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MOLECULAR CHARACTERIZATION AND DNA BARCODING OF STINGLESS BEE WORKER (*Tetragonula* spp.) POPULATIONS FROM GUJARAT, INDIA USING MITOCHONDRIAL COI GENE

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ABSTRACT

The present investigation entitled “Molecular characterization and DNA barcoding of stingless bee worker (*Tetragonula* spp.) populations from Gujarat, India using mitochondrial *COI* gene” was carried out at the Department of Plant Molecular Biology and Biotechnology, N. M. College of Agriculture, Navsari Agricultural University (NAU), Navsari, Gujarat during the period from 2025-2026. Genomic DNA extracted from adult worker bees using a modified CTAB method showed high quality, with A_{260}/A_{280} ratios ranging from 1.78 to 1.92. Mitochondrial *COI* and *COII* genes were successfully amplified and sequenced, producing fragments of ~700 bp and ~750 bp, respectively. BLASTn analysis confirmed the sequences as belonging to genus *Tetragonula* with most populations showing 99-100% similarity to

Tetragonula iridipennis, while the Anand *COI* sequence showed 99.41% similarity with *Tetragonula shubhami*. Fourteen mitochondrial sequences (seven *COI* and seven *COII*) were submitted to GenBank. Nucleotide analysis revealed an AT-rich mitochondrial genome, and sequence comparisons detected limited polymorphism, with distinct variation observed in the Anand population. Phylogenetic analysis based on *COI* and *COII* sequences grouped most Gujarat populations with *T. iridipennis* reference sequences, whereas the Anand population showed a separate clustering pattern close to *T. shubhami*. Genetic distance analysis indicated low overall divergence among populations, with comparatively higher variation in Anand. DNA barcodes generated in this study represent the first molecular barcode records of *Tetragonula* spp. from Gujarat in BOLD. Overall, this study revealed limited genetic variation among populations and suggesting the possible occurrence of *T. shubhami* in the state, pending confirmation through male morphometry and genitalia-based studies.

Key words: *Tetragonula*, stingless bee, *Tetragonula shubhami*, DNA barcoding, *COI* gene & BOLD.

Introduction

Stingless bees (Hymenoptera: Apidae: Meliponini) are an important group of eusocial, non-*Apis* bees predominantly distributed in tropical and subtropical regions. They represent the most diverse group of corbiculate bees, comprising approximately 605 described extant species belonging to 45 genera worldwide (Engel

et al., 2023). These bees are characterized by reduced wing venation, the presence of penicillium and specialized setae on the hind tibia, along with the absence of a functional sting apparatus (Wille, 1983). Due to their eusocial behaviour, adaptability, and efficient foraging activity, stingless bees play a significant role as pollinators of wild plants and agricultural crops, contributing to

ecosystem stability and sustainable crop production (Roubik, 1993; Heard, 1999; Garibaldi *et al.*, 2013).

Among Asian stingless bees, the genus *Tetragonula* is widely distributed, with *Tetragonula iridipennis* being one of the most commonly reported species in India. Stingless bee diversity in India includes species such as *T. iridipennis*, *T. bengalensis*, *T. praeterita*, *T. laeviceps*, *T. pagdeni*, *T. ruficornis* and *Lepidotrigona arcifera* (Rahman *et al.*, 2018). Recently, several new *Tetragonula* species have been described from India, including *T. vikrami*, *T. sumae*, *T. ashishi*, *T. shishirae* and *T. shubhami* (Viraktamath and Thangjam, 2021). However, accurate identification of stingless bee species remains challenging due to high morphological similarity, overlapping diagnostic characters and the presence of cryptic diversity.

DNA-based approaches have become valuable tools for resolving taxonomic uncertainties and understanding genetic diversity in stingless bees. DNA barcoding using mitochondrial cytochrome c oxidase (*COI*) gene has emerged as a reliable method for species identification and discrimination of closely related taxa (Hebert *et al.*, 2003a; Hebert *et al.*, 2003b). The application of *COI* and other mitochondrial markers has successfully revealed cryptic diversity and phylogenetic relationships in several stingless bee groups (Ndungu *et al.*, 2017; Ndungu *et al.*, 2018).

