

Surveys of stink bug egg parasitism in Asia, Europe and North America, morphological taxonomy, and molecular analysis reveal the Holarctic distribution of *Acroclisoides sinicus* (Huang & Liao) (Hymenoptera, Pteromalidae)

Giuseppino Sabbatini Peverieri¹, Mircea-Dan Mitroiu², Marie-Claude Bon³, Rammohan Balusu⁴, Luca Benvenuto⁵, Iris Bernardinelli⁵, Henry Fadamiro⁴, Martina Falagiarda⁶, Lucian Fusu², Emily Grove⁷, Tim Haye⁷, Kim Hoelmer⁸, Emily Lemke⁷, Giorgio Malossini⁵, Leonardo Marianelli¹, Matthew R. Moore⁹, Alberto Pozzebon¹⁰, Pio-Federico Roversi¹, Davide Scaccini¹⁰, Paula Shrewsbury¹¹, Glynn Tillman¹², Paola Tirello¹⁰, Rebecca Waterworth¹¹, Elijah J. Talamas^{13,14}

1 CREA – Research Centre for Plant Protection and Certification, Florence, Italy **2** UAIC – “Alexandru Ioan Cuza” University of Iasi, Faculty of Biology, Iasi, Romania **3** USDA-ARS – European Biological Control Laboratory, Montferrier le Lez, France **4** Department of Entomology and Plant Pathology, Auburn University, Auburn, AL, USA **5** ERSA – Regional Agency for Rural Development, Plant Health Service, Udine, Italy **6** Laimburg Research Centre, Vadena (Bolzano), Italy **7** CABI, Rue des Grillons 1, 2800, Delémont, Switzerland **8** USDA-ARS – Beneficial Insects Introduction Research Unit, Newark, DE, USA **9** Molecular Diagnostics Laboratory, Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville, FL, USA **10** DAFNAE – Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padua, Legnaro (Padova), Italy **11** University of Maryland, Department of Entomology, College Park, MD, USA **12** USDA-ARS – Crop Protection & Management Research Unit, Tifton, GA, USA **13** Florida State Collection of Arthropods, Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville, FL, USA **14** USDA-ARS – Systematic Entomology Laboratory, Washington, DC, USA

Corresponding author: Mircea-Dan Mitroiu (mircea.mitroiu@uaic.ro)

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Abstract

Halyomorpha halys is an invasive, widespread stink bug for which only short-term solutions are currently available for pest control worldwide. The need for long-term management solutions for *H. halys* has driven studies on augmentative and classical biological control of this species, especially by its egg parasitoids. Numerous investigations in Asia, USA, and Europe on native and exotic egg parasitoids of *H. halys*, and the effects on non-target pentatomids, have improved the global knowledge of parasitoid-host relationships, uncovered new associations, and led to the discovery of new species. This trend continues with *Acroclisoides sinicus*, a pteromalid that was described in the 1980's from Asia. In this work we report recent findings of this species in North America and Europe. Moreover, we propose that *Acroclisoides solus* **syn. nov.**, a species described originally from the USA, is conspecific with *A. sinicus* based on morphological and molecular analysis.

Keywords

brown marmorated stink bug, egg parasitoids, exotic species, biological control

Introduction

Halyomorpha halys (Stål) (Hemiptera: Pentatomidae) is a stink bug originating from eastern Asia, which in the last three decades has invaded many regions worldwide: North America in the mid-1990s, followed by Europe in the mid-2000s and, more recently, South America (Hoebeke and Carter 2003; Wermelinger et al. 2008; Faúndez and Rider 2017). In its native range, natural enemies, including egg parasitoids, are among the primary factors controlling its populations (Yang et al. 2009; Lee 2015; Leskey and Nielsen 2018). Egg parasitoids of *H. halys* have followed this species in its spread to new areas, likely transported as they develop in eggs laid on plants or other materials. *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), commonly known as the samurai wasp, was first detected in 2014 in the eastern USA, followed by a second discovery in the northwestern USA in 2016 (Talamas et al. 2015; Milnes et al. 2016). It has since spread north to Canada, where it has been present since at least 2017 (Abram et al. 2019; Gariépy and Talamas 2019). *Trissolcus japonicus* was recently detected in Europe, first in Switzerland in 2017 and then in Italy in 2018 (Sabbatini Peverieri et al. 2018; Moraglio et al. 2019; Stahl et al. 2019). Moreover, *T. japonicus* is not the only species that has followed *H. halys* in its worldwide expansion: the Asian egg parasitoid *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) was detected for the first time in Europe (northeastern Italy) in 2018 (Sabbatini Peverieri et al. 2018).

These examples of egg parasitoids following their host in the colonization of new areas are not isolated cases. There are many examples, including *Aprostocetus fukutai* Miwa & Sonan (Hymenoptera: Eulophidae) that recently followed its host *Anoplophora chinensis* (Förster) (Coleoptera: Cerambycidae) from eastern Asia to Europe (Delvare et al. 2004; Hérard et al. 2017).

However, not all adventive parasitoids are beneficial for controlling populations of *H. halys*. *Acroclisoides* Girault and Dodd (Hymenoptera: Pteromalidae) was established

in 1915 and currently comprises just over a dozen species in the Afrotropics, Australia, and South Asia. A single species with a disjunct distribution, *Acroclisoides solus* Grissell & Smith, 2006 (Hymenoptera: Pteromalidae), was described from North America (Grissell and Smith 2006; see Noyes 2019) (Table 1). *Acroclisoides* includes species that are apparently facultative or obligate hyperparasitoids, in most cases of pentatomid eggs parasitized primarily by scelionids, but also by eupelmids (Clarke and Seymour 1992; Sureshan and Narendran 2002; Grissell and Smith 2006).

We present the records of *Acroclisoides sinicus* (Huang & Liao, 1988) (Hymenoptera: Pteromalidae) from central Europe and the USA. We treat *A. solus* syn. nov. as a new junior synonym of *A. sinicus* based on morphological and molecular comparisons, revealing that *A. sinicus* is a widespread Holarctic species. Our analysis of *COI* diversity, and the distribution of other species in the genus, suggest that the European and North American populations are recent introductions.

Material and methods

Surveys in Friuli Venezia Giulia region (northeastern Italy)

Field surveys were conducted in 2018 by personnel of the local Plant Protection Service (ERSA Friuli Venezia Giulia) in Cordenons commune (46.0082N, 12.6713E) as a part of routine monitoring of *H. halys* in the region. During these surveys, more than a dozen egg masses were found to be parasitized by *T. mitsukurii* (Sabbatini Peverieri et al. 2018). In Cordenons, on August 7, three *H. halys* egg masses collected on a *Robinia pseudoacacia* L. hedgerow near an IPM apple orchard appeared to be parasitized (visually detected by the dark color of the eggs). Collected egg masses were reared in a climatic chamber (26 °C, 65% RH, 16:8 L:D) until emergence of parasitoids. Emerged parasitoid specimens were stored in ethanol for further taxonomic and molecular analysis.

