

TAXONOMIC STATUS OF STINGLESS BEE *LEPIDOTRIGONA ARCIFERA* (HYMENOPTERA: MELIPONINI) FROM THE SOUTHWEST CHINA - TIBETAN PLATEAU

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Abstract

The stingless bee, *Lepidotrigona arcifera*, which was first reported in northeast India is a pollinating insect of the tropical and subtropical regions of the world. However, little information about this species is known in terms of taxonomic status as well as detailed morphological characteristics. In this study, we discovered a stingless bee in Tibet that belongs to an Alpine climate region. The morphological comparison with the India stingless bee, confirms that this newly discovered species is *L. arcifera* from the Tibet plateau. To determine the taxonomic status of this Tibetan stingless bee, we sequenced the 16S rRNA and COI genes of samples from a dozen colonies and compared them to some other known bee species. Phylogenetic analysis shows that Tibetan *L. arcifera* is grouped with the other stingless bee species and most closely sistered to the stingless bee, *L. flavibasis*. The genetic distance between Tibetan *L. arcifera* and the other stingless bee species is approaching those between pairwise bee species belonging to other such bee genera as *Bombus* and *Apis*. Hence, our results not only offer new information on species distribution and morphological characteristics of *L. arcifera* but also confirm its taxonomic status among stingless bees for the first time, which will contribute to understanding the biodiversity of stingless bees in the future.

Keywords: COI, *Lepidotrigona arcifera*, phylogenetic relationship, stingless bee, 16S rRNA

INTRODUCTION

Stingless bees are social organisms that inhabit tropical and subtropical areas worldwide (Viraktamath & Roy, 2022; Viraktamath & Thangjam 2022). Southern China is the northern border of Asia in which this species lives, including Yunnan, Hainan, and Taiwan (Pan et al., 2019; Wang et al., 2020). Stingless bees are

a large monophyletic group comprising about 60 genera and 550 identified species (Rasmussen & Cameron, 2010; Michener, 2012; Li et al., 2021); however, many species remain undescribed (Gonzalez & Engel, 2012; Karthick et al., 2018; Makkar et al., 2018).

Trigona, the largest group, and *Melipona* are two genera of the Meliponini tribe (Vijayakumar & Jeyaraaj, 2014; Tej et al., 2017). The genus *Trigona*

can be divided into ten subgenera, among which *Lepidotrigona*, *Homotrigona*, and *Heterotrigona* occur in tropical and subtropical areas of Asia (Michener, 1990; Vijayakumar & Jayaraj, 2013). Previous studies consider that *Lepidotrigona* includes at least five species, which are *L. arcifera*, *L. ventralis*, *L. flavibasis*, *L. doipaensis*, and *L. hoozana* (Vijayakumar, 2014; Attasopa et al., 2018). However, due to few molecular evidence and the problem of sample collection (Nidup, 2021), researchers are usually confused with the morphological and taxonomic classification of *Lepidotrigona* species. Schwarz hypothesized that *L. arcifera* (Cockerell) from Testa Bridge in the Himalayas is synonymous with *L. flavibasis* (Schwarz, 1939), while Moure thought that the type of *L. arcifera* belongs to *L. hoozana* (Moure, 1961). Up to date, only nine stingless bee species have been reported in China, including *Lepidotrigona flavibasis*, *Lepidotrigona terminate*, *Tetragonilla collina*, *Tetragonilla lutea*, and *Lisotrigona carpenter* (Zheng et al., 2022). Notably, the stingless bee, *L. arcifera*, has not yet been discovered in China, due to its small size and modest morphological differences with other stingless bees. Furthermore, the evolutionary relationship between *L. arcifera* and the other stingless bees in *Lepidotrigona* genus is unclear, which restricts the general recognition of this species. Mitochondrial molecular markers usually exhibit matrilineal inheritance, which provides useful information about the evolution and phylogeny of the bee species (O'Malley, 2015). Among mitochondrial markers, the 16S rRNA gene and COI gene are highly conserved and usually used to study inter-species genetic differences and taxonomic status (Xu, 2006; Xu et al., 2019). In this study, we discovered the colonies of undetermined stingless bee from Tibetan plateau. A detailed morphometric analysis was conducted to determine whether this stingless bee belongs to *L. arcifera*. 16S

rRNA and COI genes of twelve colonies from *L. arcifera* were further sequenced to confirm its the evolutionary status in relation to the other stingless bees. Our results guide the conservation and exploitation of this particular stingless bee in Asia.

