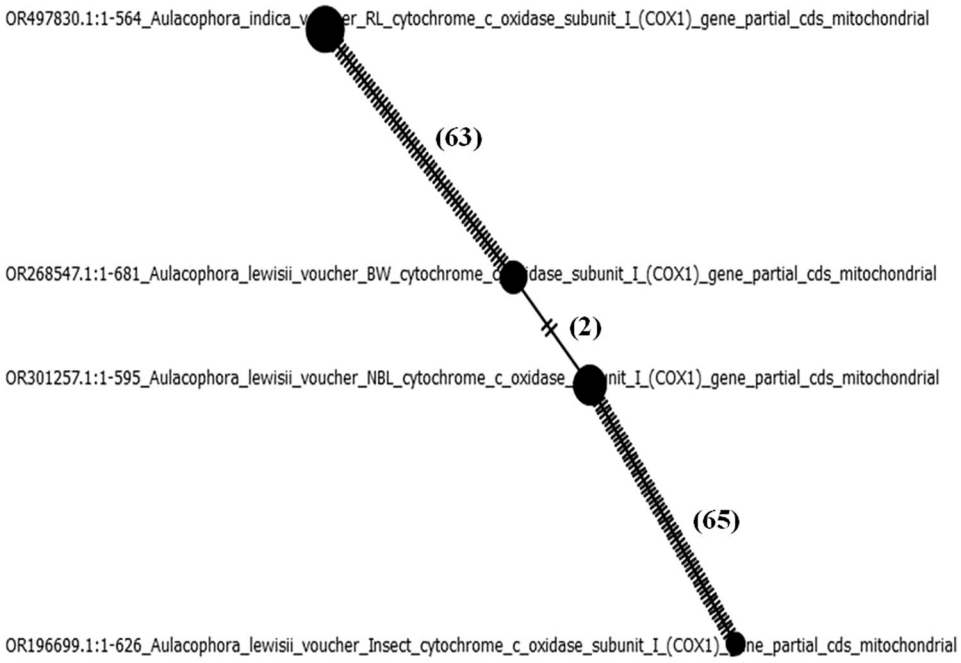

Fig. 3 Haplotype analysis was constructed by Popart 1.7 software based on TCS network. Black circles represents haplotypes. Mutational steps are indicated by hatch marks and numbers.

by 65 mutational steps from the common ancestor. In other sense, *A. lewisii* (OR196699) showed the highest number of mutational steps among the *Aulacophora* spp. in present study and genetically distant species from its common ancestors by 65 mutational steps (Fig. 3).

However, the COI gene has become an essential and widely used tool in integrative taxonomy for species identification and description (Hendrich and Balke 2011, Butcher *et al*. 2012, Aslam *et al*. 2019, Das *et al*. 2020). In the present study, molecular barcoding was employed for the identification of *Aulacophora* spp. using the mitochondrial cytochrome oxidase subunit I (mt-COI) gene region. A total of 10 samples of *Aulacophora* spp. were sequenced for the mt-COI barcode region. The analysis confirmed the presence of two species, *Aulacophora lewisii* and *Aulacophora indica*, associated with cucurbitaceous vegetable crops. The results demonstrated that COI-based molecular barcoding is highly effective for insect identification, as all species were accurately and successfully identified using this marker. This study provides a useful reference backbone for research on pest based on MT-COI gene sequence through establishing DNA barcode dataset. Similar findings were also observed by Das *et al*. (2020). Through mtDNA-COI barcode approach, they identified that three pest species of genus *Aulacophora* (*A. foveicollis*, *A. lewisii* and *A. indica*) attacking on horticultural crops in Bangladesh. The present findings are consistent with those of Dilipsundar *et al*. (2022), who successfully sequenced and analyzed the COI gene from *A. foveicollis*, *A. lewisii*, and *A. cincta* in Tamil Nadu using both phylogenetic and distance-based methods (ABGD). Their analysis accurately distinguished the species, with *A. lewisii* forming a distinct clade. Moreover, the nucleotide composition of two identified species in present study indicated Adenine and Thymine content was higher than Guanine and Cytosine. Further, the average of adenine and thiamine content (A+T=67.94%) was higher than average of guanine and cytosine content (G+C=32.58%) among identified *Aulacophora* spp. This result was supported by previous research works (Aslam *et al*. 2019, Das *et al*. 2020). In present study, there were total 64 codons observed in each *Aulacophora* spp. (Table 2). Out of 64 codons, some strong codons found between *A. lewisii* and *A. indica* which can be further utilized in translation process. A similar observation was found by Das *et al*. (2020). Furthermore, in present study, the haplotypes analysis of mitochondrial COI gene of *Aulacophora* spp. explained that *A. lewisii* and *A. indica* were disparating from their common ancestors by different mutational steps and number. This work was supported by earlier researchers (Bajpai and Tewari 2010, Asokan *et al*. 2011, Hashemi-Aghdam *et al*. 2017).

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