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Source: Proceedings of the Entomological Society of Washington, 124(1) : 1-12

Published By: Entomological Society of Washington

URL: https://doi.org/10.4289/0013-8797.124.1.1

PROC. ENTOMOL. SOC. WASH.
124(1), 2022, pp. 1–12

BRACHYPTERY ANALYSIS IN *ALLOXYSTA* (HYMENOPTERA: FIGITIDAE): SYNONYMY OF *A. CURTA* FERRER-SUAY AND PUJADE VILLAR AS THE BRACHYPTEROUS MALE OF *A. RAMULIFERA* (THOMSON) IN THE NEARCTIC

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Abstract.—Eight brachypterous species have been described within the hymenopteran genus *Alloxysta*. Intraspecific wing polymorphism linked to sex has been previously hypothesized within this genus and the aim of this work was to confirm whether the phylogenetic relationships based on morphological characters between brachypterous and macropterous species are correct using molecular analyses. This study used material collected from Minnesota (USA), with 278 specimens identified as *Alloxysta brachyptera* (Hartig, 1840), *A. curta* Ferrer-Suay and Pujade-Villar, 2017 (Ferrer-suay et al. 2017), *A. brevis* (Thomson, 1862), or *A. ramulifera* (Thomson, 1862). Twenty-three of these specimens were subjected to sequencing of the barcoding gene, Cytochrome Oxidase I. Previous analyses had identified *A. curta* as a distinct species from *A. ramulifera* based in part on shorter wing length and our current sampling from Minnesota identified only male *A. curta*. However, our molecular analyses (COI) showed that *A. curta* should instead be considered a phenotypic variant of *A. ramulifera* exhibiting male brachyptery, and we establish this

new synonymy here. *Alloxysta brevis* and *A. brachyptera* remain as valid species. These results suggest that some brachypterous taxa within the figitid subfamily Charipinae, which have been described as valid species, are actually sexually dimorphic forms from the same species with different wing morphology.

Key Words: Charipinae, wings, COI, phylogeny, hyperparasitoid

DOI: 10.4289/0013-8797.124.1.1

The evolution of flight in insects is thought to have occurred 400 million years ago and led to substantial diversification (Carpenter and Burnham 1985, Grimaldi and Engel 2005), thanks to considerable advantages in accessing resources, finding mates, and escaping predators (Denno et al. 2001). However, in virtually all orders of insects capable of flight, this mode of dispersal has been lost repeatedly, along with various levels of wing reduction (Harrison 1980, Roff 1990, Wagner and Liebherr 1992). Three important wing morphologies can be identified: brachyptery, microptery, and aptery. Brachyptery is a condition in which the wings are short enough to make flight impossible. It differs from microptery because the venation is present and visible, and from aptery which is the lack of wings altogether (New and Lienhard 2007). An inverse correlation has been posited between development of a flight apparatus and reproductive ability, understood as an energy trade-off (Denno 1994). In some species, wing morphologies are polymorphic according to sex and flightless females show higher fecundity, reproducing earlier and producing more offspring (Harrison 1980, Roff 1984, Zera 1984, Roff and Bradford 1996, Roff et al. 1999, Ikeda et al. 2008). Similarly, flightless males have been found to mate more times and father more offspring than males capable of flight (Langellotto et al. 2000). This trade-off suggests an equilibrium between wing development and reproductive success (Weis-Fogh 1952, Hocking 1953, Sotavalta and Laulajainen 1961, Roff 1977).

Brachyptery can also be linked to sex. It is more common in females, which can benefit from investing more energy in reproduction (Wagner and Liebherr 1992). Brachyptery is less common in males, often occurring when males are able to control access to the emergence sites of females (Askew 1968). This is shown in some hymenopterans like *Nasonia vitripennis* (Walker, 1836), in which males emerge before females and compete to guard the puparium from which the females emerge (Askew 1968). Similarly, males of agaonid fig wasps are non-dispersal morphs with no wings that mate with females before the latter emerge from the fig in which they developed (Weiblen 2002). In these cases, selection on wings is hypothesized to be relaxed in males because they mate locally at the emergence site (often with their sisters) with little advantage for dispersal (Hamilton 1967, King and Skinner 1991). Indeed, male brachyptery is an important component of local mate competition (LMC) theory, which explains female-biased sex ratios under conditions of inbreeding and population subdivision (Hamilton 1967).