Despite their ecological importance, molecular information on Indian stingless bees remains limited. In Gujarat, previous investigations have mainly focused on morphometric characterization of *Tetragonula* populations (Patel and Pastagia, 2016; Sharma *et al.*, 2023), whereas molecular characterization and DNA barcode resources are lacking. Therefore, the present study was undertaken to generate molecular data for stingless bee populations of Gujarat using mitochondrial *COI* and *COII* gene markers, assess their genetic diversity and phylogenetic relationships, and contribute DNA barcode resources for future taxonomic and conservation studies.

Materials and Method

Survey, Collection and Preservation of Samples

Adult worker bees of *Tetragonula* spp. were collected during 2025-2026 from naturally occurring colonies across seven districts of Gujarat, namely Navsari, Anand, Junagadh, Dantiwada, Tapi, Valsad and Narmada. Approximately 25 workers were collected from the entrance of each colony using sterile plastic vials and immediately preserved in 90% ethanol following the method of Dar and Ahmad (2017). The specimens were

transported to the laboratory and stored at -20°C until molecular analysis.

Genomic DNA extraction

Genomic DNA was extracted from 6-7 worker bees per sample using a modified CTAB (cetyl trimethyl ammonium bromide) protocol. Specimens preserved in ethanol were rinsed with double-distilled water, homogenized in liquid nitrogen and lysed in CTAB extraction buffer containing Tris-HCl, EDTA, NaCl, CTAB, β -mercaptoethanol, PVPP and SDS. Proteinase K treatment, RNase digestion and chloroform:isoamyl alcohol (24:1) extraction were performed, followed by DNA precipitation using chilled isopropanol and sodium acetate. The DNA pellet was washed with absolute ethanol, dissolved in TE buffer and stored at -20°C until further use. The detailed procedure for DNA extraction is illustrated in Fig. 1.

DNA quality assessment and PCR amplification

DNA quality was evaluated by 0.80% agarose gel electrophoresis, while DNA concentration and purity were determined using a NanoDrop spectrophotometer based on the A_{260}/A_{280} ratio. Mitochondrial cytochrome c oxidase subunit I (*COI*) and cytochrome c oxidase

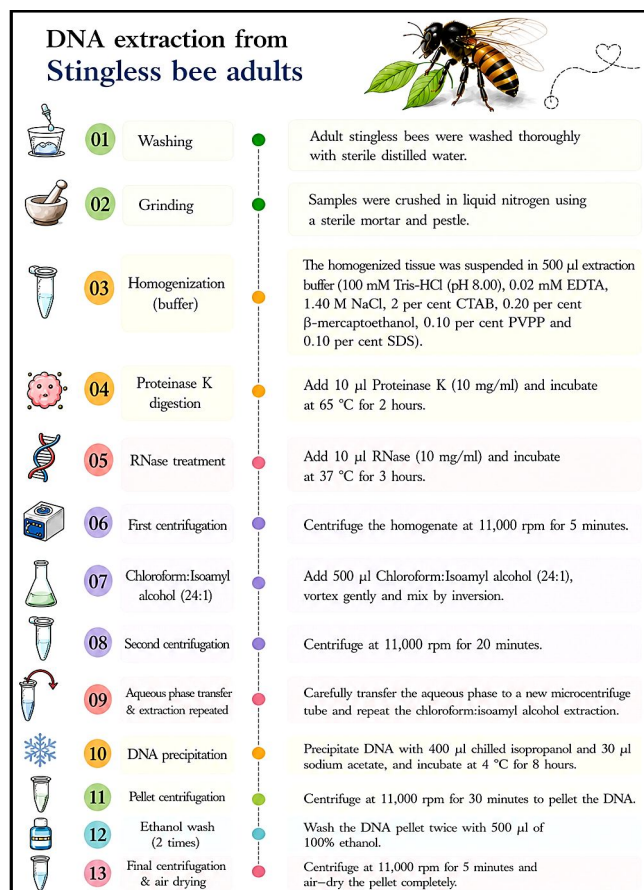


Fig. 1: Flow chart of DNA isolation from *Tetragonula* spp. using modified CTAB method.

Table 1: Details of primers used for molecular study.