Surveys in Veneto region (northeastern Italy)

In the Veneto region, field pest surveys were conducted by personnel of the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padua, during the summers of 2017 and 2018. At three sites in the vicinity of Povegliano (45.7575N, 12.1872E), Montebelluna (45.7586N, 12.0174E) and Riese Pio X (45.7170N, 11.9397E), dozens of *H. halys* egg masses were collected in apple and kiwi orchards and vineyards implementing integrated pest management and in surrounding hedgerows of *R. pseudoacacia*, *Acer campestre* L., *Sambucus nigra* L. and *Prunus* sp. Collected egg masses were reared in a climatic chamber (26 °C, 65% RH, 16:8 L:D) until emergence of parasitoids. Emerged parasitoid specimens were stored in ethanol for further taxonomic and molecular analysis.

Table 1. Worldwide *Acroclisoides* species¹.

Species	Distribution	Citations of primary host (phytophagous)	Citations of primary parasitoid host	References
<i>Acroclisoides africanus</i> Ferrière, 1940	Central Africa, Madagascar	<i>Atelocera notatipennis</i> Stål; <i>Antestopsis lactoides</i> Grathoff; <i>Antestopsis intricata</i> (Ghesquire & Carayon); <i>Antestopsis orbitalis</i> (Westwood); <i>Antestopsis thumbergii</i> (Gmelin); <i>Bathycyelia</i> <i>radhaii</i> Schouteden; <i>Bathycyelia thalassina</i> (Herrich-Schaeffer); Pentatomidae species	<i>Trisolcus</i> sp. <i>Asolcus</i> sp.	Ferrière 1940; Grissell and Smith 2006; see Noyes 2019 ²
<i>Acroclisoides bicolor</i> Luo & Qin, 1991	China	<i>Halyomorpha picus</i> (Fabricius)	n.a.	Luo and Qin 1991; Xiao and Huang 2000
<i>Acroclisoides indicus</i> Ferrière, 1931	China, India, Sri Lanka, Myanmar	<i>Placosternum dama</i> (Fabricius); <i>Erithrina</i> sp.; Pentatomidae species	n.a.	Ferrière 1931; Luo and Qin 1991; Xiao and Huang 2000; Sureshan and Narendran 2002; Grissell and Smith 2006; Sureshan 2007; see Noyes 2019 ²
<i>Acroclisoides laticeps</i> Girault & Dodd, 1915	Australia	n.a.	n.a.	Girault and Dodd 1915; see Noyes 2019 ²
<i>Acroclisoides luzonensis</i> Gahan, 1920	China, Philippines	<i>Tectocoris lineola</i> (Fabricius); Pentatomidae species; Scutelleridae species	<i>Trisolcus banksi</i> (Gahan) <i>Trisolcus</i> sp.	Gahan 1920; Grissell and Smith 2006; Xiao and Huang 2000; see Noyes 2019 ²
<i>Acroclisoides maculatus</i> Sureshan & Narendran, 2002	India	n.a.	n.a.	Sureshan and Narendran 2002
<i>Acroclisoides major</i> Girault & Dodd, 1915	Australia	n.a.	n.a.	Girault 1915; see Noyes 2019 ²
<i>Acroclisoides megacephalus</i> Girault & Dodd, 1915	Australia	<i>Aziagastus cambelli</i> Distant	<i>Anatatus</i> sp. <i>Trisolcus patineti</i> (Ferrière)	Girault 1915; Grissell and Smith 2006; see Noyes 2019 ²
<i>Acroclisoides quintus</i> Xiao & Huang, 2000	China	n.a.	n.a.	Xiao and Huang 2000
<i>Acroclisoides sinicus</i> (Huang & Liao, 1988)	China, Korea, Italy, Switzerland, USA	Pentatomidae species (China, Korea); <i>Halyomorpha halys</i> (Stål); <i>Palomena prasina</i> L.; <i>Chinavia hilaris</i> (Say); <i>Euschistus</i> sp.; <i>Brochymena</i> sp.	n.a. (China, Korea); <i>Anatatus bifasciatus</i> (Geoffroy); <i>Anatatus reduvii</i> (Howard); <i>Trisolcus edesae</i> Fovis; <i>Trisolcus</i> <i>euschisti</i> (Ashmead); <i>Trisolcus japonicus</i> (Ashmead); <i>Trisolcus mitsukurii</i> (Ashmead)	Huang and Liao 1988; Xiao and Huang 2000; Grissell and Smith 2006; Ko et al. 2018; present work
<i>Acroclisoides satina</i> Kumar & Khan, 2012	India	<i>Phymomyza atricornis</i> Meigen	n.a.	Kumar and Khan 2012
<i>Acroclisoides solus</i> Grissell & Smith, 2006	USA, Canada, Italy	n.a. (USA); <i>Acrosternum hilare</i> Say (Canada); <i>Arma custos</i> Fabricius (Italy)	n.a. (USA and Italy); <i>Trisolcus</i> sp. (Canada)	Grissell and Smith 2006; Gartepey et al. 2014; Moraglio et al. 2019
<i>Acroclisoides spilopteris</i> (Masi, 1917)	Seychelles	n.a.	n.a.	Masi 1917; Grissell and Smith 2006; see Noyes 2019 ²
<i>Acroclisoides tectacorsii</i> (Girault, 1924)	Australia	<i>Biprorilus bibax</i> Bredin; <i>Oechalia consocialis</i> (Boisduval); <i>Tectocoris banksii</i> (Donovan); <i>Tectocoris lineola</i> (Fabricius)	<i>Trisolcus biproruli</i> Girault ³ ; <i>Anatatus</i> <i>biproruli</i> Girault	Girault 1924; Grissell and Smith 2006; James 1990; see Noyes 2019 ²

¹ *Acroclisoides boneruae* (Risbec 1957) is not listed here since it needs to be transferred to another genus (Mircea-Dan Mitroiu, unpublished data).² see Noyes 2019 for additional references.³ possible hosts based on the presumption that *A. sinicus* is a hyperparasitoid of *Trisolcus* and *Anatatus*.

