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Intraspecific variation in the morphology of Alloxysta fracticornis (Thomson, 1862)(Hymenoptera: Figitidae: Charipinae)

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ABSTRACT

A new brachypterous form of *Alloxysta fracticornis* (Thomson, 1862) has been found in the Museum of Natural History of Wroclaw University (Poland). This is the first time that this morphological variation has been recorded for *A. fracticornis*. The morphological features of this new form match with this species, except for the wing length, which is characteristic of the brachypterous form, and the colour. COI sequences and phylogenetic analysis for the specimens studied (macropterous and brachypterous) confirm that the two morphologies belong to the same species, as we suspected a priori. Thus, in this study we show evidence of morphological variation in *A. fracticornis*, and demonstrate the value of studying entomological collections as well as new material to improve the taxonomic knowledge of this subfamily.

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Charipinae; *Alloxysta fracticornis*; brachyptery; intraspecific variation

Introduction

The Charipinae (Hymenoptera: Cynipoidea: Figitidae) are very small wasps (0.8–2.0 mm). They are mainly characterised by their smooth and shiny body. This subfamily is important economically because they act as secondary parasitoids of aphids via Aphidiinae (Hymenoptera: Braconidae) and Aphelininae (Hymenoptera: Aphelinidae) and secondary parasitoids of psyllids via Encyrtidae (Hymenoptera: Chalcidoidea). Charipinae contains eight valid genera: *Alloxysta* (Förster 1869) (cosmopolitan), *Phaenoglyphis* (Förster 1869) (cosmopolitan), *Lytoxysta* (Kieffer 1909) (North America), *Lobopterocharips* (Paretas-Martínez et al. 2007) (Nepal), *Dilyta* (Förster 1869) (cosmopolitan except Australia), *Apocharips* (Fergusson 1986) (Eastern Palaearctic and Neotropics), *Dilapothor* (Paretas-Martínez and Pujade-Villar 2006) (Australia) and *Thoreauana* (Girault 1930) (Australia).

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Charipinae are widely distributed in all biogeographic regions, although they are best known from the Holarctic (Ferrer-Suay et al. 2012a). The taxonomy of this subfamily has been chaotic due to the large number of species described and the scarcity of diagnostic features. In recent years numerous taxonomic revisions have been carried out, and material has been deposited in the most important natural history institutions around

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the world. Also, the type material of all Charipinae species has already been revised, which is very useful for establishing the limits between species, but some species problems remain unsolved (Ferrer-Suay et al. 2012b, 2013a, 2013b, 2013c, 2014a, 2014b).

In a previous study (Ferrer-Suay et al. 2017) the known brachypterous species of *Alloxysta* were compared with fully winged species in order to develop hypotheses regarding dimorphism of the fore wing length and shape. This analysis was based only on the morphological features distinguishing species. These species are currently considered valid, but previous molecular studies pointed out the possibility of intraspecific wing polymorphism linked to sex, with brachypterous or macropterous males or, more rarely, brachypterous females (Ferrer-Suay et al. 2017, 2018). *Alloxysta fracticornis* (Thomson, 1862) was not included in these studies because no brachypterous specimen of this species was known. However, a brachypterous form has now been found from Poland and is hypothesised to belong to *A. fracticornis*.

In this study intraspecific morphological variation in *A. fracticornis* is confirmed: females can be macropterous or brachypterous, although the reason for and cause of this variation remain unclear. We use morphological and molecular features to compare these two forms, and the resulting phylogenetic tree shows that the group formed by them belongs to the same species.

Material and methods

Samples

Alloxysta fracticornis samples used in this study:

[POLAND] 'POLONIA, BIEBRZA NP, Barwik, 53.3652 N/22.5439E sifted, river bank leg. M. Wanat': 1♂; [POLAND] 'POLONIA PROMNO ad Poznan, 1.VI.2015 leg. P. Jaloszynski': 1♂; 'PL 27.VI.2015 FVO8, 49.544 N/22.514E NE of Rapienka meadow (slope, 580 m, leg. P. Jaloszynski': 1♂ and 1♀.

Alloxysta brevis and *A. brachyptera* samples are from Ferrer-Suay et al. (2018).

