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Research Article

New Record of *Carinostigmus* Tsuneki (Hymenoptera: Crabronidae: Pemphredoninae) species in India and identity of its species using DNA barcoding

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ABSTRACT: Specimens of the aphid hunting wasp *Carinostigmus* Tsuneki (Hymenoptera: Crabronidae: Pemphredoninae) were collected from South India. Morphological identification revealed three species, and one of them, *C. griphus* Krombein, is new for India. Identification of the species is supported through COI partial gene-DNA Barcoding.

KEY WORDS: *Carinostigmus*, DNA barcoding, molecular phylogeny, sphecidae

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INTRODUCTION

The Aphid hunting wasp genus *Carinostigmus* Tsuneki 1954 belongs to the family Crabronidae, subfamily Pemphredoninae, tribe Pemphredonini, subtribe Stigmina. Species in the Stigmina are among the smallest within Crabronidae, with the adults of *Carinostigmus* varying from 4.0 to 6. 5 mm in length, apart from *Ammoplanus* and *Spilomena* of subtribes Ammoplanina and Spilomenina, respectively. The females of *Carinostigmus* prey mainly on small insects like aphids and leaf hoppers. The adults feed on nectar from generalized flowering plants, and the larvae feed on aphids provided by the mother. The nest is normally built within a burrow made in dried twigs or wooden logs. The nesting pattern of this genus was studied by Green (1903) and Iwata (1964); they found out that the adult female builds the nest by excavating the soft pith of the grass stem or dead slander twigs or sometimes even establishing the nest in wooden logs. The wasp makes a series of cells provisioned with aphids.

Carinostigmus, first proposed by Tsuneki (1954) as a subgenus, was raised to the genus level by Bohart & Menke (1976). It is characterized by the presence of the median ridge on the frons; the absence of the acetabular carinae; and the hindwing media diverging much beyond the cubito-anal cross vein. The genus was first recorded from India by Bingham (1897) based on specimens from Sikkim, as *Stigmus congruus* and *Stigmus niger*; the latter name was synonymized with *S. congruous* by Kohl (1885). Krombein (1984) revised the species of *Carinostigmus* from Sri Lanka (Ceylon) and provided a key for species identification. He described two new species, *C. costatus* and *C. griphus*. *C. costatus* was alsoreported from South India. His descriptions are based on male and female characters; however, the females of *C. congruus* and *C. griphus* are morphologically difficult to distinguish.

In this study, the authors reports the occurrence of *Carinostigmus griphus* Krombein in India for the first time and demonstrate the utility of using DNA barcodes in diagnosing females of the species that are not easily distinguished by morphology.

MATERIALS AND METHODS

Specimens were collected from 11 localities in the states of Karnataka and Tamil Nadu (Table 1) by sweep net and yellow pan traps and were processed, mounted and labeled as per standard protocol (Aguiar, 2012). Imaging was done using Leica Wild M10 stereo trinocular microscope and slight touch up made in Adobe Photoshop 7. The genus and species were identified using the keys provided by Bohart and Menke (1976) and Krombein (1984), respectively.

DNA isolation and partial COI gene sequencing

DNA was isolated from the hind leg of individual wasp using Qiagen DNeasy Blood and Tissue kit method following the manufacturer's protocol. PCR amplification of partial gene sequences of mitochondrial COI gene was done by

Table 1. Collection details of *Carinostigmus*

Sl. No.	States of India	Place of collection with Date	Geo Reference	Mode of collection	Name of the collector
	Karnataka	Hebbal, 18.x.2014	N13^{0}03'E77^{0}35'	Yellow pan traps, Sweep Net	R. G. Gracy
2.		GKVK, 18.ii.2015	N13^{0}04'E77^{0}35'	Yellow pan traps, Sweep Net	R. G. Gracy
3.		Yelahanka 6.ii.2015	N13^{0}06'E77^{0}35'	Yellow pan traps, Sweep Net	R. G. Gracy
4.		Kunigal 16.x.2014	N13^{0}40'E78^{0}06'	Yellow pan traps	Veenakumari. K
5.		Nandi Hills 18.ix.2014	N13^{0}38'E77^{0}70'	Yellow pan traps	R. G. Gracy
6.		Srirangapatna 20.x.2014	N23^{0}41'E76^{0}69'	Yellow pan traps	R. G. Gracy
7.		Kanakapura 29.xi.2014	N12^{0}55^{0}E77.41^0	Yellow pan traps	R. G. Gracy
8.		Magadi 15.x.2014	N12.95°E77.22°	Yellow pan traps	R. G. Gracy
9.		Mallur 6.viii.2014	N13.00°E 77.94°	Yellow pan traps	R. G. Gracy
10.	Tamil Nadu	Yercaud, 6.viii.2014	N11^{0}77'E78^{0}20'	Yellow pan traps	Ramesh Kumar. A
11.		Valparai 4.vii.2014	N10^{0}31'E78^{0}95'	Yellow pan traps	Ramesh Kumar. A

