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Nuclear and mitochondrial markers suggest new species boundaries in *Alloxysta* (Hymenoptera: Cynipoidea: Figitidae)

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Abstract. Due to their small size and smooth body, members of the figitid genus *Alloxysta* can be notoriously difficult to identify based on morphology alone. Moreover, several species are suspected to contain brachypterous variants that previously have been described under separate names. We used gene sequences of the mitochondrial gene cytochrome c oxidase subunit 1 (COI, DNA barcodes) and the internal transcribed spacer (ITS2) to investigate the relationships between species within the genus. Our results show that there is considerably more intraspecific variation in COI than in ITS2, but that both markers can be used to identify potential synonyms of brachypterous and fully winged species. Maximum Likelihood and Bayesian analyses of a concatenated dataset of both markers resulted in similar and fairly well supported phylogenies that indicate genetic divergence not matching morphological species boundaries for some groups. Some of the discrepancies can be explained by infections of the endosymbiont *Wolbachia*, here reported from the first time in *Alloxysta* and Charipinae. This study also presents the first records of *A. arcuata*, *A. basimacula*, *A. brachycera*, *A. brachyptera*, *A. brevis*, *A. citripes*, *A. consobrina*, *A. curta*, *A. fracticornis*, *A. halterata*, *A. marshalliana*, *A. obscurata*, *A. proxima*, *A. pusilla*, *A. ramulifera* and *A. xanthopa* from Norway.

Key words. Charipinae, *Alloxysta*, phylogeny, DNA barcodes, COI, ITS2, brachyptery, *Wolbachia*.

1. Introduction

Wasps of the family Figitidae (Hymenoptera: Cynipoidea) are biologically characterized by being parasitoids of the larvae of other insects, principally muscomorphan Diptera (Ronquist 1999), except for the subfamily Charipinae, which are parasitoids of Hymenoptera through Hemiptera. The members of this subfamily are hyperparasitoids of aphids *via* Aphidiinae (Hymenoptera: Ichneumonoidea: Braconidae) and Aphelininae (Hymenoptera: Chalcidoidea: Aphelinidae) and hyperparasitoids of psyllids *via* Encyrtidae (Hymenoptera: Chalcidoidea) (Fergusson 1986; Menke & Evenhuis 1991).

Eight genera of Charipinae are currently recognized: the cosmopolitan *Alloxysta* Förster, 1869; the Palaearctic and Neotropical *Apocharips* Fergusson, 1986; the Australian *Dilapothor* Paretas-Martínez & Pujade-Villar, 2006; *Dilyta* Förster, 1869 which is cosmopolitan except South America and Australia; *Lobopterocharips* Paretas-Martínez & Pujade-Villar, 2007b from Nepal; the North American *Lytoxysta* Kieffer, 1909; the cosmopolitan *Phaenoglyphis* Förster, 1869; and *Thoreauana* Girault, 1930 from Australia. Morphologically, the Charipinae are characterized by reductions of many characters nor-

mally found diagnostic in other figitids. They are very small (0.8–2.0 mm), generally with a shiny and smooth body (Fig. 1). The combination of these characteristics together with the great number of described species over the past 150 years have left Charipinae taxonomy in a chaotic state, making the reliable identification of species often impossible. Recently, progress has been made in resolving taxonomic issues of this subfamily (Ferrer-Suay et al. 2012a, 2013a,c,d,e, 2014b,c, 2015b), and morphological identification of European taxa is by large possible if high quality specimens are available.

Alloxysta (Fig. 1) can be differentiated from most other Charipinae genera by the combination of certain characters. Firstly, they possess a filiform antenna, where all flagellomeres are separated by constrictions. Secondly, they have a metasoma with two visible tergites of about the same length. Thirdly, they possess posterodorsal extensions of axillar strips. These traits are shared with *Phaenoglyphis*, *Lobopterocharips* and *Lytoxysta*, however, and the characters that are unique for *Alloxysta* are only visible after dissection. These include the superior flange of the petiole not protruding beyond tergite 3, and the presence of two short lateral files of setae on the metasomal ventral spine, not close to the apex (Paretas-Martínez et al. 2007a).

Accurate species identification is crucial for ecological and evolutionary analyses, but challenging to achieve in taxa with unresolved taxonomies, many undescribed species, and few morphological characteristics. The use of short standardized DNA fragments for species identification (i.e. DNA barcoding, Hebert et al. 2003) has been proposed as a tool to both meet the demand of accurate species identifications of all life stages, discover new diversity, analyse species boundaries and investigate patterns in community ecology and evolution (e.g. Kress et al. 2015). Moreover, using molecular taxonomy for classification and organization of life has been seen as an advantage over morphology in being more objective and faster (Tautz et al. 2003). Numerous studies have shown the great applicability of DNA barcoding in Hymenoptera, and DNA barcodes has been used in association of life stages (Dowton & Austin 1994; Moritz & Cicero 2004; Dasmahapatra & Mallet 2006), estimation of diversity (Smith et al. 2013; Hebert et al. 2016), discovery of cryptic taxa (e.g. Williams et al. 2012) and phylogenies (e.g. Tribull 2015). However, introgression and incomplete lineage sorting can lead to erroneous results when relying on single genetic markers (Funk & Omland 2003; Ballard & Whitlock 2004), and deep mitochondrial genetic divergence is not always accompanied by correspondingly deep differentiation in nuclear markers (e.g. Schwarzfeld & Sperling 2015). For these reasons, analyses of species boundaries using multiple loci as well as other traits often provides better evidence of genetically distinctive lineages (e.g. Roe & Sperling 2007).