In *Alloxysta* Förster, 1869, a hyperparasitoid wasp genus in the family Figitidae, brachyptery is present in eight species (Ferrer-Suay et al. 2017), where it is more frequent in the males of six species. The population structure of aphids and their primary parasitoids is often subdivided in space into distinct aphid colonies (Dixon 1985). Thus, parasitoids of aphids may be subject to conditions of partial

LMC in which much or most mating takes place in the natal patch (the aphid colony) (Mackauer and Völkl 2002). We suggest that partial LMC conditions extend to aphid hyperparasitoids and thus that selection on the production of wings in males is relaxed with respect to females, leading to a pattern of male brachyptery in *Alloxysta*.

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 for wing length and shape. This analysis was only based on the morphological features distinguishing species. These species are currently considered valid, but previous molecular experiments pointed out the possibility of an intraspecific wing polymorphism linked to sex, with brachypterous or macropterous males or more rarely brachypterous females (Ferrer-Suay et al. 2017, 2018). The aim of the current study is to test the relationship between brachypterous and macropterous species that were previously based on morphological characters. In particular, we use molecular evidence to evaluate the hypotheses that the taxon now known as *A. curta* is a male morphological (brachypterous) variant of *A. ramulifera* rather than a distinct species, and similarly that *A. brachyptera* is a brachypterous male morphological variant of *A. brevis* rather than a distinct species.

Materials and Methods

Collection, sorting, and identification.—Privately owned conventional soybean fields were sampled throughout the state (Minnesota, USA) to collect pupal cases ("mummies") of aphid parasitoids and reared to adult parasitoids as part of field sampling of the soybean aphid, *Aphis glycines* Matsumura, 1917. The survey was conducted from July 18 to August 29 in 2014, and June 24 to August 20 in 2015 throughout the major soybean growing region of the state (Kaser et al. 2014, 2015). At each of two locations within the field, 5 soybean plants per were sampled. On each plant we counted total *Aphelinus* spp. mummies and total Aphidiinae parasitoid mummies. A subsample of up to 10 mummies per plant were shipped to the University of Minnesota for identification to species level. In 2014 and 2015, 215 and 124 fields were surveyed, respectively. The main primary parasitoid attacking soybean aphid over this time period was *Aphelinus certus* Yasnosh, 1963, which is of Asian origin and had been observed in Minnesota beginning in 2011 (Heimpel et al. 2010, Ragsdale et al. 2011, Kaser and Heimpel 2018, Miksanek and Heimpel 2019). Mummies of *Aphelinus* parasitoids were collected and held individually in 0.6 mL microcentrifuge tubes, and the adults either died by drying or were killed in 95% ethanol. From 17345 *Aphelinus* mummies collected, 1365 hyperparasitoids emerged, including 546 specimens in the genus *Alloxysta*.

We studied 288 of the *Alloxysta* specimens with a stereomicroscope (Leica MZ6) directly from alcohol when possible, and measurements were made using an ocular micrometer. We only examined a subsample of all collected *Alloxysta* because these were the specimens that were in sufficiently good condition for identification. These specimens were grouped into four different species based upon morphological characters: *Alloxysta brevis* (264), *A. brachyptera* (3), *A. ramulifera* (2) and *A. curta* (19). Selected specimens were recovered from the slides for DNA analyses (see below).

Morphological terms were taken from Paretas-Martínez et al. (2007). Measurements and abbreviations included F1-F12, indicating lengths of first and subsequent antennal flagellomeres. The width of the forewing radial cell was measured from the margin of the wing to

the base of the Rs vein. Females and males were morphologically identical except for antennomere number and where indicated. *Alloxysta* species were identified using the Keys for the World Charipinae (Hymenoptera: Figitidae) (Ferrer-Suay et al. 2019) and the Interactive Charipinae Worldwide Database (Ferrer-Suay 2019).

DNA extraction and PCR.—For the molecular analysis, 23 specimens from the detected species were used (*A. brachyptera*: 2, *A. brevis*: 8, *A. curta*: 10, *A. ramulifera*: 3). From every individual we extracted DNA with the HotSHOT method (Hot Sodium Hydroxide Tris) (Truett et al. 2000), using 30 µL of NAOH-EDTA and 30 µL of Tris as reagents.