MATERIAL AND METHODS

Sample collection

Colonies of Tibetan stingless bees were typically observed nesting in withered stumps and constructing tubular entrances with beeswax. Based on these observations, the twelve colonies of stingless bees were discovered in Damu Village in Tibet, China (29°29'54.41"N 95°27'39.18"E, Fig. 1). The bee worker samples from each of the twelve colonies were separately collected and observed under the dissecting microscope. The samples were kept in 75% ethanol for subsequent DNA extraction.

The tissue DNAs of twelve samples derived from twelve colonies were extracted separately using the CTAB (cetyltrimethylammonium bromide) method (Shi et al., 2020). Using NCBI Primer-BLAST tools (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>), two pairs of specific primers across the complete sequence of the reference mitochondrial 16S rRNA gene (NC_026198.1)

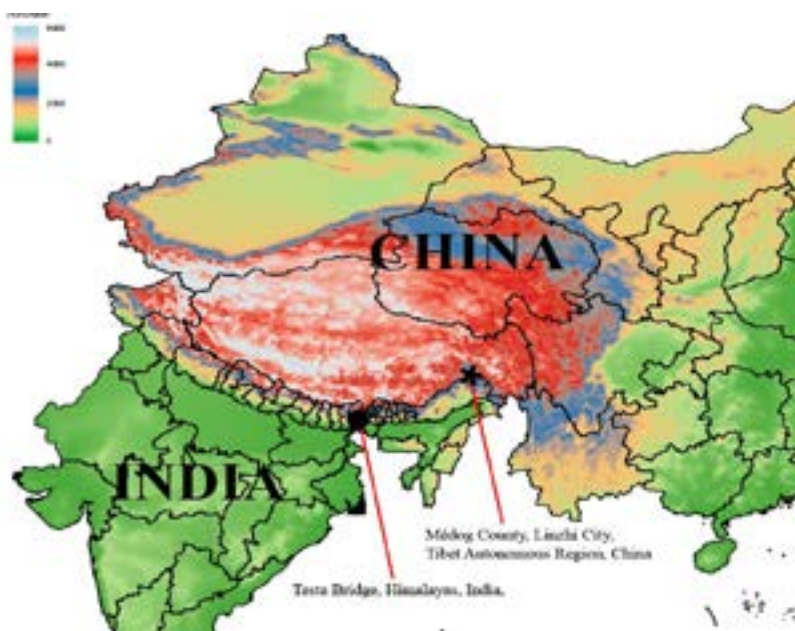


Fig. 1. The location marked with an asterisk is the sampling sites of the samples used in this study, and the circles are the geographical location of the reported *L. arcifera* (Cockerell 1929).

and COI gene (MN737481.1) were designed for synthesis. The primer sequences were as follows: 16S rRNA-F: GCAACTGCATTTTATGATATGTTTTACCATGAGGAC, 16S rRNA-R: GTGCCAGCAATAGCTGTAACTTA. COI-F: GCAAGTTTAAAGAAATGGGCAAATAATTAATTTAG, COI-R: GTATTGGAATGATAGTTCATAAAATTTCCACAG. Given the presence of high AT content for reference sequences, the PCR primers were extended to sufficient length to reach the 60°C annealing temperature. PCR amplification was carried out with a total 20 µL reaction volume that consisted of 0.5 µL (10 µM) primers, 200 ng template DNA, 0.5 µL (1.25 U/µL) Tks Gflex DNA polymerase, 10 µL 2x Gflex PCR Buffer and ddH₂O replenishment. The following PCR procedure was set as: 1) 94°C for 3 min; 2) 45 cycles of 10 s at 98°C, annealing for 15 s at 58-60°C and then 3 min at 68°C; 3) final extension for 10 min at 68°C. After the DNA electrophoresis, the PCR target products were retrieved and sent to Sangon Biotech for DNA sequencing. Then, SeqMan7.1.0(44.1) was used to assemble the sequencing results. The twelve 16S rRNA and COI sequences obtained in this study were deposited to the GenBank database with accession No.OR563636 - OR563647 and No.PP916710 - PP916721, respectively.