In the morphological phylogeny of Charipinae (Paretas-Martínez et al. 2007a), *Alloxysta* was established as monophyletic but the phylogenetic relations between species within the genus were limited by taxonomic sampling. *Alloxysta* is cosmopolitan and has been recorded from all biogeographical regions (Ferrer-Suay et al. 2012a). It is the most abundant and widespread genus within Charipinae. The main objective of this study is to present a preliminary molecular phylogeny of *Alloxysta* based on species collected in Norway, and to shed light on the species boundaries of some key taxa. Eight *Alloxysta* species were previously recorded from Norway: *A. castanea* (Hartig, 1841) by Hofsvang & Hagvar (1983), *A. fuscipes* (Thomson, 1862) by Hellén (1966), *A. macrophadna* (Hartig, 1841) by Hellén (1966), *A. pallidicornis* (Curtis, 1838) by Zetterstedt (1838), *A. pedestris* (Curtis, 1838) by Hellén (1966), *A. pilipennis* (Hartig, 1840) by Hellén (1966), *A. pleuralis* (Cameron, 1879) by Westrum et al. (2010) and *A. victrix* (Westwood, 1833) by Hellén (1966).

Fig. 1. Habitus of *Alloxysta curta* Ferrer-Suay, 2017.

2. Material and methods

2.1. Collection, sorting and identification

Specimens were sorted from Malaise trap material collected on different locations in Norway between 2009 and 2016; most were collected in 2014 and 2016. The wingless records were collected using a sweeping net at various locations (Supplementary Table S1).

Specimens were identified using relevant revisions and original descriptions (Ferrer-Suay et al. 2018 and references therein).

Morphological terms used are taken from Paretas-Martínez et al. (2007a). Measurements and abbreviations include F1–F12, indicating lengths of first and subsequent flagellomeres. The width of the forewing radial cell is measured from the margin of the wing to the base of the Rs vein. Females and males are morphologically identical except where indicated.

Table 1. Primer sequences used in PCR and sequencing.

Marker	Primer name	Direction	Primer sequence (5'–3')	Source
COI	LepF1	Forward	ATTCAACCAATCATAAAGATATTGG	Hebert et al. 2004
COI	LepR1	Reverse	TAAACTTCTGGATGTCCAAAAAATCA	Hebert et al. 2004
COI	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
COI	HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994
COI	RonMWASPdeg_t1	Forward	TGTAAAACGACGGCCAGTGGWTCWCCWGATATAKCWTTTCC	Smith et al. 2013
COI	RonIIdeg_R	Reverse	GGRGGRTARAYAGTTCATCCWGTWCC	Smith et al. 2005
COI	AMR1deg_R	Reverse	CAWCCWGTWCCKRMNCCWKCAT	Smith et al. 2005
ITS2	ITS2F	Forward	ATTCCCGGACCACGCCTGGCTGA	Veen et al. 2003
ITS2	ITS2R_Van	Reverse	CGCCTGATCTGAGGTCGTC	Veen et al. 2003
WSP	WSP81F	Forward	TGGTCCAATAAGTGATGAAGAAAC	Braig et al. 1998
WSP	WSP691R	Reverse	AAAAATTAAACGCTACTCCA	Braig et al. 1998

2.2. DNA extraction and PCR

DNA sequences, trace files and metadata are available in the Barcode of Life Data Systems (www.boldsystems.org) under the dataset DS-ALLOXYST Norwegian *Alloxysta* (DOI: dx.doi.org/10.5883/DS-ALLOXYST). DNA was extracted either from leg or whole specimens following standard protocols for insect tissue at the Canadian Centre for DNA Barcoding (CCDB). PCR and bidirectional Sanger sequencing for all markers were done at the CCDB.