using the universal COI primers (Hebert *et al*., 2004). PCR amplification was performed for a total volume of 30 µL, containing 2 µl DNA extract (20 ng), 1 µl (2mol) of each primer, 1 µl dNTP mixture (2.5 mmol for each), 2.5 µL 10 x Taq PCR reaction buffer, 3 µL 25 mM $MgCl_2^+$, and 1 unit of Taq DNA polymerase using a thermal cycler (BioRadiCyler) with the PCR cycle as follows, initial step at 94^{0}C for 1 minute and 35 cycles of the following: Denaturing 95^{0}C for 30 seconds, annealing 51^{0}C for 30 seconds, extension at 72^{0}C for 45 seconds and 4^{0}C forever (Ball and Amstrong, 2008). The PCR products size varied from 650 to 680 bp, the amplified products were confirmed by running on 1.5% agarose gel with 250bp ladder and visualized in INGENIUS^3 gel dock. The amplified products were purified using Qiagen PCR purification Kit by following the manufacture's instruction and the purified samples were sequenced using Sanger's method at M/S. Eurofins Pvt. Ltd, Bangalore, India. The sequences were annotated using NCBI Blast tools and submitted to NCBI GenBank Database as well as BOLD database and were assigned with accession number (Table 2). Using NCBI Blast, the more closely related sequences belong to two species of Crabronidae were retrieved from NCBI GenBank database and were included in the phylogeny analysis as outgroup.

Estimates of Genetic Divergence between species

Estimation of genetic distance between the species using their COI gene sequences was performed using MEGA 6 software. The numbers of base substitutions per site from between sequences are shown in Table 3. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al*., 2004). The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura and Kumar, 2002). The analysis involved 7 nucleotide sequences and was used 1st Codon positions for analysis. All ambiguous positions were removed for each sequence pair. There were a total of 224 positions in the final dataset. Genetic Divergence analysis was conducted in MEGA6 software (Tamura *et al*., 2013).

Construction of phylogeny

The sequences were converted into FASTA and consensuses were generated by Clustal-W multiple sequence alignment with default setting using MEGA 6 software. The tree was constructed using Neighbor Joining (NJ) method based on K-2 Parameter distance with the uniform rate of substitution, and the evolutionary pattern was inferred using boot strap of 1000 replicates with the Jukes Cantor model (Tamura *et al.,* 2013). Sequences of related species retrieved

from NCBI GenBank *viz., Stigmus* sp. (ALF68393) and Crabronidae (AMY29664) were used to root the tree.

Taxonomy

Genus *Carinostigmus* Tsuneki

Carinostigmus Tsuneki, 1954. Type species: *Stigmus congruus* Walker, 1860, by original designation.

Carinostigmus Tsuneki. - Bohart and Menke, 1976.

Perissostigmus Krombein, 1984, as subgenus of *Carinostigmus* Tsuneki.

Type species: *Carinostigmus bucheilus* Krombein, by original designation and monotypy.

Revision: Tsuneki, 1954 (Southeast Asia); Krombein, 1984 (Sri Lanka); Budrys, 1987 (Russian Far East).

Key to species: Krombein, 1984 (India and Sri Lanka); Tsuneki, Nozaka, Tano, Kurokawa, and Murota, 1992 (Philippines); L. Ma, X.X. Chen and Q. Li, 2012 (China).