Bioinformatic and phylogenetic analyses

MUMmer4 (Marçais et al., 2018) was used for genomic collinearity analysis and determination of single nucleotide polymorphisms (SNPs) among the 16S rRNA sequences from twelve colonies of Tibetan stingless bees. The pairwise comparison of genetic distance between bee species was performed with the use of MEGA 7.0 (Kumar et al., 2016). Both neighbor-joining and maximum-likelihood trees with 16S rRNA were constructed using IQTREE, and the bootstrap value was calculated from 1,000 replicates (Minh et al., 2020). To build the phylogenetic tree, published complete mitochondrial genomes of closely related bee species were retrieved from the GenBank database, including *Lepidotrigona flavibasis* (MN747147), *Lepidotrigona terminata* (MN737481), *Tetragonula pagdeni* (NC_066054), *Melipona scutellaris* (KP202303), *Melipona bicolor*

(AF466146), *Melipona fasciculata* (MH680930), *Bombus ignites* (DQ870926), *Bombus canariensis* (MW959771), *Bombus longipennis* (NC_057952), *Apis laboriosa* (NC_036155), *Apis dorsata* (NC_037709), *Apis florea* (KC170303), *Apis andreniformis* (NC_039709), *Apis mellifera ligustica* (L06178), *Apis mellifera intermissa* (KM458618), *Apis mellifera syriaca* (KP163643), *Apis mellifera scutellata* (KJ601784), *Apis mellifera capensis* (KX870183), *Osmia excavata* (KX494106), *Osmia pedicornis* (MT849323), *Megachile sculpturalis* (KT223644), *Megachile strupigera* (KT346366), *Drosophila melanogaster* (NC_024511), with *Drosophila melanogaster* (NC_024511) as an outgroup.

We also analyzed COI sequences from twelve colonies of Tibetan stingless bees with the same method mentioned above. Published COI genes of closely related bee species were retrieved from the GenBank database, including *Lepidotrigona arcifera* (MH347232.1), *Lepidotrigona flavibasis* (MN747147), *Lepidotrigona terminata* (MN737481), *Tetragonula pagdeni* (NC_066054), *Melipona bicolor schencki* (KC853337.1), *Melipona bicolor* (AF466146), *Melipona scutellaris* (NC_026198), *Apis laboriosa* (MH138079.1), *Apis mellifera ligustica* (AY114452.1), *Apis dorsata* (AF153113.1), *Apis florea* (MH138080.1), *Apis andreniformis* (MH138078.1), *Osmia pedicornis* (KC709846.1), *Osmia excavata* (KC709837.1), *Megachile strupigera* (KT346366), *Megachile sculpturalis* (KM585660.1), with *Drosophila melanogaster* (LC025528.1) as an outgroup.

RESULTS

Nest construction

We observe that these Tibetan wild stingless bees usually construct their nests in the holes of dead tree trunks, and a tubular entry ranging from 5 to 10 cm always appears in the nest (Fig. 2A, Fig. 2B). This particular entrance of stingless bees is commonly called a “trumpet mouth” in view of its appearance. Inside the hive, the brood and food storage areas are located in the center and separated by layer (Fig. 2C). The loose vertical layered structure made of beeswax sheets

surrounds the brood area and food storage tanks (Fig. 2C). We suspect that the peripheral sheets may act as a heat and moisture barrier to protect the beehive against the external environment.

Morphological characterization

The morphological traits of Tibetan stingless bee workers are shown detailedly in Fig. 3. The dorsal view of the bee, shown in Fig. 3A, demonstrates the following traits: the body length approximately 4 mm; the back of the thorax roughened by an

incredibly dense tessellation (yellow scales); the reddish-brown metasoma; completely black head, antenna and mesosoma. The wings are bright and iridescent (Fig. 3B). The width of the head is approximately 1.8 mm (Fig. 3C), and tiny teeth grow on the mandible (Fig. 3D). The basitarsus of the hind leg is brown, and the hair fringe on the back of the hind tibiae is plain and not branching or plumose (Fig. 3E). The forewing length is around 4.5 mm (Fig. 3F). The hind wings have six hamuli (Fig. 3G).