For COI, PCR and sequencing on most samples were run with the LepF1 and LepR1 primers (Table 1), but samples obtained through the Global Malaise Project (indicated through the BIOUGXXXXX numbers) were run with cocktails of the Lep and Folmer primers (C_LepFolF and C_LepFolR) (Hernández-Triana et al. 2014). These samples were also only sequenced in the reverse direction. For some samples where it was difficult to get high quality sequences with the Lep-primers a combination of RonMWASPdeg_t1 + LepR1 (Smith et al. 2013) or LepF1 + C_ANTMR1D (cocktail of the primers RonIIdeg_R and AMR1deg_R) (Smith & Fisher 2009) were used. In total the dataset included 216 COI sequences of which 97.2% were longer than 500 bp. PCR and sequencing of a fragment of the Internal Transcribed Spacer (ITS 2) were done using the primers ITS2F and ITS2R_Van (Table 1). ITS 2 was attempted sequenced for all specimens in the dataset, but failed for 9 specimens. Seven of 207 sequences had high quality trace files in one direction only. GenBank accessions for COI and ITS2 are listed in Supplementary Table S1. A fragment of the *Wolbachia* Surface Protein (*wsp*) was attempted sequenced from all specimens using the primers WSP81F and WSP691R (Table 1) to investigate if high intraspecific variation in COI sequences could be explained by infections of different *Wolbachia* Hertig & Burt, 1924 strains. Six specimens obtained sequences that could be matched with existing *wsp* sequences in GenBank using megablast. The sequences are deposited in GenBank under accessions MG968805-MG968810.

2.3. Sequence editing and alignment

Sequences were edited by CCDB staff (COI) or by chromatogram alignments in the software Geneious 8.1.9 (ITS2 and *wsp*). COI sequences were aligned by amino acids using the ClustalW algorithm implemented in Mega 7 (Kumar et al. 2016) with default settings. Alignment was trivial for this marker, but a 6 bp stretch of indels were observed in the alignment at positions 469–474 for the outgroup species (*Phaenoglyphis villosa* (Hartig, 1841)). The final alignment was 654 bp long. Aligning the ITS2 sequences was considerably more difficult due to high variation and considerable length differences between sequences. We used MAFFT version 7 online service tool http://mafft.cbrc.jp/ alignment/server/ (Katoh et al. 2017; Kuraku et al. 2013) and inspected the resulting alignments from different settings. The final and visually most sound alignment used E-INS-i refinement method (Katoh et al. 2005), a gap opening penalty of 2.00, an offset value 0.0, and the 20PAM/k=2 scoring matrix. The aligned matrix (681 bp) was then run through GBlocks 0.91 (Castresana 2000; Talavera et al. 2007) on the GBlocks server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) allowing smaller final blocks, gap positions in final blocks and less strict flanking regions. The software filtered out 349 sites (51%) from the original alignment that were used in downstream analysis. All alignments were inspected and converted to various file formats using the software Mesquite 3.31 (Maddison & Maddison 2017).

2.4. Phylogenetic analyses

Datasets were partitioned with PartitionFinder 2.1.1 (Lanfear et al. 2012, 2017) implementing PhyMl (Guindon et al. 2010). The best substation models and partitions for MrBayes were selected by AICc, resulting in the following partitions: For COI 1st positions the GTR+G model; for 2nd positions the HKY+G model; for 3rd positions of COI and the full ITS2 the GTR+G+I model. The two

gene alignments were concatenated using the software SequenceMatrix 1.8 (VAIDYA et al. 2011).

Bayesian phylogenetic analyses were conducted with MrBayes 3.2.6 (HUELSENBECK & RONQUIST 2001; RONQUIST & HUELSENBECK 2003) using the best fit partitions and models from PartitionFinder. MCMC analyses were run with 4 chains for 10 mill generations, sampled each 1000 generation. The first 25% of the sampled trees were discarded as burn-in. The trace files generated by MrBayes were examined with the software Tracer 1.6 (RAMBAUT & DRUMMOND 2008) to ensure sufficient mcmc generations were run to obtain stability in the posterior probabilities and acceptable Estimated Sample Size (ESS) of all parameters.

Maximum Likelihood analyses were conducted with the software RAxML (STAMATAKIS 2014) locally under the RaxmlGUI (SILVESTRO & MICHALAK 2012) with 10 runs and 500 bootstrap replicates under the thorough bootstrap option. All analyses used the GTR+G+I model and the partitions found by PartitionFinder.

Graphical interpretations of the phylogenetic trees were obtained with FigTree 1.4.3 (RAMBAUT 2016) and MEGA 7 (KUMAR et al. 2016); shallow branches within monophyletic groups that carry the same identification were collapsed to improve readability; label adjustments were made with Adobe Illustrator CC.

3. Results

Three hundred and thirteen Charipinae specimens belonging to the genera *Alloxysta*, *Dilyta* and *Phaenoglyphis* were collected. All *Alloxysta* species collected in Norway have a Palaearctic distribution; most of them have a wide distribution and are recorded from many countries. Twenty-two *Alloxysta* species were identified (Supplementary Table S1): *A. arcuata* (Kieffer, 1902), *A. basimacula* (Cameron, 1886), *A. brachycera* Hellén, 1963, *A. brachyptera* (Hartig, 1840), *A. brevis* (Thomson, 1862), *A. castanea*, *A. citripes* (Thomson, 1862), *A. consobrina* (Zetterstedt, 1838), *A. curta* Ferrer-Suay, 2017, *A. fracticornis* (Thomson, 1862), *A. fuscipes*, *A. halterata* (Thomson, 1862), *A. macrophadna*, *A. marshalliana* (Kieffer, 1900), *A. obscurata* (Hartig, 1840), *A. pallidicornis*, *A. pilipennis* (Hartig, 1840), *A. pusilla* (Kieffer, 1902), *A. proxima* Belizin, 1962, *A. ramulifera* (Thomson, 1862), *A. victrix* and *A. xanthopa* (Thomson, 1862). Sixteen of these were recorded from Norway for the first time: *Alloxysta arcuata*, *A. basimacula*, *A. brachycera*, *A. brachyptera*, *A. brevis*, *A. citripes*, *A. consobrina*, *A. curta*, *A. fracticornis*, *A. halterata*, *A. marshalliana*, *A. obscurata*, *A. proxima*, *A. pusilla*, *A. ramulifera* and *A. xanthopa*.