Morphology

Morphological terms used follow Paretas-Martínez et al. (2007). Measurements and abbreviations include F1–F12: first and subsequent antennal flagellomeres. The width of the fore wing radial cell is measured from the margin of the wing to the base of the Rs vein. Females and males are morphologically identical except for the sexual characters.

The specimens, mounted on cards, were studied with a stereomicroscope (Leica MZ6). An environmental scanning electron microscope (FEI Quanta 200 ESEM) was used for high-resolution imaging without gold-coating of the specimens.

Adults are deposited in **UV**: Universidad de Valencia, Spain (M. Ferrer-Suay coll.).

Molecular analyses

The samples were sent to AllGenetics for molecular analysis. We assembled a DNA sequence data matrix from 14 taxa of Charipinae for one molecular marker: the mitochondrial cytochrome oxidase subunit 1 gene (COI). Nine out of 14 samples yielded an

amplicon of the expected size. We include here representatives of four species of *Alloxysta*: two morphological variants of *A. fracticornis*. The data set was completed with additional sequences extracted from Ferrer-Suay et al. (2018) from a Norweigan study.

DNA isolations were carried out using a commercial kit optimised for small samples (Quick-DNA Microprep Plus kit, Zymo Research). After incubation in the kit's digestion buffer, the intact exoskeletons were transferred to new sterile tubes and preserved in 80% ethanol. DNA was isolated from the digestion buffer following the manufacturer's instructions, and re-suspended in a final volume of 10 µL. A negative control that contained no sample was included to check for contamination during the experiments.

The standard metazoan DNA barcode region, a 658-base pair (bp) fragment of the COI gene, was amplified by polymerase chain reaction (PCR) using the primer pair LCO1490 (5′ GGT CAA CAA ATC ATA AAG ATA TTG G 3′) and HCO2198 (5′ TAA ACT TCA GGC TGA CCA AAA AAT CA 3′) (Folmer et al. 1994). The PCR reactions were carried out in a final volume of 12.5 µL, containing 6.25 µL of Supreme NZYTaq Green PCR Master Mix (NZYTech), 0.5 µM of each primer, 1.25 µL of the template DNA solution, and PCR-grade water filled to 12.5 µL. The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 49°C for 30s, extension at 72°C for 1 min, and a final extension step at 72°C for 5 min.

A negative control that contained no DNA was included in the PCR to check for cross contamination during the experiments. PCR products were run on a 2% agarose gel stained with GreenSafe (NZYTech), and imaged under ultraviolet light to verify the amplicon size.

Electropherogram analysis was conducted in Geneious 8.1.9 (Biomatters Ltd). During electropherogram analysis, the primer annealing regions and the low-quality regions at both ends of each electropherogram were trimmed (error probability limit of 0.03). Sequence reads were manually checked for sequencing errors or ambiguous base calls. The final edited sequences were translated into amino acids to search for stop codons, which might indicate the amplification of nuclear mitochondrial pseudogenes (NUMTS).

To check whether the sequences obtained belonged to the target taxonomic group, they were compared to the COI barcode records available in two public reference databases: DDBJ/ENA/GenBank (International Nucleotide Sequence Database Collaboration) and BOLD, using the NCBI BLASTn tool against the Nucleotide database (nr/nt) or the BOLD Identification Engine, respectively. The accession number and the taxonomic assignment of the best match found in the reference databases, as well as the percentage of identity with the query sequence, are shown in Table 1. The sequences generated in this study are uploaded to the GenBank database. Table 3 shows the accession numbers and information about the specimens used in this phylogenetic study.

A phylogenetic analysis was performed using the COI sequences obtained in the present project and the reference sequences retrieved from Ferrer-Suay et al. (2018) (Figure 1). The sequences were aligned using Mafft v. 7.017 (Katoh and Standley 2013), as implemented in Geneious 8.1.9, and the G-INS-i algorithm. The alignments were translated into amino acids to detect possible stop codons or frame shifts, and trimmed to a final length of 581 bp.

Table 1. GenBank Database codes and published references for the sequences of *Alloxysta fracticornis* used in the molecular analyses. 1 DDBJ/ENA/GenBank or BOLD accession numbers. 2 Nucleotide identity: Percentage of nucleotides that are identical across the region where database and query sequences overlap.