Fig. 2. Nest and the entrance of *L. arcifera*. Front view of the entrance tube (A), side view of the entrance tube (B), the nest (C).

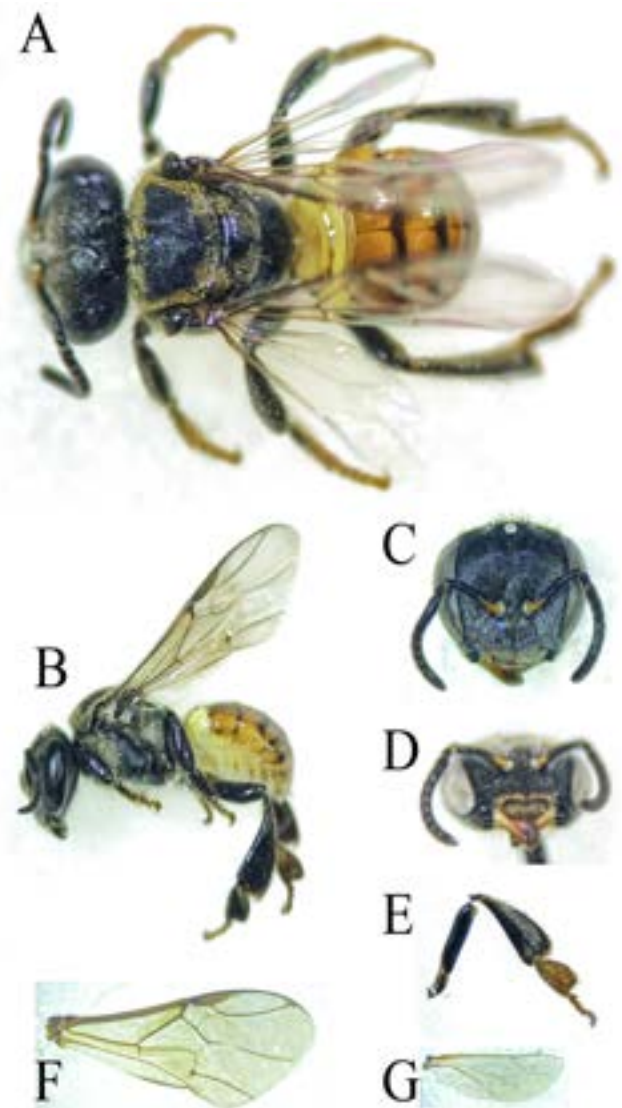


Fig. 3. The physical traits of Tibetan stingless bee workers in whole and part. Dorsal view of the whole body (A), lateral habitus of the whole body (B), frontal view of head (C), lower frontal view of the head, including mandibles (D), inner view of the right hind leg (E), right forewing (F), right hindwing (G).

We compared several morphological characteristics to those described previously for the Indian stingless bee *L. arcifera* (Cockerell, 1929; Rahman et al., 2015). Our samples shared the identical morphological indexes, including size, bottom leg colour, wing pattern, two enormous brown subbasal patches, and slender brown eyes. Based on the above morphological comparison, we concluded that the stingless bees observed in this study are the species *L. arcifera*.

Sequences variation of 16S rRNA and COI sequences

The diagram of the multiple alignments of 16S rRNA sequences showed high similarity among all the samples from twelve colonies (more than 99% identities at the level of nucleic acids). Only five single nucleotide polymorphs occurred (Fig. 4). The genetic distance of the species as well as subspecies based on 16S rRNA was calculated and shown in Fig. 5A. The value of the mean genetic distance between species *L. arcifera* and any of the other stingless bees was 0.1689, which is higher than the mean distance between the interspecies of genus *Apis* (0.1298), *Osmia* (0.0899) as well as *Bombus* (0.0959), but is slightly lower than that of genus *Megachile* (0.1865). Within the genus *Trigona*, *L. arcifera* shows the

smallest genetic distance to *L. flavibasis* (0.0679) of all the stingless bees, implying that these two species have a closer relationship.