Eight morphology-based species are retrieved as separate monophyletic entities in the results of analyses of the concatenated dataset (Fig. 2): *A. castanea*, *A. citripes*, *A. fuscipes*, *A. obscurata*, *A. pallidicornis*, *A. pilipennis*, *A. proxima* and *A. pusilla*. All of these, except *A. citripes*, also form monophyletic groups in the COI tree (Fig. 3), and almost all show deep splits suggesting strongly divergent mitogenomes between species. The results from the analyses of the ITS2 dataset are not directly comparable since some specimens lack sequences; however, *A. basimacula*, *A. castanea*, *A. fuscipes*, *A. pallidicornis*, *A. pilipennis* and *A. pusilla* are retrieved as monophyletic (Fig. 4).

For the concatenated dataset (Fig. 2), *A. ramulifera* and the brachypterous *A. curta* are present in a well-supported and well-defined clade with little internal divergence. Similarly, specimens of *A. victrix* are present within a well-defined clade of *A. consobrina*, and the brachypterous *A. halterata* groups with fully winged specimens of *A. brachycera*. Specimens morphologically fitting the descriptions of *Alloxysta fracticornis* and the brachypterous *A. brachyptera* are nested within a clade of quite divergent genetic groups of *A. brevis*, while specimens of *A. basimacula* and *A. macrophadna* each form two groups interrelated with *A. proxima* and *A. fuscipes*. Similar relationships are found in the COI-tree (Fig. 3) and in the ITS2-tree (Fig. 4), although genetic divergences in general are considerably lower in the latter.

Sequences of the *Wolbachia* outer surface protein (*wsp*) were obtained for six specimens: NOFIG1025 (*A. brevis*); NOFIG66, NOFIG1102 (*A. citripes* – 2 specimens); NOFIG85, BIOUG16335-D02 (*A. ramulifera* – 2 specimens); NOFIG1101 (*P. villosa*).

4. Discussion

There is considerable concordance between morphological identifications and molecular groups for several species. Sequences from all specimens of *A. castanea* form well-supported monophyletic groups for both markers. *Alloxysta castanea* is the most abundant species collected in this study and is currently recorded from all biogeographic regions (own observation). It was first described from Germany, but over the past few years *A. castanea* has been found in large numbers in collections around the world (FERRER-SUAY et al. 2013b, 2014a, 2015a, 2018). Our molecular data only includes Norwegian populations of *A. castanea* and it is possible that genetic divergence within this species is detected if more populations are sampled. Nevertheless, there are sequences in BOLD from Bulgaria, Canada, Germany and the UK that group with sequences from our Norwegian specimens.

Alloxysta citripes was first described from Sweden, but has later been recorded from the Neotropical region (Jamaica) and Nearctic region (Iowa, USA) (FERRER-SUAY et al. 2014a). There is considerable genetic divergence among the specimens we have sequenced from Norway, and ITS2 and COI sequences of specimen NOFIG1152 do not group with the remaining sequences in the individual gene trees. They do group together in the results