PCR amplification was done with 3 µL of extracted DNA. A 710 bp fragment of the 5' region of the mitochondrial cytochrome c oxidase subunit 1 (COI) was amplified using primers LCO1490 and HCO2198 (Folmer et al. 1994). PCR conditions for COI amplification were as follows: 94°C for 1 min; 35 cycles of 94°C for 30 s, 48°C for 1 min and 68°C for 1 min; a final extension step of 7 min at 68°C was included after cycling. Then, PCR products were purified by ammonium precipitation and reconstituted in 10 µL of LTE buffer (10 mM Tris, 0.1 mM EDTA). Sequencing with Sanger method and PCR primers was conducted using the Big Dye Terminator v3. Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's instructions.

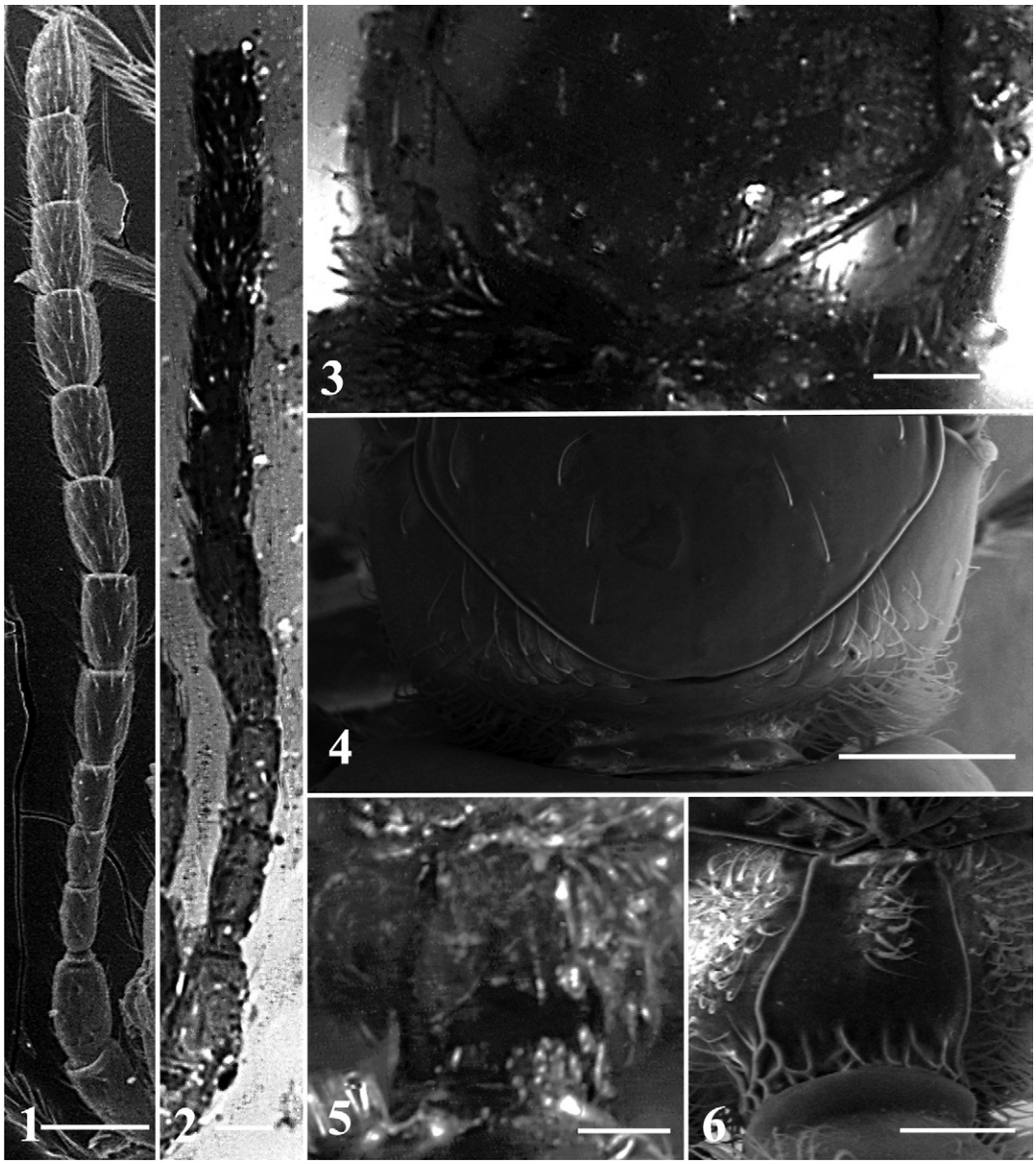
Sequence editing, alignment, and phylogenetic analysis.—Sequences corresponding to each sample were assembled using Staden package v2.0.0 (Staden et al. 1998). Multiple alignments were carried out with Clustal X v1.81 (Thompson et al. 1997) with gap opening and gap extension penalties of, respectively, 10.0 and 0.2, and then manually revised.