Surveys in Trentino-Alto Adige/Südtirol region (northern Italy)

In 2018, during monitoring of *H. halys*, naturally laid egg masses of *H. halys* were collected in a parking area in the municipality of Ora (46.3620N, 11.2985E), in the province of Bolzano. Several species of maple trees (*Acer platanoides* L., *Acer negundo* L. and *Acer pseudoplatanus* L.), ailanthus [*Ailanthus altissima* (Mill.) Swingle] and linden (*Tilia platyphyllos* Scop.) were sampled for egg masses once or twice per week. Egg masses were collected between September 10 and October 26. Field-collected eggs were reared in a climatic chamber at 25 ± 1 °C, $65 \pm 5\%$ RH, 16:8 L:D until *H. halys* nymphs or parasitoids emerged. Emerged parasitoids were stored in 70% ethanol for further analysis.

Surveys in Zurich city (northeastern Switzerland)

In 2019, natural egg masses of *H. halys* (n = 11) and *Palomena prasina* L. (Hemiptera: Pentatomidae) (n = 6) were collected in an urban park area at the lake of Zurich (district Seefeld, 47.355912N, 8.550674E), where adventive *H. halys* populations were first detected in Europe in 2007 (Wermelinger et al. 2008). Trees inspected for eggs included *Catalpa bignonioides* Walter, *Paulownia tomentosa* (Thunb.) Steud., *Tilia platyphyllos*, and *Liriodendron tulipifera* L. Egg masses were collected on three occasions (June 28, July 2 and 10). Field collected egg masses were kept in small Petri-dishes and stored at 25 ± 1 °C, 60% RH, 16:8 L:D until nymphs and parasitoids had emerged. Emerged parasitoid specimens were stored in ethanol for further taxonomic and molecular analysis.

Surveys in Alabama and Georgia (southeastern USA)

In 2017 and 2018, during monitoring activities of stink bugs and their egg parasitoids, two sites yielded naturally laid stink bug egg masses from which *Acroclisoides* emerged. One was an egg mass of *H. halys* collected from a pecan tree [*Carya illinoensis* (Wangenh.) K. Koch] close to a commercial organic blueberry farm in Auburn (32.5325N, 85.4316W), Lee County, Alabama. Three egg masses of *Chinavia hilaris* (Say) (Hemiptera: Pentatomidae) were collected from a mimosa tree (*Albizia julibrissin* Durazz) in Irwin County, Georgia (31.3339N, 83.1926W). The egg masses were reared in a walk-in environmental chamber at 25 ± 2.0 °C, $50 \pm 10\%$ RH, and 12:12 L:D photoperiod for parasitoid emergence.

Surveys in Maryland (mid-Atlantic USA)

In 2017 and 2018, Project ‘Stink-be-Gone’, a citizen science project in collaboration with University of Maryland Extension and Master Gardeners, was established in cen-

tral and western Maryland to monitor for stink bug egg masses, including *H. halys*. Sampling occurred for an average of one hour per week for six weeks (July and August 2017; late June to early August 2018). Participants recorded the time spent searching and collected GPS coordinates and general habitat characteristics (e.g., “private yard” or “park”) for all survey periods regardless of collection results. If egg masses were found, the host plant was identified to genus or species. Following collection, all egg masses and collection data were immediately sent to researchers at the University of Maryland, College Park for processing. Samples were placed in a growth chamber (Model 36LLVL, Percival Scientific, Perry, Iowa, USA) at 25 °C and 16:8 L:D. Egg masses were monitored daily for emergence of either bug nymphs or parasitoids. Parasitoids were transferred to 70% ethanol for later identification to species (all *Trissolcus* Ashmead and *Telenomus* Haliday and females of *Anastatus* Motschulsky) or genus (*Anastatus* males, see Burks 1967). After six weeks, unhatched eggs were dissected to ascertain their fate (e.g., unemerged bug nymph or parasitoid adult, partially developed parasitoid, infertile bug egg). Egg masses were identified to genus (unpublished key by Dieckhoff and Hoelmer). Master Gardeners collected egg masses of eight stink bug genera throughout both summers.

Origin of other material examined

Other samples of *Acroclisoides* specimens used in the molecular analysis were collected in China and South Korea by Kim Hoelmer from 2014–2017 and by Lucian Fusu during field trips conducted in 2016 (see results, Table 4). Paratypes of *A. solus* were provided on loan from the National Museum of Natural History, Smithsonian Institution, Washington, D.C. The remaining sequences analyzed here were mined from GenBank.

Morphological analysis and material examined

The following keys and taxonomic works were used for the identification of *Acroclisoides* species: Grissell and Smith (2006), Sureshan and Narendran (2002), Xiao and Huang (2000), Huang and Liao (1988), Masi (1917), Girault and Dodd (1915).

The examined material listed below (all initially unidentified Pteromalidae, except paratypes of *A. solus*), including vouchers used in the molecular analyses, is deposited in the following institutions: CABI, Delémont (Switzerland); CREA, Florence (Italy); DAFNAE, University of Padua, Padova (Italy); EBCL, Montferrier le Lez (France); ERSA, Udine (Italy); FSCA, Florida State Collection of Arthropods, Florida (USA); Laimburg RC, Laimburg Research Centre, Vadena (Italy); MICO, Mitroiu Collection, Iași (Romania); NHMB, Natural History Museum Bern (Switzerland).

Italy: 19♀♀, 9♂♂, Cordenons, Friuli V. G., 46.0082N, 12.6713E, 8.viii.2018, Iris Bernardinelli, Giorgio Malossini & Luca Benvenuto leg., on *Halyomorpha halys*

eggs on *Robinia pseudoacacia* (6♀♀, 3♂♂, CREA; 9♀♀, 6♂♂, ERSA; 1♀, FSCA; 3♀♀, MICO); 1♀, 8 unsexed, Riese Pio X, Veneto, 29.viii.2017, Paola Tirello and Davide Scaccini leg., on *Halyomorpha halys* eggs on *Vitis vinifera* (1♀, MICO; 8 unsexed DAFNAE); 3♀♀, 3 unsexed, Montebelluna, Veneto, 29.viii.2017, Paola Tirello and Davide Scaccini leg., on *Halyomorpha halys* eggs on *Vitis vinifera* (1♀, 3 unsexed, DAFNAE; 2♀♀, MICO); 4♀♀, 5 unsexed, Povegliano, Veneto, 16.viii.2017, Paola Tirello and Davide Scaccini leg., on *Halyomorpha halys* eggs on *Actinidia* sp. (1♀, 5 unsexed, DAFNAE; 3♀♀, MICO); 12♀♀, 2 unsexed, Ora, Trentino-Alto Adige/Südtirol, 46°21'43.3"N, 11°17'54.6"E, 27.ix.2018, Martina Falagiarda, on *Halyomorpha halys* eggs on *Acer* sp. (10♀♀, 2 unsexed, Laimburg RC; 2♀♀, MICO).