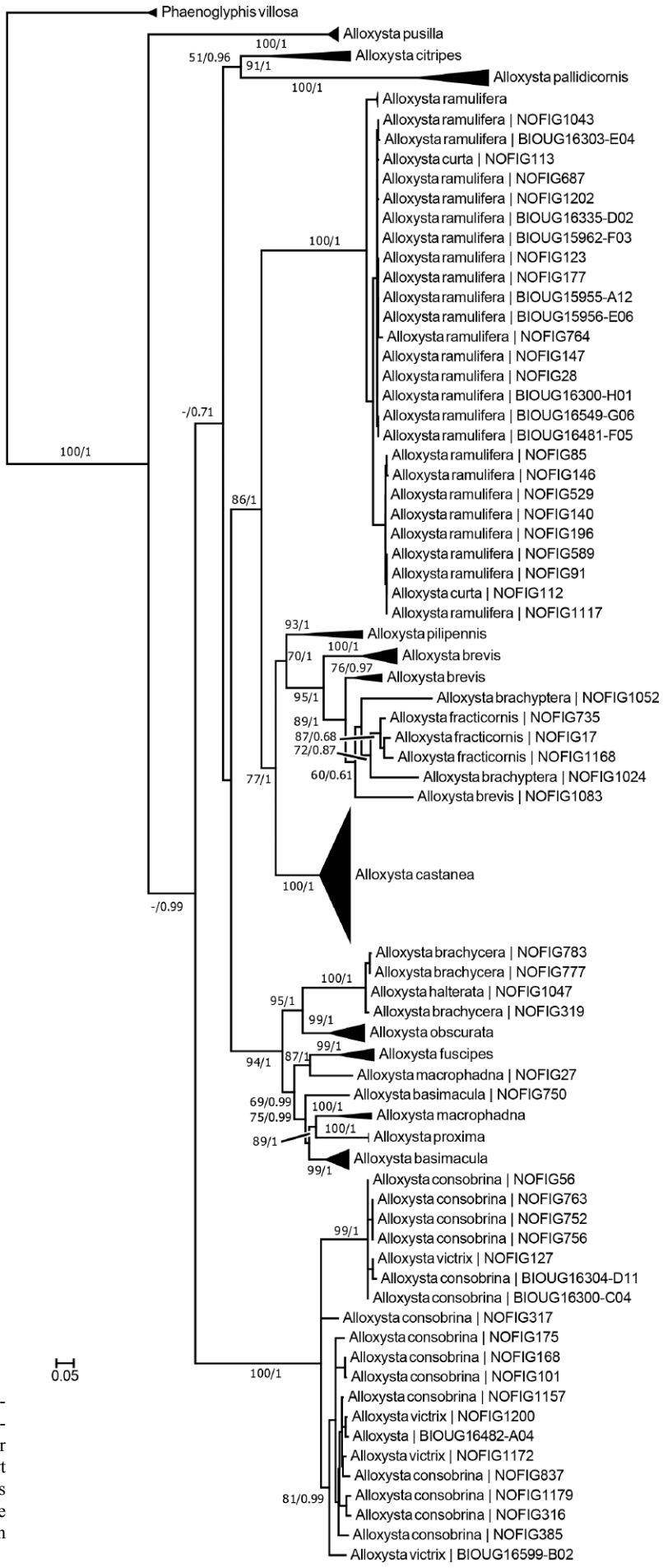


Fig. 2. Maximum likelihood tree from concatenated ITS2 and COI sequences. Groups containing specimens without conflicting identification or deep divergences are collapsed. Bootstrap-support and posterior probabilities from Bayesian analysis on branches, only values > 50 included. * indicate different relationships in results from Bayesian analyses.

from the concatenated dataset, however. Almost identical *Wolbachia* sequences were obtained from two closely grouped specimens, but not from NOFIG1152. Thus, there is a possibility that the observed intraspecific divergence is caused by endosymbiont infections in some populations. There are no other sequences of *A. citripes* in BOLD.

Alloxysta fuscipes was originally described from Sweden, but has later been recorded from different countries in northern Europe (Ferrer-Suay et al. 2012a). In BOLD, COI barcodes from Norway group tightly with those from several sites throughout Canada, indicating a wide northern Holarctic distribution of this species.

Alloxysta obscurata was first described from Germany, but has later also been found in the Nearctic and Neotropical regions (Andrews 1978; Ferrer-Suay et al. 2012b). Sequences from the Norwegian specimens cluster with one sequence from UK identified as *A. semiaperta* in BOLD. This is a sequence mined from GenBank (Accession JX507460), originally published by Elias et al. (2013). We have not examined the voucher, but *A. obscurata* and *A. semiaperta* are morphologically very similar and can only be separated by the length ratios between antennal flagellomeres: F2–F4 subequal in *A. semiaperta*, but F2 longer than F3 and F3 shorter than F4 in *A. obscurata*.

Alloxysta pallidicornis was originally described from England. It is characterized by having a completely open radial cell, pronotal and propodeal carinae, and a very long flagellomere F1. DNA barcodes from Norwegian populations match that of one specimen from southern Germany. Otherwise, there are no records of this species in BOLD.

Alloxysta pilipennis was originally described from Germany and it has been also recorded in the Nearctic and Neotropical regions (Andrews 1978; Ferrer-Suay et al. 2012b). DNA barcodes of Norwegian specimens do not group with any other barcodes in BOLD.

Alloxysta proxima was first described from the Medny Island east of the Kamchatka peninsula, but is later reported from USA (Ferrer-Suay et al. 2014a) and France (Ferrer-Suay et al. 2015a). Our specimens of this species are collected from Spitsbergen, near Longyearbyen (78°N) and constitute the most northern record of the species (perhaps of any *Alloxysta*). The specimens fit the description of the species despite being found geographically very distant from previous records. The DNA barcodes of our specimens do not group with any other sequences in BOLD, and are the only sequences of *A. proxima* in this database as well as in the NCBI GenBank. There are only three confirmed resident aphid species on Spitsbergen (Coulson et al. 2014): *Acyrthosiphon svalbardicum* Heikinheimo, 1968, *Sitobion calvulum* (Ossiannilsson, 1958) and *Pemphigus* sp., possibly the widespread *P. groenlandica* (Rübsamer, 1898). It is not known which might be the host of *A. proxima*, but two recently described Braconidae, *Diaeretellus svalbardicum* Chaubert, 2012 and *Aphidius leclanti* Chaubert, 2012, are known parasitoids of *A. svalbardicum*, and *A. svalbardicum* and *S. calvulum*, respectively (Coulson et al. 2014). Both of these braconid species have been recorded from Longyearbyen near the two localities where our *A. proxima* specimens were collected and are potential intermediate hosts.