Sr.no.	Primer name	Sequences (5'-3')	Annealing temp (°C)	Reference
1.	<i>MCOX-I</i> (Forward)	GGTCAACAAATCATAAAGATATTG	60°C	Lunt <i>et al.</i> , (1996)
2.	<i>MCOX-I</i> (Reverse)	TAAACTTCAGGGTGACCAAAAAATCA		
3.	<i>MCOX-II</i> (Forward)	TACAGTTGGAATAGACGTTGATAC		
4.	<i>MCOX-II</i> (Reverse)	TCCAATGCACTAATCTGCCATATTA		
5.	LepF1	ATTCAACCAATCATAAAGATATTGG	50°C	Sheffield <i>et al.</i> , (2009)
6.	LepR1	TAAACTTCTGGATGTCCAAAAAATCA		

subunit II (*COII*) genes were amplified using published primer pairs (Lunt *et al.*, 1996; Sheffield *et al.*, 2009) (Table 1). PCR amplification was performed in a 60 μ l reaction volume under the following conditions: initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 45 s, with a final extension at 72°C for 7 min. Amplified products were resolved on 1.60% agarose gels stained with ethidium bromide and visualized using a Bio-Rad Gel Documentation System.

DNA sequencing and sequence analysis

PCR products were purified and sequenced using

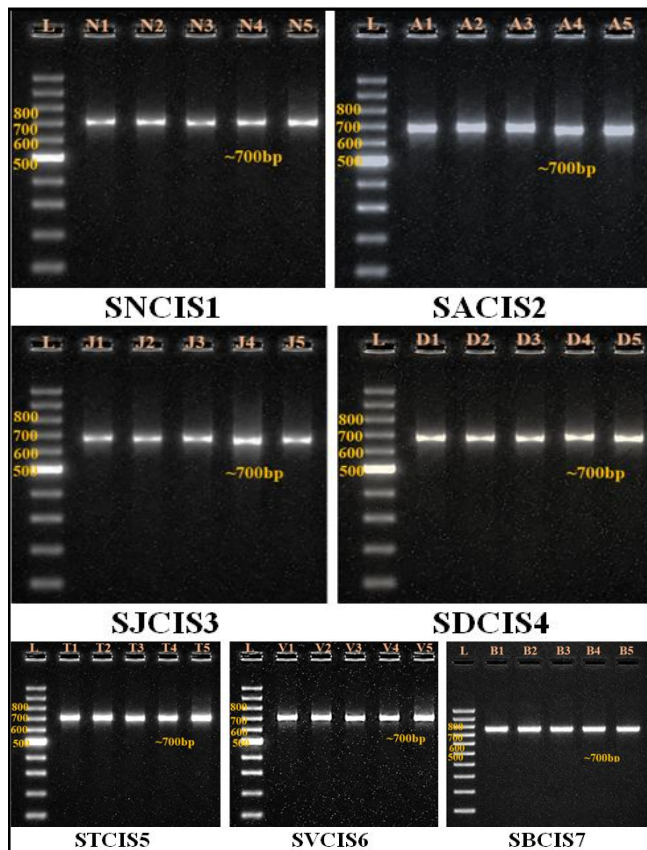


Fig. 2: PCR amplification of *COI* gene of *Tetragonula* spp. collected from Navsari, Anand, Junagadh, Dantiwada, Tapi, Valsad and Narmada location. Representative sample shown: Ladder (L: 100bp), Navsari (N1 to N5), Anand (A1 to A5), Junagadh (J1 to J5), Dantiwada (D1 to D5), Tapi (T1 to T5), Valsad (V1 to V5) and Narmada (B1 to B5).

the Sanger sequencing method on an ABI 3730xl Genetic Analyzer with the BigDye Terminator v3.1 Cycle Sequencing Kit. Sequence chromatograms were edited and assembled using Sequencher, and sequence quality was verified in BioEdit. Sequence identity was confirmed using the BLASTn program, and validated sequences were submitted to the NCBI GenBank database through the Bank It portal. Multiple sequence alignment was performed using ClustalW.