Sample ID	Allgenetics ID	Species	Subproject	Sequencing results	GenBank	Taxon	Nucleotide identity (%)
1	FRACTI 01	*A. fracticornis*	FRACTI	Complete (658 bp)	MG940551.1	*A. fracticornis*	99.24 (653/658)
2	FRACTI 02	*A. fracticornis*	FRACTI	Complete (658 bp)	MG940551.1	*A. fracticornis*	99.39 (654/658)
3	FRACTI 03	*A. fracticornis* (brachyptera)	FRACTI	Complete (658 bp)	MG940644.1	*A. fracticornis*	97.72 (643/658)
4	FRACTI 04	*A. fracticornis* (brachyptera)	FRACTI	Complete (658 bp)	MG940551.1	*A. fracticornis*	99.39 (654/658)

Table 3. Data of the specimens used in the phylogenetic analysis.

Species	GenBank accession number	Authors
Alloxysta brachyptera	MG940626	Ferrer-Suay et al. 2018
Alloxysta brachyptera	MG940508	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940473	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940574	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940545	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940466	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940558	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940476	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940507	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940448	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940588	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940557	Ferrer-Suay et al. 2018
Alloxysta brevis	MG940441	Ferrer-Suay et al. 2018
Alloxysta fracticornis	MG940644	Ferrer-Suay et al. 2018
Alloxysta fracticornis	MG940470	Ferrer-Suay et al. 2018
Alloxysta fracticornis	MG940551	Ferrer-Suay et al. 2018
Alloxysta fracticornis (brachyp.)	–	This study
Alloxysta fracticornis (brachyp.)	–	This study
Alloxysta fracticornis (brachyp.)	–	This study
Alloxysta fracticornis (brachyp.)	–	This study

From each alignment, a matrix of nucleotide identities for every pair of sequences was estimated in Geneious. The best-fit partitioning schemes and nucleotide substitution models were selected using PartitionFinder v. 2.1.1 (Lanfear et al. 2012, 2017) under the corrected Akaike information criterion (AICc). Bayesian phylogenetic analyses were carried out with MrBayes v. 3.2.6 (Ronquist and Huelsenbeck 2003) using the appropriate models of nucleotide substitution selected with PartitionFinder. The analyses were run with four incrementally heated Markov chains for 10×10^6 generations in two independent runs with samplings at intervals of 500 generations. Stationarity was assessed using the software Tracer v. 1.7 (Rambaut et al. 2018). Samples obtained during the first 25% of generations were discarded as burn-in, and the remaining data were used to generate a majority-rule consensus tree, where the percentage of samples recovering any particular clade of the consensus tree represented the clade's posterior probability.