The diagram of the multiple alignments of COI sequences also showed high similarity among all the samples from twelve colonies (more than 99% identities at the level of nucleic acids), and fourteen single nucleotide polymorphs occurred (Fig. 6). The genetic distance of the species as well as subspecies based on COI was calculated and shown in Fig. 7A. The value of the mean genetic distance between species *L. arcifera* and any of the other stingless bees was 0.1760, which is higher than the mean distance between the interspecies of genus *Apis* (0.0916), *Osmia* (0.0634) as well as *Megachile* (0.1094). Within the genus *Trigona*, *L. arcifera* from Tibet shows the smallest genetic distance to *L. arcifera* (0.1045) from the GenBank database of the stingless bees.

Phylogenetic analysis of 16S rRNA and COI sequences

With the use of *Drosophila melanogaster* as an outgroup, the phylogenetic tree based on 16S rRNA sequences was constructed as shown in Fig. 5B. The species *L. arcifera*, *L. terminata*, *L. flavibasis*, *T. pagdeni* constitute a separated *Trigona* clade that sistered to the clades of

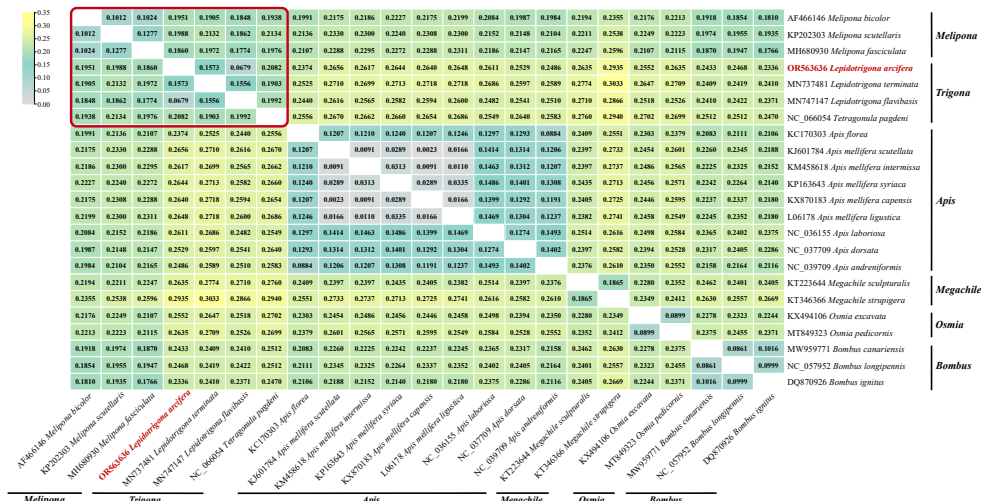
Snp Loci	277	454	736	1113	1114	1115	1322					
OR563636	...	A	...	G	...	T	...	T	T	...	G	...
OR563637	...	A	...	T	...	T	...	T	T	...	T	...
OR563638	...	A	...	T	...	T	...	T	T	...	T	...
OR563639	...	A	...	G	...	T	...	T	T	...	G	...
OR563640	...	A	...	G	...	T	...	T	T	...	G	...
OR563641	...	A	...	G	...	T	...	T	T	...	G	...
OR563642	...	A	...	G	...	T	...	—	—	...	G	...
OR563643	...	A	...	G	...	T	...	T	T	...	G	...
OR563644	...	—	...	G	...	T	...	T	T	...	T	...
OR563645	...	A	...	G	...	T	...	T	T	...	G	...
OR563646	...	A	...	G	...	T	...	T	T	...	G	...
OR563647	...	A	...	G	...	—	...	T	T	...	T	...
Consensus	...	A	...	G	...	T	...	T	T	...	G	...

Fig. 4. The diagram of the multiple sequence alignment of 16S rRNA genes from twelve colonies and generation of a consensus sequence. (The dots represent the same bases, and the dashes represent the missing bases.)

Melipona genus. Within the *Trigona* clade, *L. arcifera* is closely grouped with *L. flavibasis*, suggesting that *L. arcifera* is more closely related to *L. flavibasis* than the other *Trigona* species. This result is consistent with the conclusion drawn by the genetic distance calculation above.