Switzerland: 5♀♀, 3♂♂, Zurich city, Canton Zurich, Lat. 47.351708 Long. 8.559493, 2.vii.2019, Tim Haye and Emily Grove leg., ex eggs of *Halyomorpha halys* on *Tilia platyphyllos* (ZP4), (1♀, MICO; 1♀, NHMB, 3♀♀, 3♂♂, CABI); 9♀♀, 6♂♂, Zurich city, Canton Zurich, Lat. 47.353213 Long. 8.553968, 10.vii.2019, Emily Lemke and Emily Grove leg., ex eggs of *Halyomorpha halys* on *Liriodendron tulipifera* (ZP6), (1♀, MICO; 3♀, NHMB; 5♀♀, 6♂♂, CABI); 12♀♀, 8♂♂, Zurich city, Canton Zurich, Lat. 47.353213 Long. 8.553968, 10.vii.2019, Emily Lemke and Emily Grove leg., ex eggs of *Palomena prasina* on *Liriodendron tulipifera* (ZP7), (1♀, 1♂, MICO; 1♀, 1♂, NHMB; 10♀♀, 6♂♂, CABI); 6♀♀, 6♂♂, Zurich city, Canton Zurich, Lat. 47.354905 Long. 8.535045, 10.vii.2019, Emily Lemke and Emily Grove leg., ex eggs of *Palomena prasina* on *Catalpa bignonioides* (ZP8), (1♀, 1♂, MICO; 1♀, 1♂, NHMB; 4♀♀, 4♂♂, CABI).

South Korea: 5♀♀ [GB] Gyeongsan-si, Daehak-ro, 280, Yeungnam Univ., 35°49'11.6"N, 128°45'53.6"E, 14.viii.2016, L. Fusu (MICO); 1♀ S. Korea: Chungbuk, Okcheon-gun, Bougimyeon, Soesan-li, 150 m, Malaise trap, 10.ix–03.x.2004, 36°16.594'N, 127°36.742'E, Tripotin rec. (MICO).

USA: 2♀♀ paratypes of *A. solus*, VA: Fairfax Co. near Annandale 38°50'N, 77°12'W Aug. 17–20 2004 Malaise trap David R. Smith; 2♀♀ Alabama, Lee Co., Auburn, ex BMSB eggs on pecan, R. Balusu and G. Tillman 25.vii.2018 (FSCA); 1♀ Georgia, Irwin Co., ex *Chinavia hilaris* eggs on mimosa tree, G. Tillman 24.vii.2017 (FSCA); 3♀♀ Georgia, Irwin Co., ex *Chinavia hilaris* eggs on mimosa tree, G. Tillman 21.vii.2017 (FSCA).

Abbreviations of morphological terms: F, funicular segment; GT, gastral tergite; MT, metasomal tergite; MV, marginal vein; OOL, ocello-ocular line; PV, postmarginal vein; POL, posterior ocellar line; SM, submarginal vein; SV, stigmal vein.

Molecular analysis

The DNA extraction, PCR amplification and sequencing of all the specimens listed in Table 4 were conducted in three different laboratories: the USDA-ARS – European Biological Control Laboratory (EBCL), the Florida Department of Agriculture

and Consumer Service – Florida State Collection of Arthropods (FSCA), Division of Plant Industry (FDACS-DPI), and the Research Group in Invertebrate Diversity and Phylogenetics at the University of Iasi (UAIC). Genomic DNA was nondestructively isolated from the entire specimen using the Qiagen DNeasy kit (Hilden, Germany) at EBCL and FSCA as described in Sabbatini Peverieri et al. (2018) and at UAIC following the protocol developed by Cruaud et al. (2019). The barcode region of the mitochondrial Cytochrome Oxidase Subunit I (*COI*) was amplified using the universal barcoding primers LCO1490 and HCO2198 (Folmer et al. 1994). Amplification and sequence editing were done at EBCL and FSCA as described in Sabbatini Peverieri et al. (2018) and at UAIC as reported in Fusu and Ribes (2017).

Two *A. solus* samples required troubleshooting for successful *COI* barcoding. The samples USNMENT01335770 (MN018863.1) and FSCA00090246 (MN018864.1) both yielded *Wolbachia* *COI* sequences when using the universal barcoding primers (Folmer et al. 1994) and LEP-F1/LEP-R1 (Hebert et al. 2004) (see Smith et al. 2012 for an explanation of this phenomenon). These samples were alternatively amplified and sequenced with the primer pair C1-J-1632/C1-N-2191 (Kambhampati and Smith 1995; Simon et al. 1994). The thermocycling conditions for C1-J-1632/C1-N-2191 were: 1) initial denaturing at 95 °C for 2 minutes, 2) 98 °C for 20 seconds, 3) 40 °C for 30 seconds, 4) 72 °C for 30 seconds [steps 2–4 repeated for 30 cycles], and 5) final extension at 72 °C for 7 minutes. Slightly shorter (432 bp and 484 bp) *COI* barcode sequences were generated from these samples using this primer pair.

All sequences generated from this study are deposited in GenBank and all residual DNAs are archived at the place of the DNA extraction (Table 4). Voucher specimens which have been reexamined following the molecular analysis are presently deposited in public collections or in laboratory collections (Table 4). All barcode sequences were translated into amino acids to check for stop codons. The 45 sequences obtained were compared with sequences present in GenBank using the Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLASTn>) and aligned with the four barcode sequences of *A. solus* previously available. The alignment was performed using Clustal Omega (Sievers and Higgins 2014), implemented in Seaview version 4 (Gouy et al. 2010). Polymorphism information, haplotype diversity (*H_d*), nucleotide diversity (π) and number of haplotypes were determined using DnaSP version 6 (Rozas et al. 2017). The phylogenetic relationships among haplotypes were depicted using statistical parsimony in TCS (Clement et al. 2000) implemented in PopART (Leigh and Bryant 2015) which allows visualization of the frequency and geographical distribution of haplotypes.

The molecular distances between sequences of all individuals from different countries were calculated by the standard Kimura 2-parameter (K2P) measure (Kimura 1980) using Mega 6 (Tamura et al. 2013). The range of *COI* K2P distances found between *A. sinicus* haplotypes was compared to distances in the subfamily Pteromalinae as a whole using i) intraspecific distances, and ii) congeneric distances. The Pteromalinae DNA sequences used in this study were downloaded from GenBank and searched locally using software from the Blast+ toolkit. To create the Pteromalinae database used

in this study, we targeted only genera represented by at least two identified species, and each species was represented by at least two sequences. The qualifying sequences were then extracted and aligned with the barcode sequence [MK188331.1](#) of *A. solus*. The aligned Pteromalinae sequences were checked by eye and the edges trimmed using Seaview version 4 and imported into Mega 6 to calculate the molecular distances. The molecular distances were then split into intraspecific observations and interspecific (congeneric) observations.