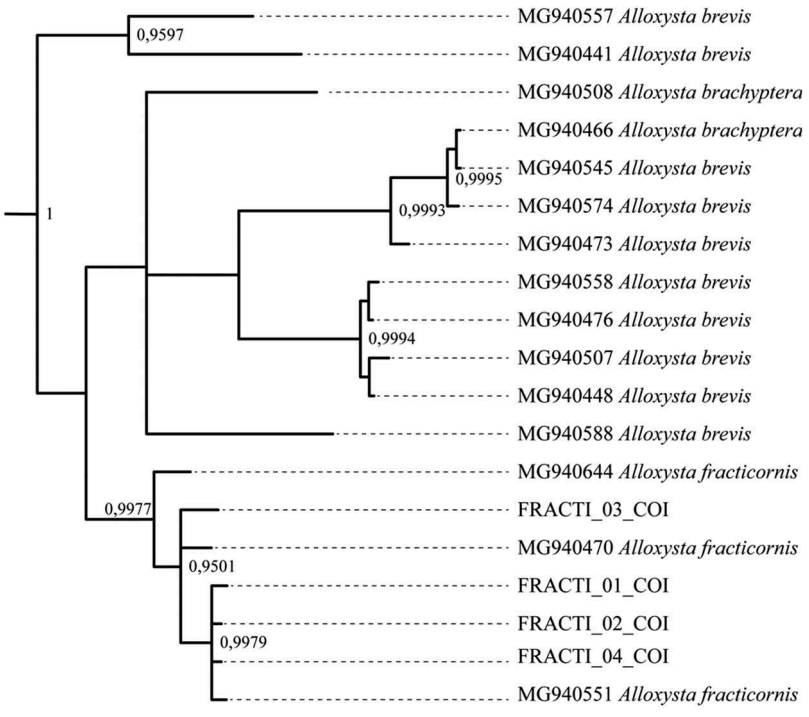
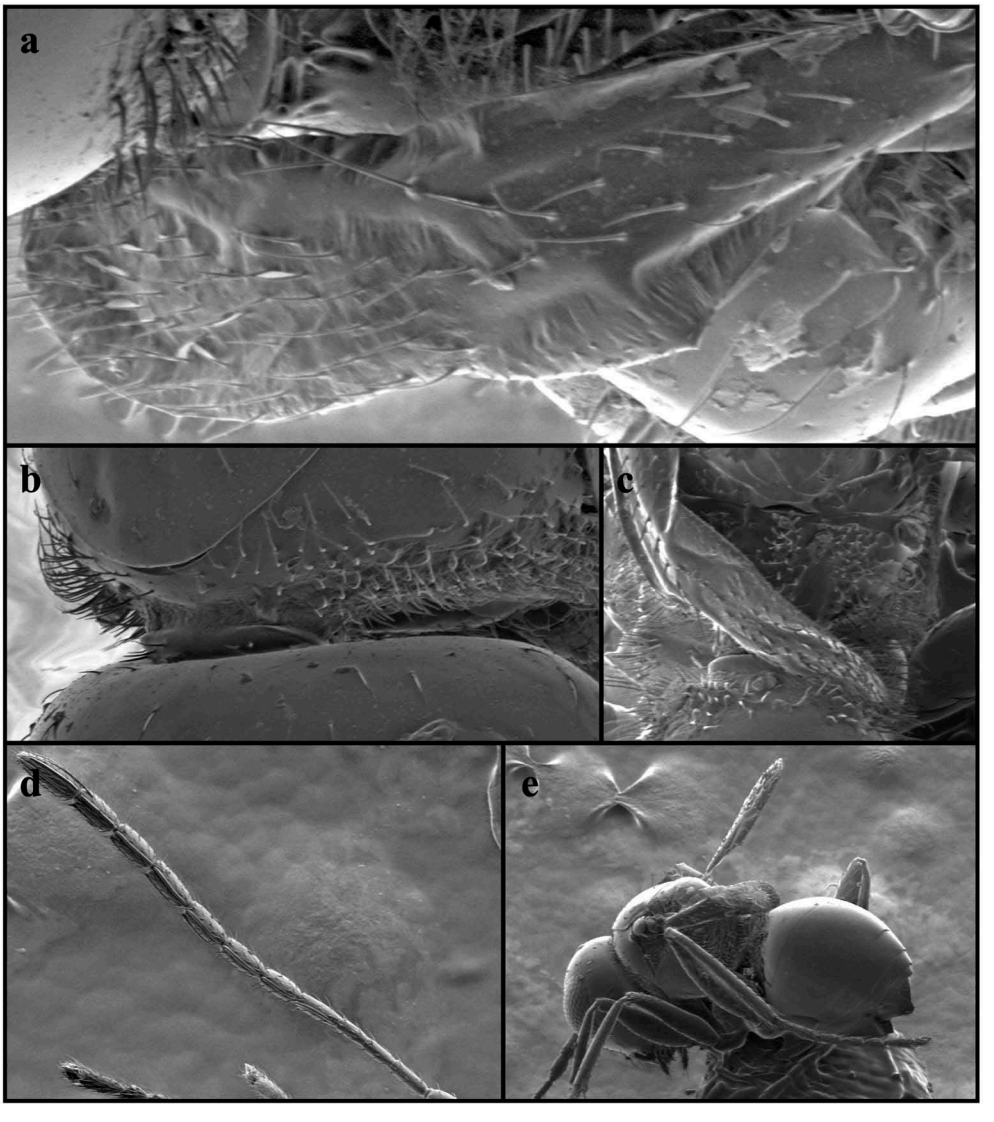


Figure 1. Bayesian tree inferred from the GenBank and FRACTI COI sequences (03 and 04 represent brachypterous specimens; 01 and 02 represent winged specimens). Posterior probability values are shown on branches (only values ≥ 0.95 are included).