Using *Drosophila melanogaster* as an outgroup, the phylogenetic tree based on COI sequences was constructed as shown in Fig. 7B. The species *L. arcifera* from Tibet, *L. arcifera* from northeast India, *L. terminata*, *L. flavibasis*, *T. pagdeni* constitute a separated *Trigona* clade that sistered to the clades of *Melipona*

A



B

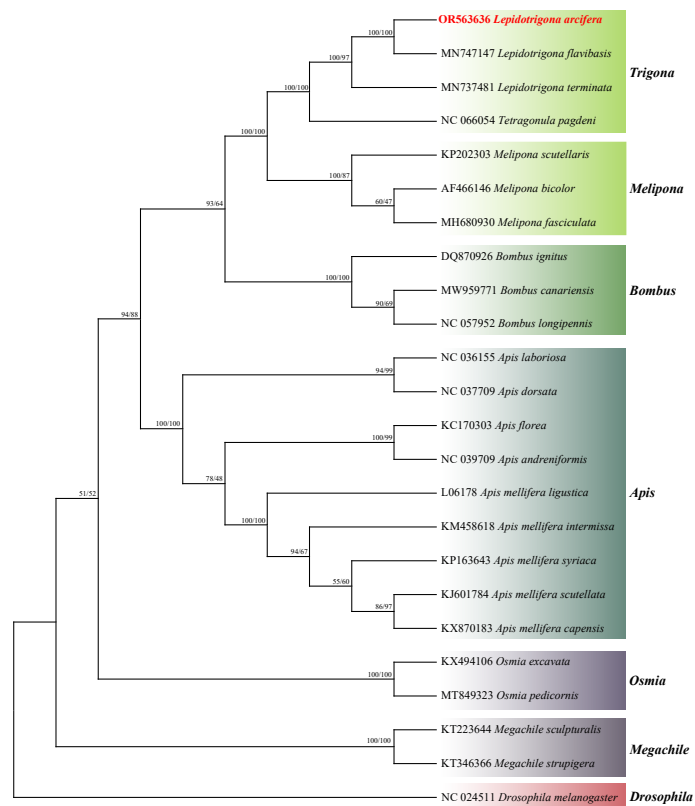


Fig. 5. Pairwise comparison of genetic distance based on 16S rRNA gene (A), the red boxes are labeled with genetic distances between stingless bee genera. Phylogenetic relationship based on 16S rRNA gene (B), bootstrap values present in the above branch using neighbor-joining and maximum-likelihood methods, the species *Drosophila melanogaster* was used as the outgroup.



Fig. 6. The diagram of the multiple sequence alignment of COI genes from twelve colonies and generation of a consensus sequence. (The dots represent the same bases).