Alloxysta pusilla was first described from France, but has also been found in the Neotropical Region (Ferrer-Suay et al. 2018). Our DNA barcodes in BOLD do not match barcodes from other regions and our sequences represent the only DNA barcodes of *A. pusilla* in BOLD.

The remaining morphospecies are in some sort of conflict with the results from the molecular analyses. *Alloxysta basimacula* is paraphyletic in our trees with sequences of *A. macrophadna* and *A. proxima* nested within the same clade. *Alloxysta basimacula* was first described from Scotland, while *A. macrophadna* was described from Germany, this species has been widely recorded in the Palaearctic region and also in the Nearctic: USA and Canada. The specimens causing the conflict are NOFIG750 (*A. basimacula*) and NOFIG27 (*A. macrophadna*), which show considerable genetic divergence compared to the other members of their species, and group with different clades in the trees (Figs. 2–4). *Alloxysta basimacula* can be separated from both *A. macrophadna* and *A. proxima* by having a completely open (as opposed to partially closed) radial cell. *Alloxysta macrophadna* is similar to *A. fuscipes* for this character, but can be separated by not having subequal flagellomeres F2–F4. No specimens of either of these species were positively diagnosed with *Wolbachia*. Although the effect of endosymbionts cannot be ruled out completely (only leg tissue was used to obtain DNA), we suspect that there might be cryptic species hidden within both of these names.

Specimens of *A. brachycera* and *A. halterata* group tightly in all trees (Figs. 2–4), and have almost identical DNA barcodes. *Alloxysta brachycera* was originally described from Finland, and has previous to this study only been recorded from France (Ferrer-Suay et al. 2015a). The dimorphic *A. halterata* was first described from Sweden, but later recorded from different countries in Europe (Hellén 1963; Hübner et al. 2002; Cameron 1886). Studies of wing dimorphism in *A. halterata* have shown that males of this species are always short-winged, while the females occur in both short-winged and long-winged forms (van Veen, pers. comm). Fully winged morphs of *Alloxysta halterata* can be confused with the fully winged species *A. crassa* (Cameron, 1889), and *A. crassa* and *A. barchycera* are very similar in having an open radial cell and pronotal carinae, but missing propodeal carinae and rhinaria, and having a club-shaped proximal end of flagellomere F4. Our results indicate that the three species could be the same, but a more detailed morphological and molecular study with broader sampling is necessary to resolve the taxonomy of this species complex.

Genetic groups within the species *Alloxysta brevis* are genetically deeply divergent in our results, and the species is also paraphyletic with specimens of *A. fracti-*