We used MEGA7 (Kumar et al. 2016) to carry out multiple alignments and phylogenetic analysis, building a phylogenetic tree (Neighbor joining, Kimura 2-parametres, 500 bootstraps). Sequences from previous studies or found in NCBI (Elias et al. 2013, Ferrer-Suay et al. 2018) were used. We chose *Phaenoglyphis villosa* (accession number: MG342266.1) (Figitidae: Charipinae) as an outgroup. Moreover, we used two sequences of *Alloxysta brachyptera* to study the relationship with our *Alloxysta brevis*. They were already in our possession from previous studies (Ferrer-Suay et al. 2018). We followed the techniques of Hebert et al. (2003) to hypothesize whether two species were the same or not using genetic distance.

Our *Alloxysta* sequences are available in GenBank with the following accession numbers: OK562103.1, OK562081.1, OK562082.1, OK562079.1, OK562080, OK586758.1, OK586708.1, OK586757.1, OK586709.1, OK586704.1, OK586706.1, OK586705.1, OK586699.1, OK586698.1, OK586702.1, OK586707.1, OK586703.1, OK586701.1, OK586696.1, OK586697.1, OK586700.1, OK586622.1.

Results

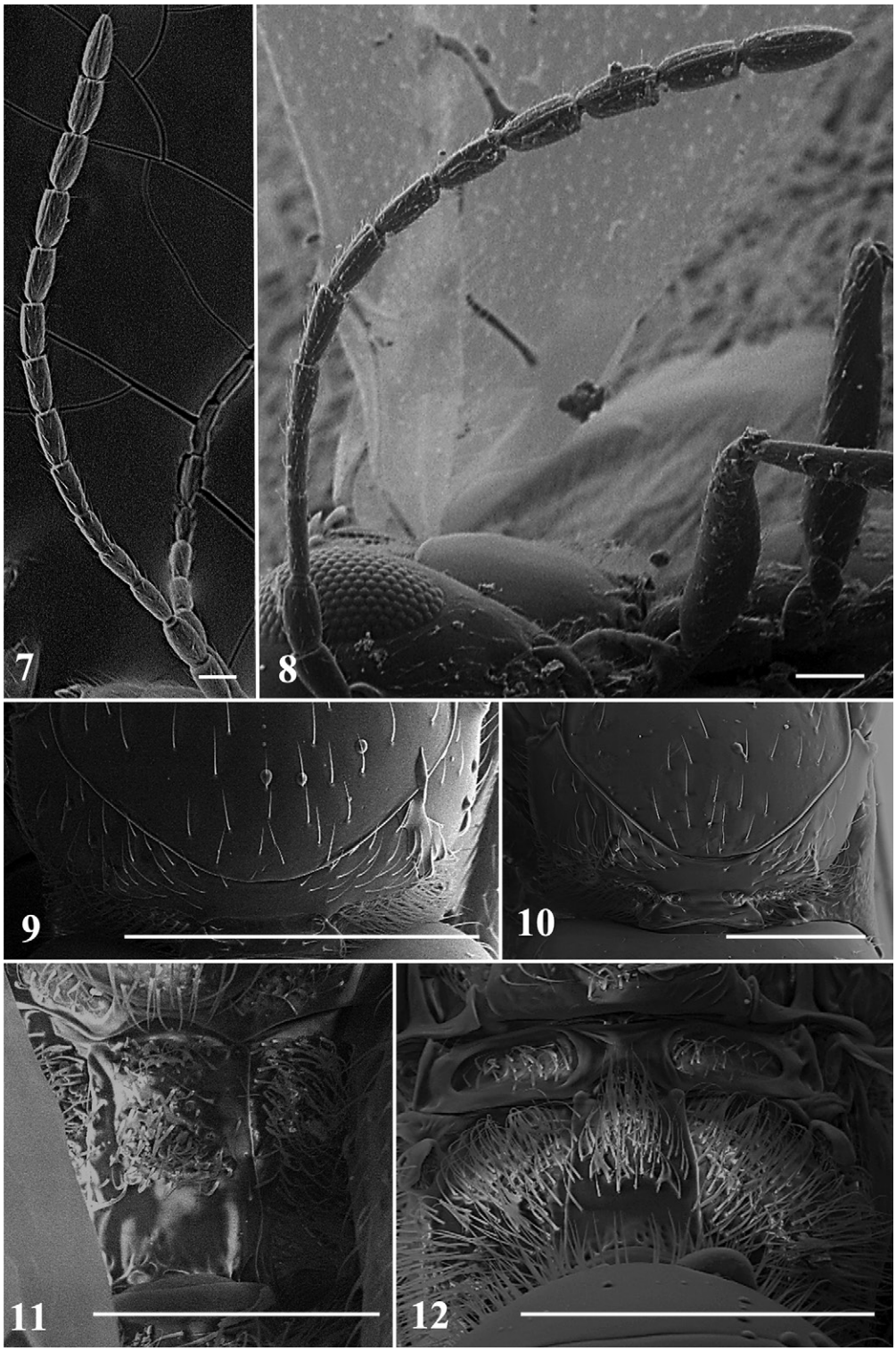
Through morphological analysis, four species of the genus *Alloxysta* were identified: two brachypterous species (*A. brachyptera* and *A. curta*) and two macropterous species (*A. ramulifera* and *A. brevis*) (Figs. 1–12). In total 288 specimens were examined from those four species (Table 1). Three specimens identified as *A. brachyptera* were collected, two of which were males and one of which was a female (Table 1), but COI amplification showed these two males to belong to *A. ramulifera* (Table 2, Fig. 13). Similarly, all 19 field-collected specimens originally identified as *A. curta* were males (Table 1). COI amplification of all 10 of these specimens subjected to molecular analysis also