Results

Surveys in Friuli Venezia Giulia region (northeastern Italy)

Our taxonomic studies determined that the *Acroclisoides* specimens found in Cordenons in 2018 belong to the species *A. sinicus*. From the three collected *H. halys* egg masses a total of 28 specimens of *A. sinicus* emerged (Table 2). Specimens of *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae), a well-known European native parasitoid that can parasitize eggs of *H. halys* (Haye et al. 2015; Roversi et al. 2016), also emerged. Detailed observation of the parasitized eggs revealed consistent differences in the exit holes produced by *A. sinicus* and *An. bifasciatus*, enabling eggs to be associated with the species that parasitized them.

Males of *An. bifasciatus* are visibly smaller than females (Bolivar y Pieltain 1935), and the circular exit holes that they produce are smaller than those of the larger females (Fig. 1A, B). *Acroclisoides sinicus*, independent of sex, produced exit holes on *H. halys* eggs of similar size to those of females of *An. bifasciatus*, but with much more irregular margins (Fig. 1C, D). *Trissolcus mitsukurii* (associated with *A. sinicus* in the Veneto region), produced round exit holes similar in size to females of *An. bifasciatus*, but with less serrate margins and a conspicuous dark ring on the upper margin of the parasitized eggs, just below the exit hole and the operculum (Fig. 1E). These observations permitted us to assess the number and species of parasitoids that emerged prior to the field collection of the egg masses (see Table 2). Emergence holes produced by *T. japonicus* (associated with *A. sinicus* in Zurich city, see results of Switzerland) are similar to those produced by *T. mitsukurii*, except there is no dark ring below the operculum (Fig. 1F).

Surveys in Veneto region (northeastern Italy)

Acroclisoides specimens found at the three localities of Povegliano, Montebelluna and Riese Pio X belong to the species *A. sinicus* as confirmed by our taxonomic study. In 2017, 24 *A. sinicus* specimens emerged from three collected *H. halys* egg masses. Egg masses parasitized by *A. sinicus* were collected on August 16 (one egg mass from Povegliano site on *Actinidia* sp.) and August 29 (two egg masses, one from Montebelluna

Table 2. Parasitoids that emerged from stink bug eggs collected in Cordenons and Ora (Italy) and in Zurich city (Switzerland).

Site	Pentatomid host species	Plant host genus/species	N of eggs/mass	N of eggs hatched	N of <i>A. sinicus</i> emerged	N of other parasitoids emerged	N of eggs unhatched, with no parasitoids emerged
Cordenons	<i>H. halys</i>	<i>Robinia pseudoacacia</i>	15	0	8 (5♀♀, 3♂♂)	2 <i>An. bifasciatus</i> (2♂♂)	5 (unidentified content ¹)
		<i>Robinia pseudoacacia</i>	28 ²	0	20 (14♀♀, 6♂♂)	3 <i>An. bifasciatus</i> (2♂♂, 1♀♀)	12 (unidentified content ³)
			14 ²	0			0
Ora	<i>H. halys</i>	<i>Acer</i> sp.	14	0	14 (12♀♀, 2 unsexed)	0	0
Zurich city	<i>H. halys</i>	<i>Tilia platyphyllos</i>	28	0	8 (5♀♀, 3♂♂)	1 <i>T. japonicus</i> (1♀♀)	19 (2 parasitized by <i>T. japonicus</i> , 1 scelionid pupa, 16 unidentified content)
		<i>Liriodendron tulipifera</i>	20	0	15 (9♀♀, 6♂♂)	0	5 (1 parasitized by <i>T. japonicus</i> , 4 unidentified content)
	<i>P. prasina</i>	<i>Liriodendron tulipifera</i>	27	0	20 (12♀♀, 8♂♂)	0	7 (1 parasitized by <i>A. sinicus</i> , 6 unidentified content)
		<i>Catalpa bignonioides</i>	26	0	12 (6♀♀, 6♂♂)	12 <i>T. japonicus</i> (1♂, 11♀♀)	2 (unidentified content)

¹ One egg was suspected to be parasitized by *T. mitsukurii* due to the presence of a black ring below the operculum of the egg.
² The two egg masses were found on the same leaf and reared together in the same vial. From these two egg masses, 7 parasitoids emerged in the field prior to collection; an estimation of total number of egg parasitoids emerged assessed through analysis of exit holes revealed that from the two egg masses collected, the following egg parasitoids emerged: 12 *A. sinicus* and 4 *An. bifasciatus* (1♀, 3♂♂) in the first egg mass, and 10 *A. sinicus* and 4 *An. bifasciatus* (3♀♀, 1♂) in the second egg mass.
³ Suspected to be all parasitized by *T. mitsukurii* due to the presence of a black ring below the operculum of the eggs.

and one from Riese Pio X both on *Vitis vinifera* L.). Parasitism of single egg masses by different parasitoid species was also observed: from the egg mass containing six *A. sinicus* individuals in Montebelluna, 15 individuals of *T. mitsukurii* also emerged; from the egg mass in Povegliano from which nine *A. sinicus* individuals emerged, 10 *An. bifasciatus* also emerged; but from the *H. halys* egg mass collected in Riese Pio X, only *A. sinicus* emerged (nine individuals). No *Acroclisoides* individuals were found in the monitoring campaign of 2018.

Surveys in Trentino-Alto Adige/Südtirol region (northern Italy)

From the *H. halys* egg masses collected in Ora, only one egg mass (collected on September 27, 2018) containing 14 eggs produced 14 specimens of *A. sinicus*. No other parasitoid species or *H. halys* nymphs emerged from the egg mass.

Surveys in Zurich city (northeastern Switzerland)

Acroclisoides individuals were reared from two *H. halys* egg masses and two egg masses of the native stink bug, *P. prasina* (Table 2). The first *A. sinicus* emerged from a single *H. halys* egg mass collected from *Tilia platyphyllos* on July 2, 2019. From the same egg mass a single female of *T. japonicus* was reared (Fig. 1F). On July 10, 2019 single *H. halys* and *P. prasina* egg masses were found on the same *L. tulipifera* tree, both generat-