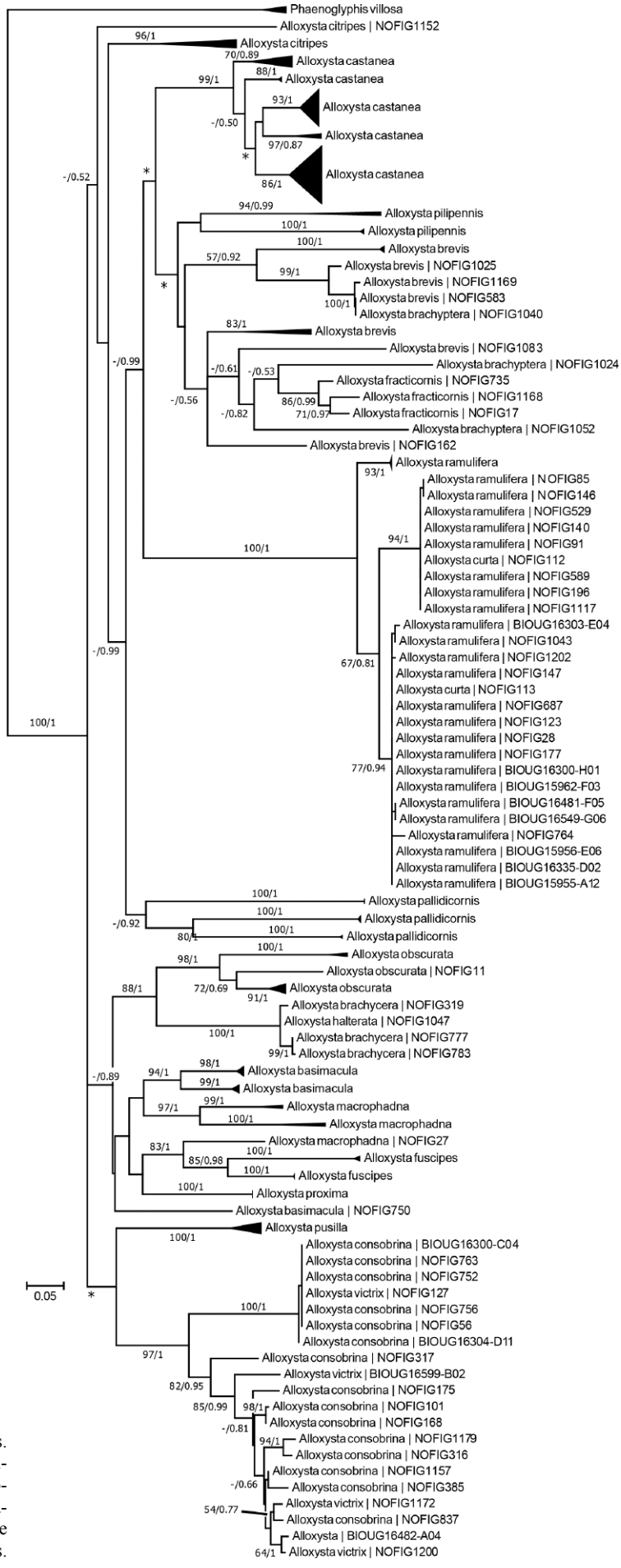
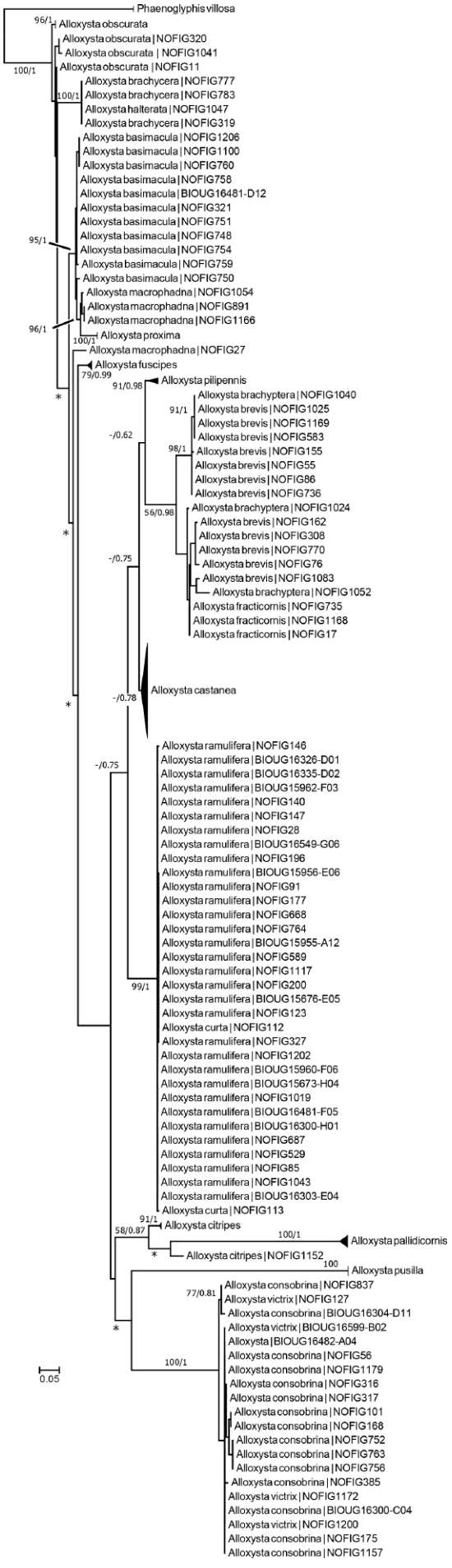


Fig. 3. Maximum likelihood tree from COI sequences. Groups containing specimens without conflicting identification or deep divergences are collapsed. Bootstrap-support and posterior probabilities from Bayesian analysis on branches, only values > 50 included. * indicate different relationship in results from Bayesian analyses.



cornis and *A. brachyptera* nested within fairly well-supported clades in the concatenated- and ITS-trees (Figs. 2, 4). *Alloxysta brevis* and *A. fracticornis* are both Palaearctic in distribution, and originally described from Sweden. However, *A. brevis* is considered to be cosmopolitan as it has been recorded from all biogeographical regions except Antarctica (FERRER-SUAY et al. 2017). *Alloxysta brevis* and *A. fracticornis* are morphologically very similar in having a small and closed radial cell, no pronotal carinae, and propodeal carinae forming a plate. However, they can be separated based on the relationship of the antennal flagellomeres: F1 is longer than pedicel and F2 in *A. fracticornis*, and F1 is shorter than pedicel and subequal to F2 in *A. brevis*. The two species are undoubtedly closely related, but a thorough taxonomic revision, combining morphological and molecular characteristics, should be conducted before eventual synonymization. *Alloxysta brachyptera* was first described from Germany, but has been recorded throughout Europe (FERRER-SUAY et al. 2017). *Alloxysta brevis* and *A. brachyptera* have been established as related (FERRER-SUAY et al. 2017). One specimen of *A. brevis* tested positive for *Wolbachia*. Thus, endoparasitism can possibly explain the genetically divergent pattern observed in this species.