Figs. 1–6. Comparison between *A. brachyptera* and *A. brevis*. 1, Antenna of *A. brevis*. 2, Antenna of *A. brachyptera*. 3, Pronotum of *A. brachyptera*. 4, Pronotum of *A. brevis*. 5, Propodeum of *A. brachyptera*. 6, Propodeum of *A. brevis*. (Scale bar = 50 μm).

showed them to belong to *A. ramulifera* (Table 2, Fig. 13).

Alloxysta brachyptera and *A. curta* had previously been considered valid brachypterous species, but Ferrer-Suay et al. (2017) suggested that the brachypterous habitus could be a phenotypic variant of a macropterous species. The relationships hypothesized in 2017 were: *A. curta* was a morphological variant of *A. ramulifera* and *A. brachyptera* a morphological variant of *A. brevis*. Following the techniques



Figs. 7–12. Comparison between *A. curta* and *A. ramulifera*. 7, Antenna of *A. curta.* 8, Antenna of *A. ramulifera.* 9, Pronotum of *A. curta.* 10, Pronotum of *A. ramulifera.* 11, Propodeum of *A. curta.* 12, Propodeum of *A. ramulifera*. (Scale bar = 50 μm).

of Hebert et al. (2003), the phylogenetic tree allowed us to establish that *A. curta* and *A. ramulifera* were the same species, with a genetic distance of less than 0.2% (Fig. 13). However, *A. brachyptera* and *A. brevis* show a genetic distance typical of separate species and equivalent to the distance between *A. brachyptera* and *A. ramulifera* (9.3–10.7%).

Table 1. *Alloxysta* species identified based on morphological characters.

Species	Female	Male
A. brevis	263	1
A. brachyptera	1	2
A. ramulifera	2	0
A. curta	0	19

Discussion

During the earliest attempts to resolve the taxonomy of the subfamily Charipinae, the brachypterous species were clustered in the genera *Pezophycta* and *Nephycta* (Förster, 1869). However, taxonomists started to doubt the validity of the dichotomy between macropterous and brachypterous species within the Charipinae in the mid-20th century. For example, Hellén (1963) synonymized these two genera with *Alloxysta* using morphological characters and suggested that *A. discreta* Förster, 1869, a brachypterous species, might represent the male of *A. ramulifera*, due to the lack in the literature of females in the former and males in the latter species (van Veen 1999). This was considered a case

Table 2. *Alloxysta* specimens used in the molecular analysis of this study, indicating initial morphological analyses and molecular determination, and whether the specimen was brachypterous or macropterous.