Genus description (Krombein, 1984)

The wasps belonging to *Carinostigmus* are small in size, varying from 4 to 6.5 mm, with predominantly black glossy integument as in three species discussed below; frons with median carina, with T-like projection at base; antenna 12-segmented in female, 13-segmented in male, in male with short erect fringe-like setae beneath the segments. Mandible bidentate (male) or tridentate (female) apically in subgenus *Carinostigmus*, butbidentate in subgenus *Perissostigmus*; clypeus in most species without dense silvery setae; labrum varying from subtriangular to narrowly rounded at apex; maxillary palpus with 6 segments; labial palpus with 4 segments; eyes separated and having narrow grooves along orbits; ocelli normal; occipital carina and hypostomal carina present; notauli developed, parapsidal lines present; pronotal lobe conically produced, white to ivory; omaulus present, acetabular carina absent; female fore tarsus without rake; hind tibia without series of posterior spines; stigma enlarged, as long as first submarginal cell, two submarginal and two discoidal cells present; hind wing cu-a positioned about half way from the wing base to origin of media; petiole long, slender, much longer than twice its diameter; and longer than hind coxa; female pygidial plate present, oval to teardrop-shaped in outline.

Species descriptions

Carinostigmus (Carinostigmus) congruus (Walker)

Male maxillary palpi normal; underside of head with a few weak rugae, a large median area with moderately close punctures more smoother space laterally towards eye margin; labrum honey colour; clypeal lobe glossy, convex, produced in front and bidentate; antenna, all trochanters, fore and mid tibiae and tarsi, and mandibles testaceous to light yellowish brown; interocular distance at anterior ocellus 1.4 × that at antennal insertions; vertical rugae absent or evanescent,irregular rugae present on declivous surface of pronotum anterior to transverse ridge; pronotal lobe ivory; pronotal ridge weaker laterally, not emarginated mesally.

***Carinostigmus (Carinostigmus) griphus* Krombein**

Male maxillary palpi normal; underside of head with vertical rugae mesally in smalarea, smooth and moderately punctate laterally; clypeal lobe strongly shagreened in male and less so in female, less convex than *C. congruus*, not well produced; palpi, underside of scape, underside of pedicel, mandible, trochanters and fore tarsi testaceous; interocular distance at anterior ocellus 1.7 × that at antennal insertions; anterior pronotal ridge prominent but weaker than in *C. costatus,* slightly emarginated mesally in male, lateral angles more predominant laterallydeclivous slope anterior to pronotal ridge with vertical ridges.

***Carinostigmus (Carinostigmus) costatus* Krombein**

Underside of head with close longitudinal ridges; female maxillary palpi normal, in males conspicuously elongated, flattened, fringed on the inner margin with long curled setae and extending up to fore coxa; female mandible with ivory streak; trochanters black or dark; anterior pronotal ridge more prominently produced laterally and emarginated mesally.

RESULTS AND DISCUSSION

A study of specimens of *Carinostigmus* collected in Karnataka and Tamil Nadu revealed that three species, *Carinostigmus (Carinostigmus) congruus* (Walker), *Carinostigmus (Carinostigmus) costatus* Krombein, and *Carinostigmus (Carinostigmus) griphus* Krombein (Fig. 1). This constitutes the first record of *C. griphus* for India.

Since no male *C. congruous* has been collected, the author used a molecular approach (Hebert *et al.,* 2004) to establish the female identity of *C. congruous* as it is difficult to identify them based on morphological character as mentioned by Krombein, 1984. The DNA barcoding of three species using partial mitochondrial COI gene was carried out and the sequences from respective males and female specimens were analyzed Table 2. This analysis revealed that the gene sequence from male and female of the same species matches 100% with each other. Based on the sequence similarity, the females of *C. griphus* and *C. congruous* were distinguished from each other. The pairwise genetic divergence between and within (male vs female) the species was estimated using MEGA6. software. The result clearly showed that the genetic distance within species (male and female) was 0.004, wherein