Results and discussion

According to Ferrer-Suay et al. (2012a) there are 111 species of *Alloxysta*, only six of which are brachypterous: *A. apteroidea* Hellén, *A. brachyptera* (Hartig), *A. glebaria* Hellén, *A. halterata* (Thomson), *A. marshalliana* (Kieffer) and *A. pedestris* (Curtis). The older genera *Pezophycta* Förster and *Nephycta* Förster were described to accommodate these brachypterous species. *Pezophycta* included species with a short fore wing without a radial cell present, and *Nephycta* included the species with a radial cell present but fore wing shorter, not longer than metasoma (Kieffer 1902). Later, Hellén (1963) synonymised these two genera with *Alloxysta* on the basis of their general morphological features. According to van Veen (pers. comm.), the division of the genus *Alloxysta* into fully winged and brachypterous species may seem obvious, but the possibility of intraspecific wing polymorphism is thereby ignored (Ferrer-Suay et al. 2017).

The genetic results reproduce the topology obtained by Ferrer-Suay et al. (2018). All the terminals of both brachypterous and macropterous samples of *A. fracticornis* are recovered in a single monophyletic clade with an unresolved relationship to the rest of the species. The brachypterous forms are nested within the *A. fracticornis* clade with well-supported nodes, albeit with some unresolved terminal relationships (Figure 1). The distance matrix supports the distinctiveness of *A. fracticornis* for the gap between the



Q7 **Figure 2.** Morphological features of *Alloxysta fracticornis* (Thomson, 1862), brachyptery form: (a) fore
Q8 wing, (b) pronotum in dorso-lateral view, (c) propodeum, (d) antennae, (e) lateral habitus.

intraspecific and interspecific genetic distances of the *A. fracticornis* clade (Table 2). The intraspecific divergence of *A. fracticornis* ranges between 0.3 and 2.5%, while the distance between species is more than 5.7%. The recorded divergence is consistent with the between-species divergence threshold in Cynipoidea and other Hymenopterans (Sheffield et al. 2009; Nicholls et al. 2012, 2018; Cerasa et al. 2018; Nicholls and Pujade-Villar 2020; Pujade-Villar et al. 2020). Thus, the molecular data support the morphological variation of wings within *A. fracticornis*.

Figure 3. Forms of *Alloxysta fracticornis* (Thomson, 1862): (a) brachypterous, (b) macropterous.

The terminals assigned to *A. brachyptera* and *A. brevis* are recovered as polyphyletic, with low-supported nodes, similar to the results of the 2018 study. Two main lineages divide part of the terminals of *A. brachyptera* and *A. brevis* from the rest of the terminals including *A. fracticornis* (Figure 1). The molecular markers used by Ferrer-Suay et al. (2018) and in this study may be poor at resolving the interspecific relations among the three species. *Alloxysta brachyptera* and *A. brevis* are two species that require morphological and phylogenetic revision. The lack of consistent diagnostic characters to differentiate these two species may have led to the misidentification of specimens, or could suggest possible synonymy. The low resolution of these two species directly affects the support for the monophyly of *A. fracticornis*; however, the morphological limits of *A. fracticornis* are clear and it is always recovered in a monophyletic clade. Future studies should try different markers or more robust methodologies to assess the resolution of those groups.

We have found in the Charipinae material deposited in the Museum of Natural History of Wroclaw University (Poland) the first record of a brachypterous form of *A. fracticornis*. The features of this new form match with this species except for the wing length (Figures 2 and 3). The COI sequence has confirmed our hypothesis in this case.

The macropterous form of *Alloxysta fracticornis* is characterised by having a closed radial cell 2.2 times as long as wide, pronotal carinae absent, propodeal carinae present, male and female with the beginning of rhinaria in F3, F1–F3 subequal in length, and F3 curved in male. The brachypterous form of *A. fracticornis* shares these morphological features except for the wing length (Figure 1) in females; brachypterous males have not been found yet. *Alloxysta fracticornis* is similar to *A. mullensis* but they can be differentiated by the relation between F1 and pedicel: F1 is longer than the pedicel in *A. fracticornis* while F1 is subequal to the pedicel in *A. mullensis*; F1–F3 are subequal in length in *A. fracticornis* but F1 is longer than F2 and F2 is subequal to F3 in *A. mullensis*; F3 is curved in the *A. fracticornis* male but without any flagellomere curved in the *A. mullensis* male.