	Identification			
Brachypterous (B) - Macropterous (M)	Morphological	Molecular	Sample Reference	Locality
B	*A. brachyptera* (♂)	*A. ramulifera*	10	Saint Paul MN
B	*A. brachyptera* (♂)	*A. ramulifera*	16	Saint Paul MN
M	*A. brevis* (♀)	*A. brevis*	45	Saint Paul MN
M	*A. brevis* (♀)	*A. brevis*	48	Saint Paul MN
M	*A. brevis* (♀)	*A. brevis*	44	Pope County MN
M	*A. brevis* (♀)	*A. brevis*	45	Pope County MN
M	*A. brevis* (♀)	*A. brevis*	50	Douglas County MN
M	*A. brevis* (♀)	*A. brevis*	61	Todd County MN
M	*A. brevis* (♀)	*A. brevis*	67	Scott County MN
M	*A. brevis* (♀)	*A. brevis*	59	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	2	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	6	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	7	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	8	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	9	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	13	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	42	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	67	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	70	Saint Paul MN
B	*A. curta* (♂)	*A. ramulifera*	71	Saint Paul MN
M	*A. ramulifera* (♀)	*A. ramulifera*	32	Saint Paul MN
M	*A. ramulifera* (♀)	*A. ramulifera*	42	Saint Paul MN
M	*A. ramulifera* (♀)	*A. ramulifera*	103	Saint Paul MN

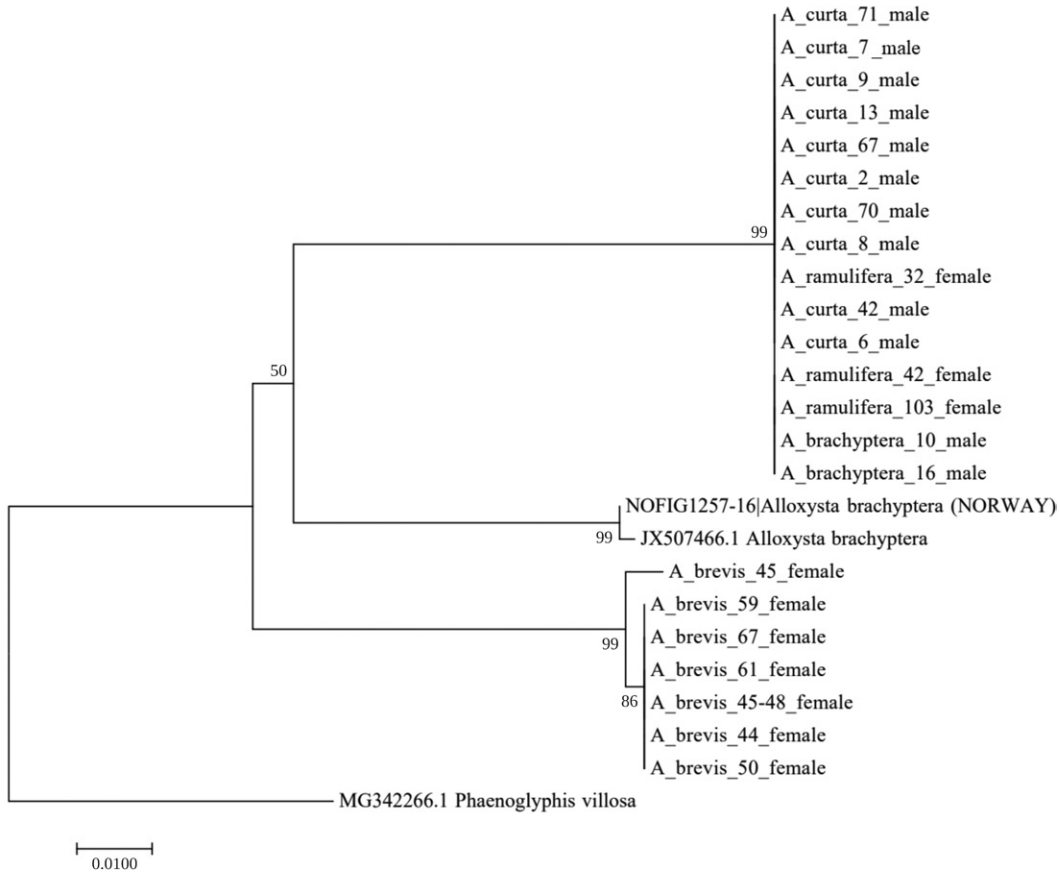


Fig. 13. Neighbor joining phylogenetic tree (COI) for four putative species of *Alloxysta* and the outgroup *Phaenoglyphis villosa* (Figitidae: Charipinae). Names followed by reference number are based on the morphologies in Table 2. Numbers on the branches indicate bootstrap values.