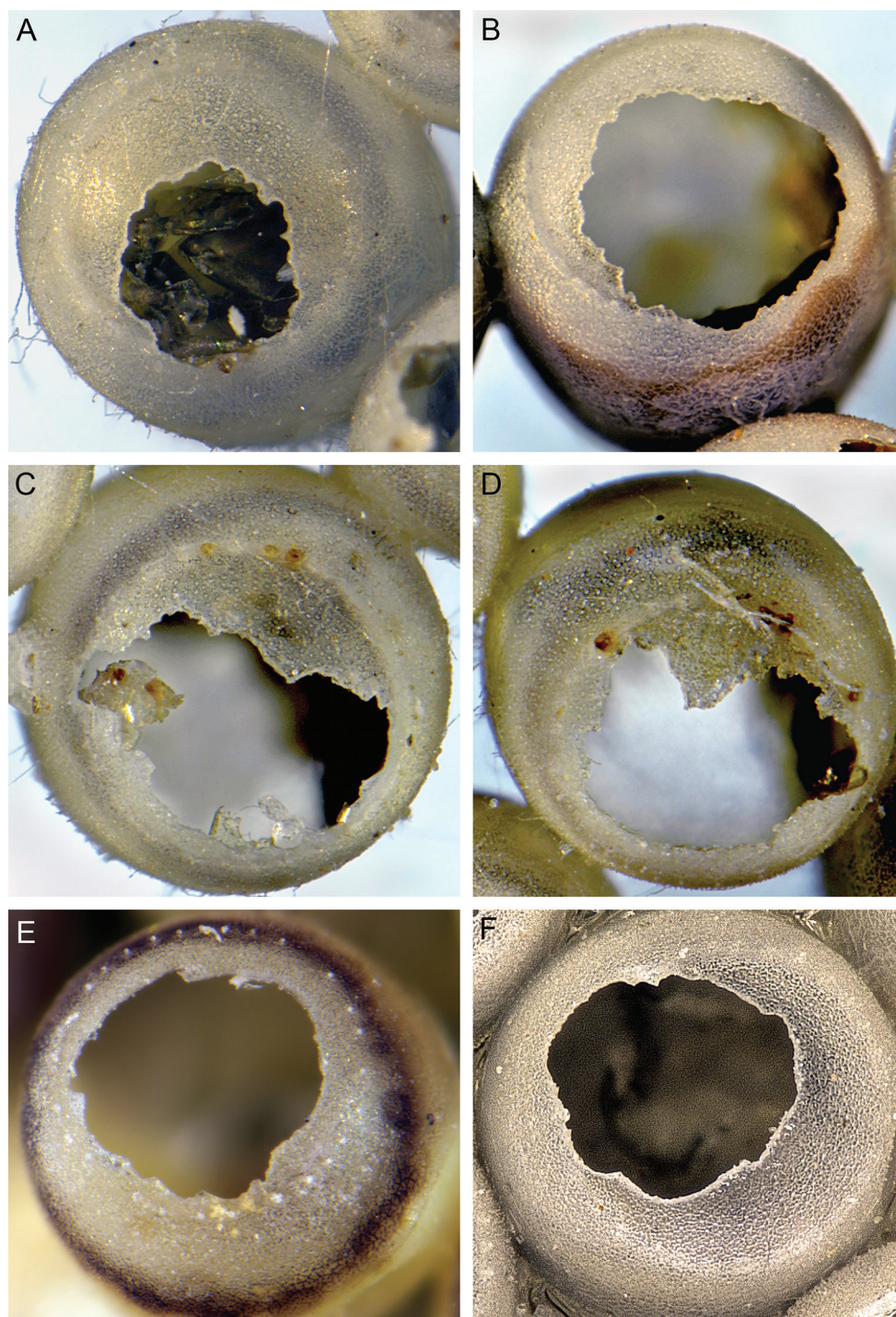


Figure 1. Typical shapes of exit holes produced by egg parasitoids of *Halyomorpha halys* in Europe: *Anastatus bifasciatus* male (A) and female (B) (NE-Italy); *Acroclisoides sinicus* (C,D) (NE-Italy); *Trissolcus mitsukurii* (E) (NE-Italy) and *Trissolcus japonicus* (F) (Switzerland).

ing *Acroclisoides* individuals. From an additional *P. prasina* egg mass collected on the same date on *C. bignonioides*, 12 *A. sinicus* emerged along with 12 *T. japonicus*. No *Acroclisoides* individuals emerged from egg masses collected June 28, 2019.

Surveys in Alabama and Georgia (southeastern USA)

Of the three *H. halys* egg masses collected in Auburn, AL, during the summer of 2018, one egg mass collected from pecan on July 25 produced six specimens of *A. sinicus* (Table 3). Nine *Trissolcus euschisti* (Ashmead) (Hymenoptera: Scelionidae) and seven *Anastatus reduvii* (Howard) (Hymenoptera: Eupelmidae), both of which are common indigenous parasitoids that sometimes parasitize *H. halys* in the USA (Cornelius et al. 2016), also emerged from this egg mass. One scelionid pupa died; four eupelmid late instars died. Of the nine *C. hiliaris* egg masses collected in Irwin County, GA, during the summer of 2017, three egg masses collected from mimosa produced *A. sinicus*. Fifteen *A. sinicus* emerged from one *C. hiliaris* egg mass collected from mimosa on July 21, 2107, and from this egg mass emerged 49 *Trissolcus edessae* Fouts (Hymenoptera: Scelionidae) and two *An. reduvii*. The second *C. hiliaris* egg mass collected from this plant on the same date produced 31 *A. sinicus* and no other parasitoids. From the third egg mass of *C. hiliaris*, collected on mimosa on July 24, 2017, one *A. sinicus* and 53 *T. edessae* emerged.

Surveys in Maryland (mid-Atlantic USA)

Acroclisoides sinicus emerged from two egg masses of *Euschistus* sp. (Hemiptera: Pentatomidae) collected on August 15 and 27, 2017 in Montgomery (39.2063N, 77.2079W)

Table 3. Parasitoids that emerged from stink bug egg masses collected in Alabama and Georgia (southeastern USA) and in Maryland (mid-Atlantic USA).

Site	Pentatomid host species	Plant host genus/ species	N of eggs/ mass	N of eggs hatched	N of <i>A. sinicus</i> emerged	N of other parasitoids emerged	N of eggs unhatched, with no parasitoids emerged
Auburn, AL	<i>H. halys</i>	<i>Carya illinoensis</i>	32	5	6 (4♀♀, 2♂♂)	9 <i>T. euschisti</i> (9♀♀); 7 <i>An. reduvii</i> (3♀♀, 4♂♂)	5 (1 scelionid pupa, 4 eupelmid late instars)
Irwin Co., GA	<i>C. hiliaris</i>	<i>Albizia julibrissin</i>	71	5	15	49 <i>T. edessae</i> ; 2 <i>An. reduvii</i> (2♀)	0
			56	22	31	0	3 (unidentified content)
			56	2	1	53 <i>T. edessae</i>	0
Montgomery Co., MD	<i>Euschistus</i> sp.	<i>Cornus</i> sp.	14	0	5 (3♀♀, 2 unsexed)	0	9 (all eggs parasitized but only 2 unemerged <i>A. sinicus</i> identified)
Frederick Co., MD	<i>Euschistus</i> sp.	<i>Cercis canadensis</i>	14	0	2	0	12 (9 eggs parasitized, 7 by <i>Telenomus</i> sp. and 2 unidentified, 3 eggs predated)
			13	0	12 (8♀♀, 2♂♂, 2 unsexed)	0	1 (parasitized, unidentified)
Allegany Co., MD	<i>Brochymena</i> sp.	<i>Lonicera japonica</i>	12	0	6 (4♀♀, 2 unsexed)	0	6 (5 parasitized by <i>A. sinicus</i>)