Phylogenetic and genetic diversity analyses

Nucleotide composition (AT and GC content),

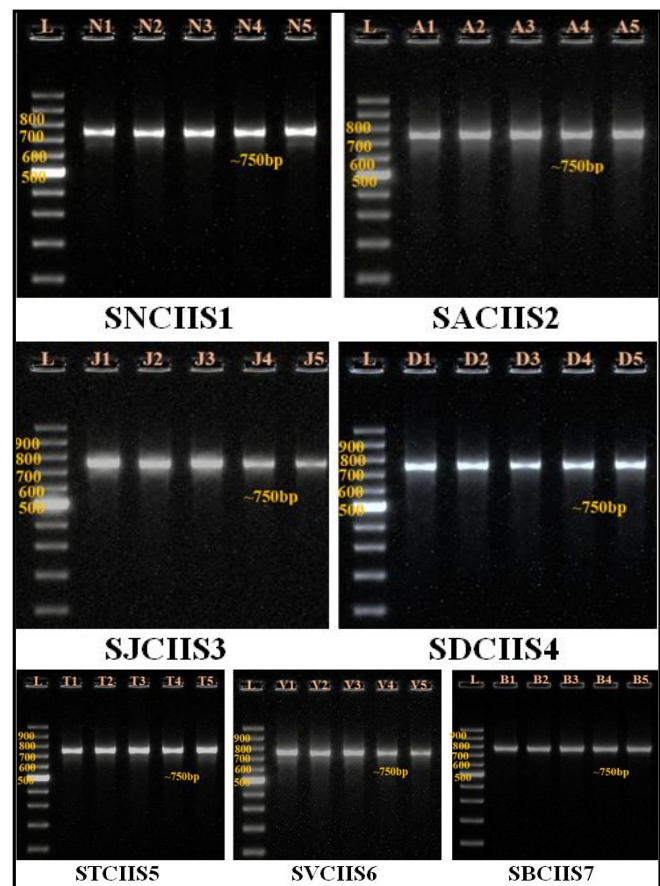
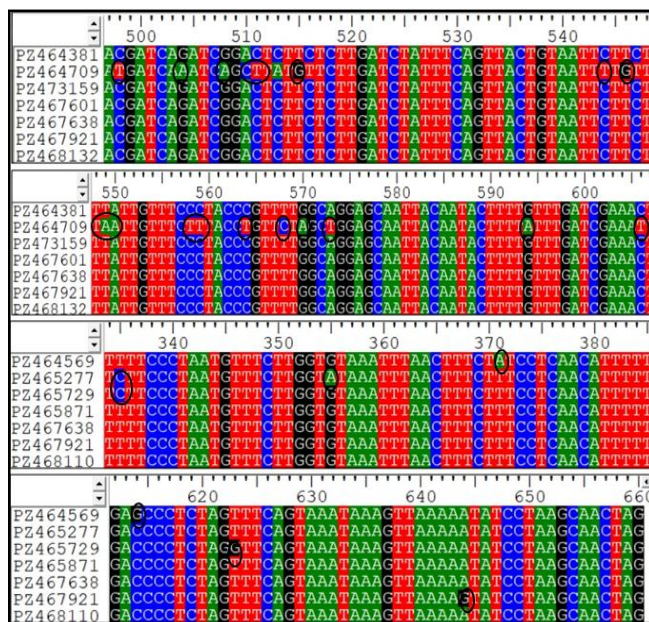


Fig. 3: PCR amplification of *COII* gene of *Tetragonula* spp. collected from Navsari, Anand, Junagadh, Dantiwada, Tapi, Valsad and Narmada location. Representative sample shown: Ladder (L: 100bp), Navsari (N1 to N5), Anand (A1 to A5), Junagadh (J1 to J5), Dantiwada (D1 to D5), Tapi (T1 to T5), Valsad (V1 to V5) and Narmada (B1 to B5).

Table 2: Percentage composition of AT and GC nucleotide in partial *COI*, *COII* gene sequences of *Tetragonula* spp.

Accession no.	% composition of different nucleotide bases					
	T	C	A	G	A+T	G+C
PZ464381.1	40.03	20.09	26.18	13.70	66.21	33.79
PZ464709.1	43.36	16.96	26.11	13.57	69.47	30.53
PZ473159.1	40.03	20.09	26.18	13.70	66.21	33.79
PZ467601.1	40.18	19.94	26.03	13.85	66.21	33.79
PZ467638.1	39.73	20.24	26.33	13.70	66.06	33.94
PZ467921.1	39.88	20.09	26.48	13.55	66.36	33.64
PZ468132.1	39.73	20.09	26.48	13.70	66.21	33.79
PZ464569.1	37.27	19.09	28.18	15.45	65.45	34.55
PZ465277.1	36.97	19.55	28.48	15.00	65.45	34.55
PZ465729.1	36.97	19.39	28.03	15.61	65.00	35.00
PZ465871.1	37.27	19.24	28.18	15.30	65.45	34.55
PZ465958.1	37.12	19.24	28.03	15.61	65.15	34.85
PZ467700.1	36.92	19.23	28.15	15.69	65.08	34.92
PZ468110.1	37.03	19.06	28.28	15.63	65.31	34.69
Average	38.76	19.45	27.22	14.57	65.97	34.03