Alloxysta ramulifera was originally a Palaearctic species, first described from Sweden. It is widely recorded throughout the Palaearctic region (Ferrer-Suay et al. in prep.) and now also from the Nearctic (New Mexico, USA) (FERRER-SUAY et al. 2014a). In our results, *A. ramulifera* groups with the recently described *A. curta* Ferrer-Suay et al., 2017. *Alloxysta curta*, and the possibility that this species is the brachypterous form of *A. ramulifera*, was discussed in the original description (FERRER-SUAY et al. 2017). Our results indeed indicate that this is the case, but we would like to await a more thorough morphological analysis before suggesting a formal synonymy. One specimen of *A. ramulifera* tested positive for *Wolbachia.* Although such endosymbiont infections can lead to shared mitochondria between species (SMITH et al. 2012), we doubt this is the case here since the exact same relationship is found in the nuclear ITS2 data.

Alloxysta consobrina is a cosmopolitan species, very widely distributed in all biogeographical regions, and very common in collections around the world (own observation). The species was formerly known as *A. fuscicornis* (FERRER-SUAY et al. 2013a). In our results, three specimens have been identified as *A. victrix*, they are nested within a well-supported clade of *A. consobrina*. The two species have very similar morphologies, and their possibly close relationship was discussed by FERRER-SUAY et al. (2013b). Both species have closed radial cells, pronotal carinae, and no propodeal carina. FER-

← **Fig. 4.** Maximum likelihood tree from ITS2 sequences. Groups containing specimens without conflicting identification or deep divergences are collapsed. Bootstrap-support and posterior probabilities from Bayesian analysis on branches, only values > 50 included. * indicate different relationship in results from Bayesian analyses.

GUSSON (1986) recognized the similarity between *A. fuscicornis* and *A. victrix*, and synonymized these species without any detailed discussion of characters. However, MENKE & EVENHUIS (1991) found that they are quite distinct when reared from the mummies of *Macrosiphum rosae* (Linnaeus, 1758) and related species, as well as *Brevicoryne brassicae* (Linnaeus, 1758) (MENKE & EVENHUIS 1991). *Alloxysta fuscicornis* and *A. victrix* differ in the size of the radial cell, the length of the flagellomeres and the colour of the head. For these reasons, MENKE & EVENHUIS (1991) resurrected *A. fuscicornis* from the synonymy with *A. victrix*, but mentioned that the taxonomy of the two species needed further study. More recently, VAN VEEN et al. (2003) sequenced a variable nuclear gene region (ITS2) for several *Alloxysta* species. The authors showed that each species possesses a unique allele with no intraspecific variation, concluding that *A. victrix* and *A. fuscicornis* should be considered as different species. The ITS2 sequences that we obtained from specimens identified as *A. consobrina* and *A. victrix* do not show a similar pattern, as there are no species-specific alleles for either species (manual inspection of full alignment). Although more variable, the COI sequence-data show the same, and specimens of *A. victrix* do not group together in any of the trees (Figs. 2–4). It could be that we have a different morphological interpretation of these species than VAN VEEN et al. (2003), and we have certainly sampled different populations. We argue, however, that our results support a more thorough taxonomic review of this species complex including a broader sampling throughout the distribution ranges of these species.

Wolbachia infections are well-known to occur in various groups of Hymenoptera, including parasitoids, and also reported from Figitidae (e.g. DAVIES et al. 2013; WACHI et al. 2015). Our results apparently are the first to report infections of this endosymbiont in *Alloxysta* and also within Charipinae. The infection rate of the investigated specimens in our study was low (2.7%), but this is likely biased by the fact that most of our DNA isolates originate from extractions of leg-tissue only. We would expect a higher infection rate if full bodies were used in the extractions as *Wolbachia* concentrations are known to be higher in the abdomen of infected specimens.

5. Conclusions

Our study provides some new insights into the molecular characterisation of species in the genus *Alloxysta*. While some morphological species are well supported by mitochondrial and nuclear markers, others are questioned and will require more thorough morphological and molecular analysis to be taxonomically resolved. Most of the subfamily Charipinae lacks molecular data for detailed analyses of relationships between and within its species. Thus, more studies like this are necessary to clarify the species boundaries and phylogenetic relationships within the subfamily. We show that *Wolbachia* infections are present in *Alloxysta*, but our data is too scarce and scattered to conclude on the influence this endoparasitism might have in shaping the evolutionary history of the genus.

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Electronic Supplement File

at http://www.senckenberg.de/arthropod-systematics

File 1: ferrersuay&al-figitidaealloxysta-asp2018-electronicsupplement-1.pdf — **Table S1.** List of examined specimens with associated ID-numbers in BOLD and GenBank accessions.