of a sexually dimorphic species that was resolved by Ferrer-Suay et al. (2012) when *A. discreta* became a synonym of *A. ramulifera*. For *A. curta*, only brachypterous males were known in the original description of Ferrer-Suay et al. (2017). They proposed that *A. curta* represents a phenotypic, sexual variant of *A. ramulifera*, based on morphological features like the small and closed forewing radial cell and the presence of pronotal and propodeal carinae (Figs. 7–12). They also proposed the linkage of another brachypterous species, *A. brachyptera,* to a macropterous one, *A. brevis*. One of the most important diagnostic characteristics, the forewing radial cell, is not present in brachypterous species like *A. brachyptera*, that possess wings shorter than the mesosoma, but *A. brachyptera* and *A. brevis* share the rest of the morphological features, such as rhinaria, club-shaped F4 antennomeres, and the presence of only propodeal carinae which form a plate (Ferrer-Suay et al. 2017) (Figs. 1–6).

In the current study, we used molecular analyses to demonstrate phenotypic variation linked to sex, because macropterous males of *A. ramulifera* were never found and all the known specimens of *A. curta* were males. Because of this, we can assert that *A. curta* is the brachypterous male of

the species *A. ramulifera*, updating a new synonymy. It is possible that the life history of *A. ramulifera* includes wing reduction in males because only the females need flight to find new hosts for future offspring, as has been proposed for many fig wasps and parasitoid species including *Nasonia vitripennis* (Hamilton 1967, King and Skinner 1991). Aphid parasitoids (both primary and secondary) may be exposed to patchy host environments and thus experience LMC-like conditions, which have been shown to favor male brachypterism (Hamilton 1967, Mackauer and Völkl 2002).

In many hymenopterans, a COI sequence divergence higher than 8% is enough to define two species as distinct (Hebert et al. 2003). *Alloxysta brevis* and *A. brachyptera* show a genetic distance greater than 9.3%, consistent with the hypothesis that they are different species. In the case of *A. brachyptera*, more investigations are required to understand the actual phylogenetic relationships to other species of *Alloxysta*. The morphological identification of micro-Hymenoptera like these can be very difficult, especially when the identification keys include details like the presence of carinae or wing venation and can be further complicated by wing loss for characters found on the wings themselves. In this situation, molecular analysis is particularly helpful, because it not only clarifies the hypothetical relationships between species, but can also correct identification errors. Charipinae taxonomy has often shown problems of misidentification or cryptic complexes (Pujade-Villar et al. 2007, Ferrer-Suay, Selfa, and J. Pujade-Villar 2012). More studies with other molecular markers would be required to clarify the taxonomical situation within *A. brachyptera* and *A. brevis*.

Our work supports the relationships hypothesized by Ferrer-Suay et al. (2017) for the species *A. ramulifera*, but *A. brevis* and *A. brachyptera* remain distinct. Importantly, the brachypterous phenotypes were, until now, only known from the Palearctic region (Ferrer-Suay et al. 2017). Thus, the material studied here provides the first record of a brachypterous phenotypical variant of the genus *Alloxysta* in the Nearctic region.

Acknowledgments

Alice Casiraghi benefited from an Erasmus+ scholarship during the year 2018-2019, to do her Master thesis at the University of Valencia. The investigation in Spain was conducted in the context of Project CGL2015-68188-P, funded by "Ministerio de Economia, Industria y Competitividad" (MIMECO) of Spain. We thank Nicholas Padowski and Kelton Welch for sampling as well as funding by the University of Minnesota Rapid Agricultural Response Fund, the University of Minnesota Agricultural Experiment Station, the Minnesota Invasive Terrestrial Plant and Pest Center, the Minnesota Soybean Research and Promotion Council and the U.S. Dept. of Agriculture Sustainable Agriculture Research and Education program.

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