barcode gap analysis and pairwise genetic distances were estimated from the aligned *COI* and *COII* sequences. Phylogenetic trees were constructed in MEGA 12 using the Maximum Likelihood method with the Kimura two-parameter (K2P) substitution model and 1,000 bootstrap replications. Pairwise genetic distance matrices were generated in MEGA 12, and lower triangular heatmaps illustrating genetic divergence among populations were prepared using R Studio. DNA barcode records, together with specimen metadata and sequence information, were submitted to the Barcode of Life Data System (BOLD).

**Fig. 4:** SNP variation for Barcode gap analysis of mitochondrial *COI* and *COII* sequences of *Tetragonula* spp. collected from different agro-climatic zone of Gujarat.

Result and Discussion

Survey, Collection and Preservation of Samples

Stingless bee (*Tetragonula* spp.) worker specimens were collected during 2025-2026 from seven agro-climatic regions of Gujarat, namely Navsari, Anand, Junagadh, Dantiwada, Tapi, Valsad and Narmada, GPS coordinates were recorded. Feral colonies were found inhabiting diverse nesting sites, including tree trunks, rock crevices, mud pots and collapsed stone walls, demonstrating their adaptability to both natural and artificial habitats. The entrance tubes of all colonies were observed to be short, irrespective of their geographical location. Similar nesting preferences and colony characteristics in stingless bees have been reported by Rahman *et al.*, (2018), Singh and Khan (2019) and Jarpla (2025). The collected specimens were preserved in alcohol and used for molecular characterization.

Genomic DNA Extraction, Quality and Quantity Assessment

High-quality genomic DNA was successfully extracted from *Tetragonula* worker bees using the modified CTAB method. Agarose gel electrophoresis showed intact DNA bands with negligible degradation. The extracted DNA showed A_{260}/A_{280} ratios ranging from 1.78 to 1.92, confirming good purity and suitability for PCR amplification and downstream molecular analyses. Whereas highest concentration recorded from Tapi (1486.50 ng/μl) and the lowest from Junagadh (663.80 ng/μl). Similar CTAB-based DNA extraction approaches have been successfully used for molecular characterization of insects (Rana, 2019; Pathak, 2025).

PCR Amplification and DNA Sequencing

The mitochondrial *COI* and *COII* gene regions were successfully amplified from all seven geographical isolates, producing amplicons of ~700 bp and ~750 bp, respectively (Fig. 2 and Fig. 3). Clear amplification without non-specific products confirmed the efficiency of the selected primers. Similar PCR amplification and

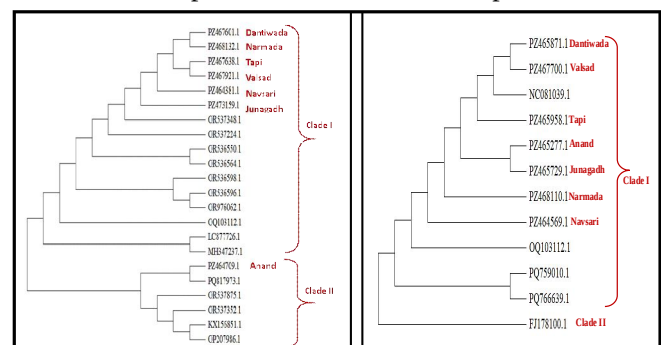
**Fig. 5:** Neighbor-Joining (NJ) phylogenetic tree, based on partial mitochondrial *COI* and *COII* gene sequences.