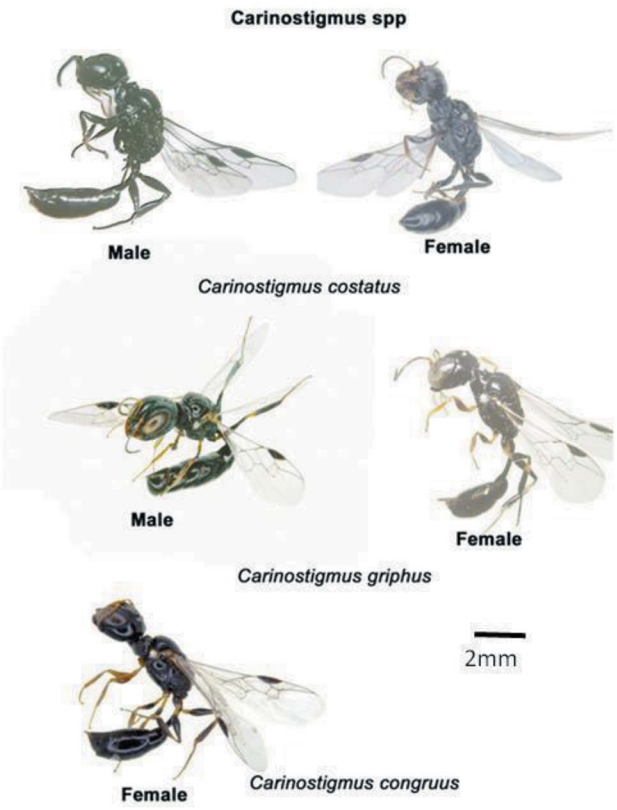

Fig.1. *Carinostigmus* spp.

it was higher between species and varied from 0.07 to 0.09. The distance between genera (outgroups) varied from 0.16 to 0.11 Table 3. The females of *C. congruus* and *C. griphus* had the genetic distance varying from 0.033 to 0.028. This shows these two species were more closely related than each of them was to *C. costatus*.

The molecular phylogeny tree was constructed using seven sequences of the closely related species by MEGA 6. software. The tree clearly indicates the presence of three species as they are diverging and forming different clades, wherein the males and females of the same species are together in the tree (Fig. 2). The related *Stigmus* sp. forms a separate clade and is aligned with the outgroup from Crabronidae. *C. griphus* and *C. congruus* were grouped in the same clade, which confirmed that they are more closely related to each other than to *C. costatus*. The molecular phylogeny and genetic distance confirm the proximity of the C. *griphus* and *C. congruus*.

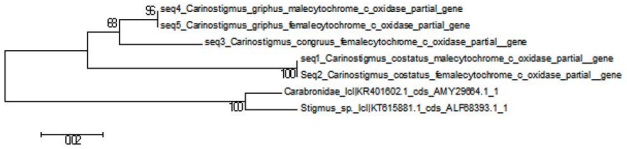

Fig. 2. Molecular Phylogeny of *Carinostigmus* spp.

CONCLUSION

The females of *Carinostigmus congruus* and *C. griphus*

Table 2. Sequence details of three species of *Carinostigmus* deposited in various databases.

Sl. No.	Species		GenBank accession numbers	BOLD Accession Numbers
1.	*C. congruus*	♀	KT070204	BOLD:ACD90874
2.	*C. griphus*	♂	KT070205	BOLD:ACV20062
		♀	KT070206	
3.	*C. costatus*	♂	KT070202	BOLD:ACV20072
		♀	KT070203.1	

Table 3. Pairwise genetic divergence among the three species of *Carinostigmus*

Species	Sp. ID	1	2	3	4	5	6	7
*Carinostigmus costatus*_male	1	0.000						
*Carinostigmus costatus*_female	2	0.004	0.000					
*Carinostigmus congruus*_female	3	0.091	0.091	0.000				
*Carinostigmus griphus*_male	4	0.076	0.077	0.028	0.000			
*Carinostigmus griphus*_female	5	0.075	0.071	0.033	0.004	0.000		
*Stigmus*_sp. {outgroup}	6	0.156	0.161	0.121	0.115	0.124	0.000	
Carabronidae_{1}	7	0.149	0.154	0.121	0.110	0.119	0.019	0.000

differ only in the clypeus shiny and slightly more produced in the latter species. These characters are more subjective and there is a chance for the misidentification of the females. The present study clearly demonstrates the use of the molecular method in the identification. The partial COI gene sequence comparison with their male counterpart yielded the proper identify that has been further confirmed with the help of the molecular phylogeny.

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