Alloxysta fracticornis was previously recorded from Poland by Kierych (1979, p. 14), and here we confirm its presence. This species has a mainly Palaearctic distribution (Ferrer-Suay et al., in prep.). It was described by Thomson from Sweden in 1862. Its type material

Table 2. Pairwise identity matrix of the COI gene.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	MG940644_Alloxysta fracticornis voucher NOFIG735																				
2	MG940551_Alloxysta fracticornis voucher NOFIG17	98,1																			
3	FRACTI_04_COI	98,1	99,3																		
4	FRACTI_02_COI	98,1	99,3	99,7																	
5	FRACTI_01_COI	98,1	99,3	99,7	99,7																
6	FRACTI_03_COI	97,8	97,5	97,8	97,8	97,8															
7	MG940470_Alloxysta fracticornis voucher NOFIG1168	98,1	97,8	98,1	98,1	98,1	98,1														
8	MG940557_Alloxysta brevis voucher NOFIG76	93,8	93,8	93,8	94	93,6	93,9	94													
9	MG940441_Alloxysta brevis voucher NOFIG308	93,1	92,8	92,9	92,9	92,6	92,7	93,2	94,3												
10	MG940508_Alloxysta brachyptera voucher NOFIG1024	94,3	94,1	94,1	94,1	94,1	93,4	94,1	91,2	90,5											
11	MG940574_Alloxysta brevis voucher NOFIG1169	92,3	91,4	92,1	91,7	91,9	92,2	92,5	90	89,3	90,4										
12	MG940545_Alloxysta brevis voucher NOFIG583	92,1	91,2	91,9	91,6	91,7	92	92,3	90,2	89,5	90,4	99,7									
13	MG940466_Alloxysta brachyptera voucher NOFIG1040	92,1	91,2	91,9	91,6	91,7	92	92,3	90,2	89,5	90,4	99,7	100								
14	MG940473_Alloxysta brevis voucher NOFIG1025	92,9	91,9	92,6	92,3	92,4	92,3	93,2	91	90	90,7	98,1	98,1	98,1							
15	MG940558_Alloxysta brevis voucher NOFIG86	92,6	92,3	92,6	92,6	92,6	93	93,1	92,1	90,7	91,7	93,5	93,3	93,3	93,8						
16	MG940476_Alloxysta brevis voucher NOFIG155	92,8	92,4	92,8	92,8	92,8	93,2	93,2	92,3	90,9	91,9	93,6	93,5	93,5	94	99,8					
17	MG940507_Alloxysta brevis voucher NOFIG55	92,9	92,4	92,8	93,1	92,8	93,2	93,4	92,1	91,4	91,9	93,3	93,1	93,1	93,6	99,1	99,3				
18	MG940448_Alloxysta brevis voucher NOFIG736	92,8	92,5	92,8	92,8	92,8	93,4	93,3	92,3	91,1	92	93,3	93,2	93,2	93,7	99,5	99,7	99,4			
19	MG940588_Alloxysta brevis voucher NOFIG1083	93,3	92,3	92,8	92,6	92,6	93	93,5	92,6	91,9	92,4	91,9	92,1	92,1	91,9	92,9	93,1	92,9	93		
20	MG940626_Alloxysta brachyptera voucher NOFIG1052	91,4	90,4	90,7	90,7	91	91,1	91,3	89,2	89,3	89,8	88,8	89	89	89,5	89	89,2	89	89	90,2	

was revised by Ferrer-Suay et al. (2013a); that work contains a complete redescription of *A. fracticornis* as well as images of type material. This information is also presented on the website of the subfamily Charipinae (www.charipinaedatabase.com).

It is important to draw attention to the necessity of continuing with the collection of new material, as well as the correct identification of specimens, in order to improve the knowledge of this important subfamily. Also, it is necessary to make a major effort to link molecular sequence data with type specimens and modern taxonomy. Charipinae need an integrated taxonomic solution, or they will remain in chaos.

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