Table 3: Genetic distance matrix of different *Tetragonula* spp. individuals based on partial mitochondrial COI gene sequences.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
A																						
B	0.20																					
C	0.00	0.20																				
D	0.00	0.21	0.00																			
E	0.00	0.21	0.00	0.01																		
F	0.00	0.21	0.00	0.01	0.00																	
G	0.00	0.21	0.00	0.01	0.01	0.01																
H	0.00	0.20	0.00	0.00	0.00	0.00	0.00															
I	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00														
J	0.02	0.20	0.02	0.02	0.02	0.02	0.02	0.02	0.02													
K	0.25	0.15	0.25	0.26	0.26	0.26	0.26	0.25	0.25	0.25												
L	0.02	0.20	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.25											
M	0.04	0.20	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.25	0.04										
N	0.04	0.20	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.25	0.04	0.00									
O	0.04	0.20	0.04	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.25	0.04	0.01	0.01								
P	0.19	0.16	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.18	0.22	0.18	0.19	0.19	0.19							
Q	0.25	0.14	0.25	0.26	0.26	0.26	0.26	0.25	0.25	0.25	0.00	0.25	0.25	0.25	0.25	0.22						
R	0.20	0.01	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.19	0.14	0.19	0.19	0.19	0.19	0.15	0.13					
S	0.18	0.14	0.18	0.19	0.19	0.19	0.19	0.18	0.18	0.18	0.24	0.18	0.18	0.17	0.18	0.09	0.24	0.13				
T	0.24	0.15	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.01	0.23	0.24	0.24	0.25	0.22	0.00	0.14	0.23			
U	0.19	0.17	0.19	0.20	0.20	0.20	0.20	0.19	0.19	0.20	0.06	0.20	0.20	0.20	0.23	0.23	0.05	0.16	0.23	0.05		
V	0.08	0.19	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.10	0.26	0.10	0.10	0.10	0.12	0.15	0.26	0.18	0.15	0.24	0.19	

A = PZ464381.1, B = PZ464709.1, C = PZ473159.1, D = PZ467601.1, E = PZ467638.1, F = PZ467921.1, G = PZ468132.1, H = OR537348.1, I = OR537224.1, J = OR536550.1, K = KX156851.1, L = OR536564.1, M = OR536596.1, N = OR536598.1, O = OR976062.1, P = LC877726.1, Q = OP207986.1, R = PQ817973.1, S = MH347237.1, T = OR537352.1, U = OR537875.1 and V = OQ103112.1.

sequencing results have been reported for stingless bees (Vamshikrishna, 2021) and bumble bees (Rana, 2019). The amplified products were sequenced using the Sanger method, and high-quality consensus sequences were generated for further analysis.

Sequence Homology Analysis using BLASTn

BLASTn analysis of the *COI* and *COII* sequences confirmed the identity of *Tetragonula* specimens from Gujarat. *COI* sequences from Navsari, Junagadh, Dantiwada, Tapi, Valsad and Narmada showed 99-100% similarity with *T. iridipennis*, whereas the Anand isolate showed the highest similarity (99.41%) with *T. shubhami* (PQ817973.1), indicating a distinct species. In contrast, *COII* sequences of all populations showed high similarity (99.20-99.80%) with *T. iridipennis*. The discrepancy between *COI* and *COII* may be attributed to the conserved nature of the *COII* region and limited reference sequences for *COII* region of *T. shubhami* in NCBI GenBank. Similar marker-specific variation has been reported by Singh (2020) and Pathak (2025), while the effectiveness of BLASTn for species identification was demonstrated by Hebert *et al.*, (2003a).

Estimation of Nucleotide Composition

Nucleotide composition analysis revealed an AT-rich mitochondrial genome in the studied *Tetragonula* populations. The *COI* sequences showed AT content ranging from 66.06 to 69.47%, whereas *COII* sequences exhibited 65.00 to 65.45% AT content, with the highest AT content recorded in the Anand *COI* sequence (69.47%) (Table 2). The higher proportion of adenine and thymine compared to guanine and cytosine indicates a conserved mitochondrial genome structure, which is a characteristic feature of insect mitochondrial genomes. Similar AT-rich nucleotide composition has been reported by Pathak (2025) in *Spodoptera litura* and Singh (2020) in *Apis cerana*. The uniform nucleotide composition further supports the conserved nature of mitochondrial gene regions and their utility in comparative genetic studies.

Barcode Gap Analysis

COI and *COII* gene sequences of *Tetragonula* populations from different agro-climatic regions of Gujarat were aligned using the ClustalW algorithm in BioEdit v7 (Hall, 1999) to assess genetic variation. Both gene regions showed high sequence conservation with limited SNPs. The Anand *COI* isolate (PZ464709.1) exhibited

Table 4: Genetic distance matrix of different *Tetragonula* spp. individuals based on partial mitochondrial COII gene sequences.