and Frederick (39.4760N, 77.2703W) Counties, respectively (Table 3). In Montgomery County, five *A. sinicus* emerged from the egg mass collected on *Cornus* sp. in a private home site with a wooded landscape of mixed deciduous trees, shrubs and herbaceous plants. In Frederick County, 14 *A. sinicus* emerged from two egg masses collected from *Cercis canadensis* L. located in the Fountain Rock Park and Nature Center (landscape has a mix of deciduous trees and shrubs and herbaceous plants). Six *A. sinicus* emerged from a *Brochymena* sp. (Hemiptera: Pentatomidae) egg mass collected in Allegany County (39.6786N, 78.7372W) (July 7, 2018) on *Lonicera japonica* Thunb. in the demonstration garden of the University of Maryland Extension (landscape with fruiting trees, managed and unmanaged trees, flowering herbaceous plants and shrubs, vegetable plants and mown turf). Fifteen of the emerged specimens were females, two were males, and the sex of the remainder could not be determined. No other parasitoids emerged during rearing and no stink bug nymphs emerged from any of the three egg masses.

Morphological analysis and redescription of the species

Acroclisoides Girault & Dodd, 1915

Acroclisoides Girault & Dodd, 1915: 344.

Neocoruna Huang & Liao, 1988: 426; synonymy by Xiao and Huang (2000): 94.

Diagnosis. BOTH SEXES: antenna 11263 (Figs 2E, 3B, D), inserted very high on face (Figs 2B, 3B, D); head much wider than high (Figs 2B, 3B, D), with strong occipital carina (Fig. 2C); gena with very large hollow at mouth margin (Fig. 2G); mandibles large (Figs 2B, 3B); notauli complete (Fig. 2F); fore wing with MV slightly thickened (Fig. 2D); metasoma with petiole subquadrate, smooth; gaster with GT1 narrow (Fig. 3A). FEMALE: ovipositor sheaths short, mostly concealed under terminal gastral tergites (Fig. 3A).

Acroclisoides sinicus (Huang & Liao, 1988)

Neocoruna sinica Huang & Liao, 1988: 427.

Acroclisoides sinicus (Huang & Liao, 1988); new combination by Xiao and Huang (2000): 95.

Acroclisoides solus Grissell & Smith, 2006: 925; syn. nov.

Diagnosis. BOTH SEXES: clypeal margin emarginate (Figs 2B, 3B, D); antenna with F6 whitish, occasionally also F5, the latter especially in males (Figs 2E, 3B, D); MV 0.9–1.1× SV and distinctly shorter than PV (Fig. 2D). FEMALE: fore wing usually with brownish infuscated spot, ranging from faint to pronounced, behind SV; spot

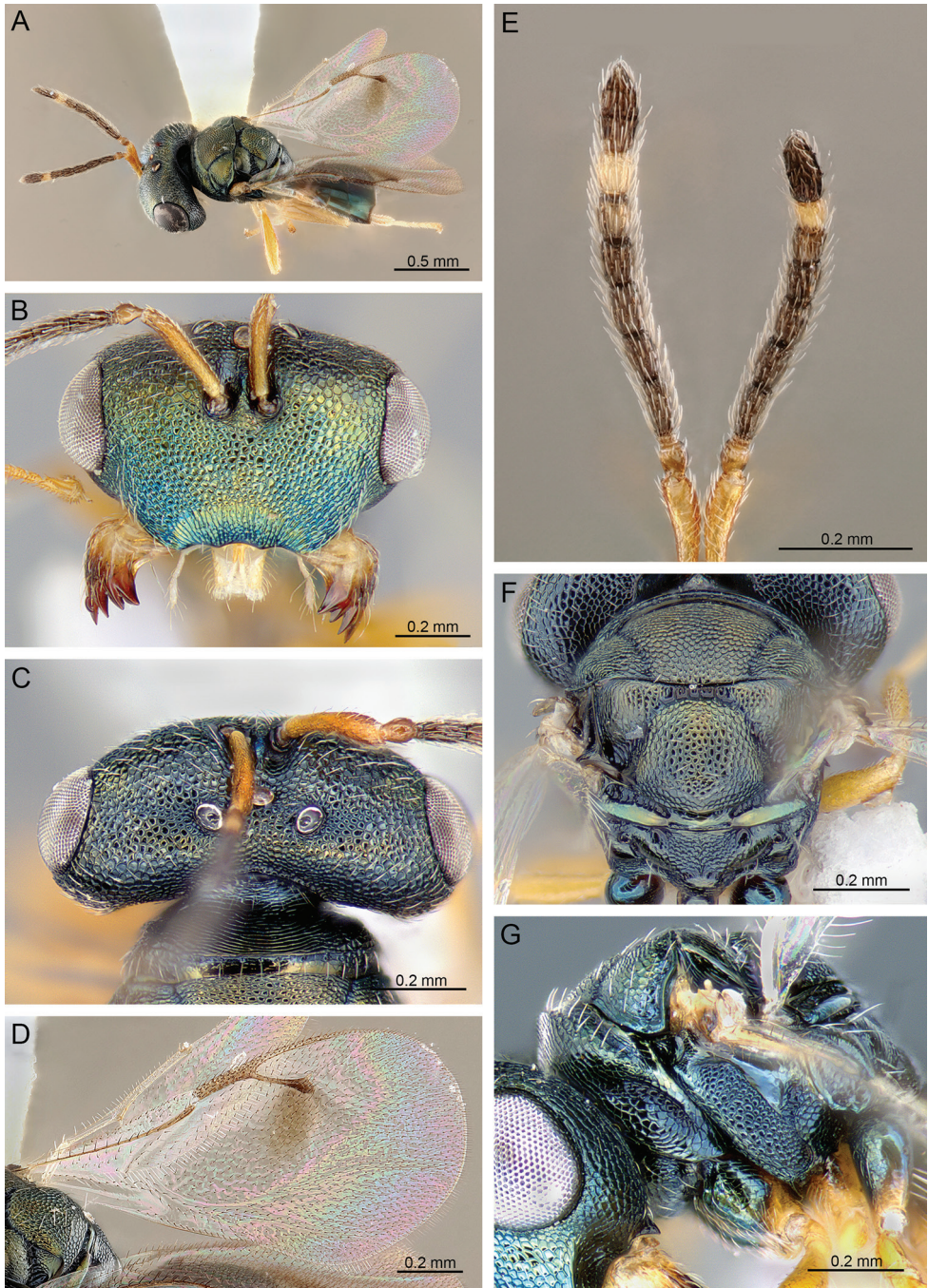


Figure 2. *Acroclisoides sinicus*, ♀ (Italy, Cordenons): habitus in dorso-lateral view (A); head in frontal view (B); head in dorsal view (C); fore wing (D); antennae (E); mesosoma in dorsal view (F); mesosoma in lateral view (G).