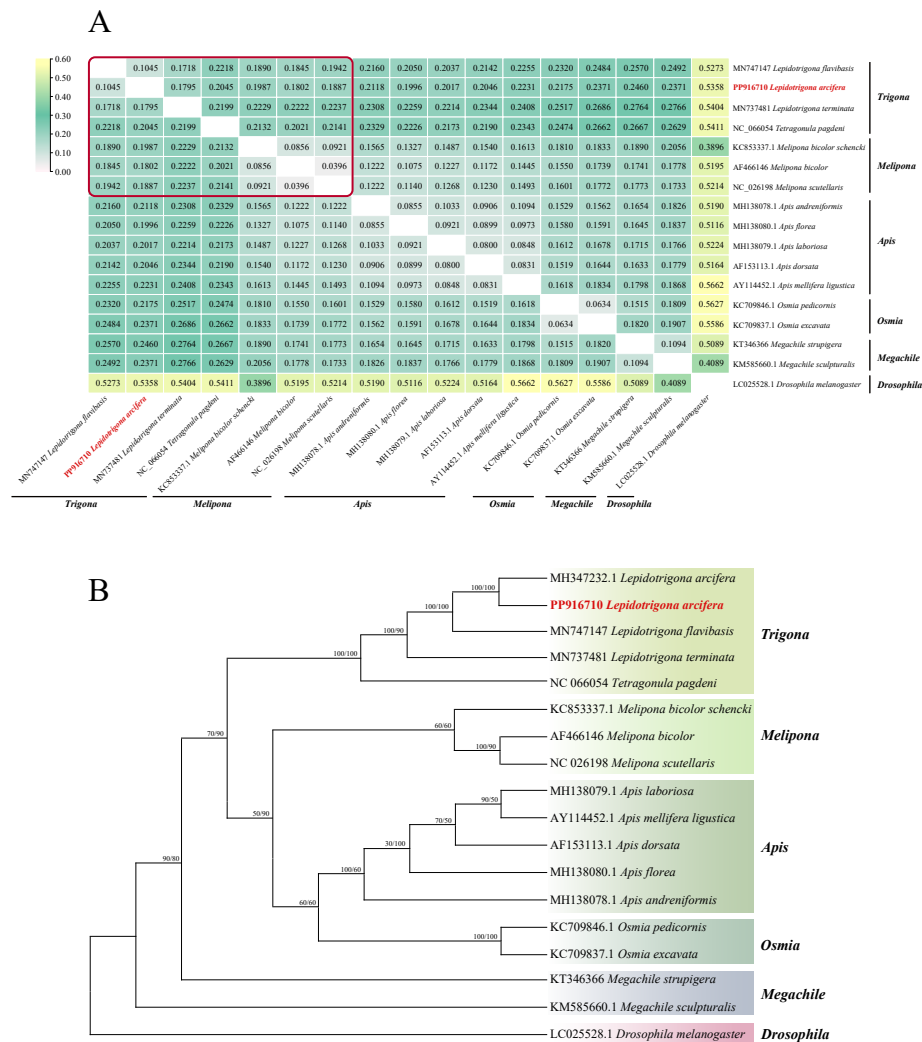


Fig. 7. Pairwise comparison of genetic distance based on COI gene (A), the red boxes are labeled with genetic distances between stingless bee genera. Phylogenetic relationship based on COI gene (B), bootstrap values present in the above branch using neighbor-joining and maximum-likelihood methods, the species *Drosophila melanogaster* was used as the outgroup.

genus. Within the *Trigona* clade, *L. arcifera* from Tibet is closely grouped with *L. arcifera* from northeast India, which confirms that the Tibetan stingless bee is *L. arcifera* rather than other *Trigona* species.

DISCUSSION

In this study, we observed a stingless bee, *L. arcifera* in the relatively cold area of Mêdog, Tibet of Southwest China for the first time, even though stingless bees are typically found in tropical and subtropical climates (Silva-Junior et al., 2019). This signifies that some lesser-known stingless bee species can occur in regions that were never considered suitable habitats for stingless bees. Although Indian *L. arcifera* had been reported previously, the morphological characteristics and taxonomic status had not been well studied at that time. As a consequence, the taxonomic position of *L. arcifera* has been ambiguous for a long time (Vijayakumar, 2014). Therefore, we sequenced the complete mitochondrial 16S rRNA and COI gene of Tibetan *L. arcifera* and combined available molecular data, and then confirmed that it had the closest relationship to the known *L. arcifera* from the GenBank database. As molecular markers, both 16S rRNA and COI genes are suitable for studying the phylogenetic relationship of stingless bee species. In the public GenBank database, only three stingless bee species present had the available complete COI sequences (Silvestre & Arias, 2006; Silvestre et al., 2008; Cristiano et al., 2012). In comparison, nine species had complete 16S rRNA sequences. Up to now, no 16S rRNA data, and only fourteen partial COI sequences of *L. arcifera* were available in public databases. In this study, we obtained the complete 16S rRNA and COI sequences of *L. arcifera*, which were used for taxonomic localization of the Tibetan stingless bee. In the future, with the molecular information accumulating, more mitochondrial markers, including 16S rRNA and COI genes, need to be involved in studying the interspecies or intraspecies phylogenetic relationship among *Lepidotrigona* species.

In conclusion, our studies provide new knowledge on the geographic distribution, biological characteristics and phylogenetics of *L. arcifera*. This first report on the occurrence of *L. arcifera* species in China will attribute to establishing a local stingless bee nursery in Tibet for resource preservation and artificial propagation, and then facilitate the conservation of this valuable stingless bee.

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Author Contributions: TXy analyzed the data and presented the data graph. YYx conceived the study and wrote the manuscript. LYh has made significant contributions to PCR amplification. JY contributed to the picture beautification. CXl, ZL, and WWf have contributed to the sample collection, and all authors have contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships.

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