	A	B	C	D	E	F	G	H	I	J	K	L
A												
B	0.01											
C	0.01	0.01										
D	0.01	0.01	0.01									
E	0.01	0.01	0.00	0.00								
F	0.01	0.01	0.01	0.00	0.00							
G	0.01	0.01	0.01	0.01	0.00	0.01						
H	0.00	0.01	0.00	0.00	0.00	0.00	0.00					
I	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00				
J	0.07	0.07	0.06	0.06	0.06	0.06	0.06	0.04	0.15			
K	0.07	0.07	0.06	0.06	0.06	0.06	0.06	0.04	0.15	0.00		
L	0.99	0.98	0.99	0.97	0.99	0.99	0.98	0.99	0.99	1.03	1.03	

Where, A = PZ464569.1, B = PZ465277.1, C = PZ465729.1, D = PZ465871.1, E = PZ465958.1, F = PZ467700.1, G = PZ468110.1, H = OQ103112.1, I = NC_081039.1, J = PQ759010.1, K = PQ766639.1 and L = FJ178100.1.

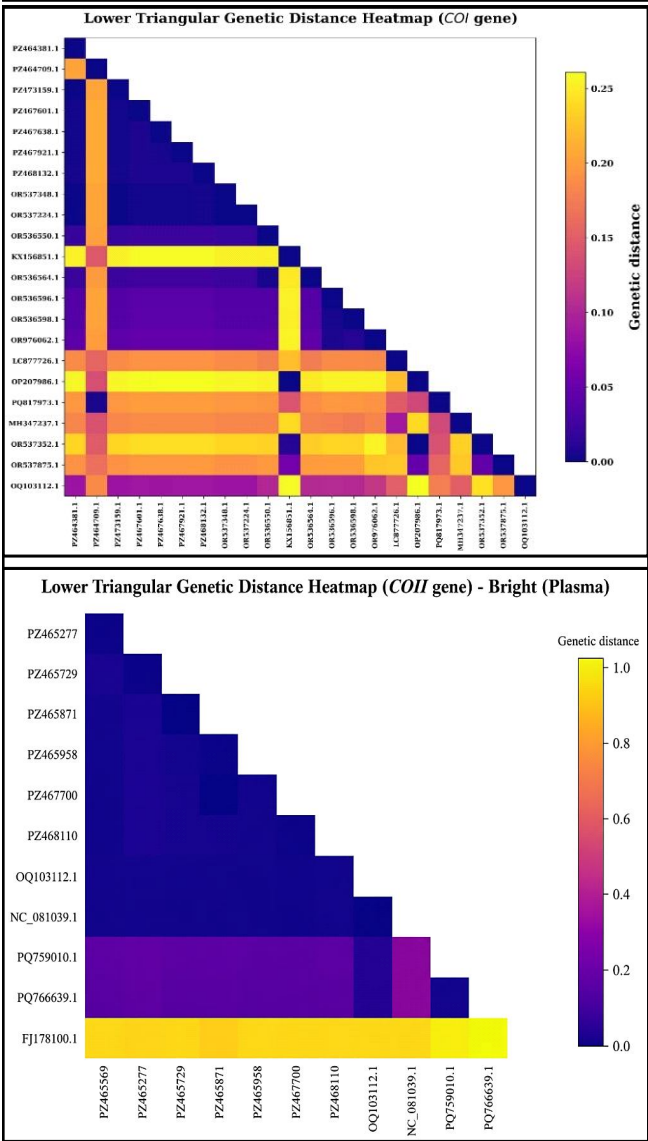


Fig. 6: Heatmap of pairwise genetic distances among *Tetragonula* spp. based on *COI* and *COII* gene sequences.

comparatively higher nucleotide substitutions, while *COII* sequences showed only few scattered variations among populations (Fig. 4). The low level of sequence divergence indicates limited intraspecific variation and genetic similarity among *Tetragonula* populations. Similar low barcode divergence has been reported in insects, confirming the effectiveness of mitochondrial markers for species identification and genetic analysis (Hebert *et al.*, 2003; Pathak, 2025).

Phylogenetic Analysis and Genetic Distance

Phylogenetic analysis based on *COI* sequences separated the populations into two major clades. Navsari, Junagadh, Dantiwada, Tapi, Valsad and Narmada clustered with *T. iridipennis* reference sequences retrieved from GenBank, whereas Anand formed a separate cluster with *T. shubhami*. *COII* analysis showed

Table 5: List of accession numbers and process ID generated for the sequences deposited in NCBI and BOLD respectively.

Sr. no.	Sample ID	Gene	Accession number	Process ID (BOLD)
1	SNCIS1	<i>COI</i>	PZ464381.1	SDP1222023-26
2	SACIS2	<i>COI</i>	PZ464709.1	SDP1222037-26
3	SJCIS3	<i>COI</i>	PZ473159.1	SDP1222027-26
4	SDCIS4	<i>COI</i>	PZ467601.1	SDP1222029-26
5	STCIS5	<i>COI</i>	PZ467638.1	SDP1222031-26
6	SVCIS6	<i>COI</i>	PZ467921.1	SDP1222032-26
7	SBCIS7	<i>COI</i>	PZ468132.1	SDP1222035-26
8	SNCOIIS1	<i>COII</i>	PZ464569.1	SDP1222024-26
9	SACOIIS2	<i>COII</i>	PZ465277.1	SDP1222038-26
10	SJCOIIS3	<i>COII</i>	PZ465729.1	SDP1222028-26
11	SDCOIIS4	<i>COII</i>	PZ465871.1	SDP1222030-26
12	STCOIIS5	<i>COII</i>	PZ465958.1	SDP1222034-26
13	SVCOIIS6	<i>COII</i>	PZ467700.1	SDP1222033-26
14	SBCOIIS7	<i>COII</i>	PZ468110.1	SDP1222036-26

closer clustering of all Gujarat populations with *T. iridipennis*, indicating the conserved nature of this marker (Fig. 5). Similar phylogenetic patterns have been reported in other *Tetragonula* studies (Vamshikrishna, 2021).

Pairwise genetic distances based on the *COI* gene ranged from 0.00 to 0.21, Anand population exhibited comparatively higher genetic distances (0.21) from the remaining Gujarat populations, reflecting its distinct phylogenetic placement (Table 3). Similarly, *COII*-based genetic distances ranged from 0.003 to 0.011, indicating low genetic divergence and high mitochondrial sequence conservation across the studied populations (Table 4). Lower triangular heatmaps generated from pairwise distance matrices visually demonstrated the close genetic relationships among populations and highlighted comparatively higher divergence in the Anand population (Fig. 6). Similar patterns of low mitochondrial genetic variation and heatmap representation were reported by (Pathak, 2025).

BOLD and GenBank Submission

All *COI* and *COII* sequences generated in the present study were successfully submitted to the NCBI GenBank and the Barcode of Life Data System (BOLD), along with specimen metadata, taxonomic information, collection details, specimen images and trace files. A total of 14 sequences (7 *COI* and 7 *COII*) were deposited and assigned unique GenBank accession numbers and BOLD Process IDs (Table 5). As of 17 June 2025, the BOLD database contained 50 records of *T. iridipennis*, including 12 sequences generated in the present study, while only one record of *T. shubhami* was available, to which this study contributed two additional barcode sequences. These submissions represent the first DNA barcode records of *Tetragonula* spp. from Gujarat in BOLD and provide valuable molecular resources for future taxonomic, phylogenetic and biodiversity studies. Similar barcode submissions have been reported by Arunima (2017), Namitha (2020) and Pathak (2025).

Conclusion

The present study successfully characterized *Tetragonula* spp. populations from seven agro-climatic regions of Gujarat using mitochondrial *COI* and *COII* gene markers. High-quality genomic DNA enabled successful PCR amplification, Sanger sequencing and generation of reliable consensus sequences. BLASTn analysis confirmed the identity of most populations as *T. iridipennis*, whereas the Anand population showed the highest sequence similarity with *T. shubhami*. Nucleotide composition analysis revealed an AT-rich mitochondrial genome, while barcode gap analysis indicated high

sequence conservation with limited intraspecific variation. Phylogenetic analysis, pairwise genetic distance and heatmap visualization further supported the close genetic relationship among Gujarat populations and distinguished the Anand population based on the *COI* gene. The generated sequences were successfully submitted to NCBI GenBank and the Barcode of Life Data System (BOLD), representing the first DNA barcode records of *Tetragonula* spp. from Gujarat. Overall, the study demonstrates the usefulness of mitochondrial *COI* and *COII* markers for species identification, molecular characterization and genetic diversity studies in stingless bees, while providing molecular evidence for the possible occurrence of *Tetragonula shubhami* in Gujarat.

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