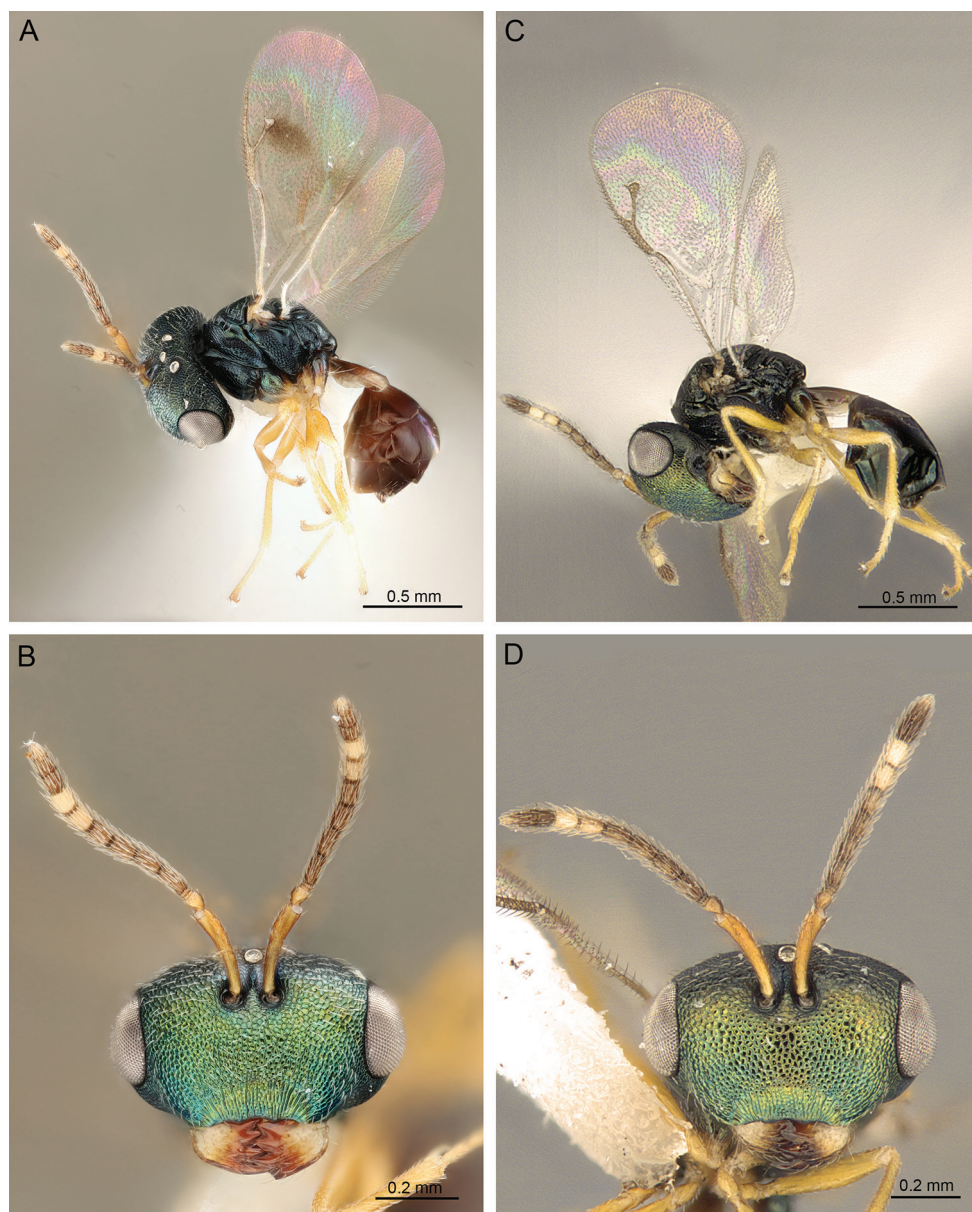


Figure 3. *Acroclisoides sinicus*: paratype of *A. solus* ♀ (U.S.A.), habitus in lateral view (A); idem, head in frontal view (B); ♂ (Switzerland), habitus in lateral view (C); idem, head in frontal view (D).

round to oval and not projecting beyond SV (Figs 2D, 3A). MALE: fore wing always hyaline (Fig. 3C).

Description. Female (Figs 2A–G, 3A, B). **Body length.** 1.7–2.5 mm (n = 10). **Color.** Head in frontal view bright green, with golden reflections (Figs 2B, 3B); frons,

vertex and occiput dark olive-green (Fig. 2C, F). Antenna (Figs 2E, 3B) with scape and pedicel yellowish-brown; funicle and clava brown to dark brown, except F6 and sometimes ventral side of F5 whitish. Mandible with basal half whitish-yellow to yellowish-brown, distal half including teeth reddish-brown (Figs 2B, 3B). Mesosoma in dorsal view mainly dark olive-green (Fig. 2F); mesosoma in lateral view dark blue-green (Fig. 2G). Legs (Fig. 3A) with fore and hind coxae dark green at least dorsally, apices and sometimes ventral part yellowish to yellowish-brown; mid coxa mainly yellowish-brown, basally and dorsally at least slightly darker; the rest of leg parts yellowish-brown, except tarsal apices dark brown. Fore wing hyaline, usually with distinct brownish spot behind SV, which may be very faint or absent to very distinct (Figs 2D, 3A); hind wing hyaline (Fig. 3A); tegula and venation dark brown. Metasoma with petiole black; gaster mainly dark metallic green to brown (Figs 2A, 3A).

Head. Clypeus broadly emarginate (Fig. 2B). Occipital carina (Fig. 2C) ventrally terminating in a conspicuous tooth, visible in lateral view of the head (Fig. 2G). Antennal scrobes conspicuous, separated by interantennal crest not reaching median ocellus (Fig. 2B). Clypeal region striate-reticulate; face reticulate, alveolae getting smaller towards vertex (Fig. 2B). Width of head in frontal view about $1.6\times$ height; in dorsal view width $2.05\text{--}2.40\times$ length. OOL $1.4\text{--}1.6\times$ POL. Minimum distance between eyes $2.3\text{--}2.4\times$ eye height. In lateral view eye height $1.1\text{--}1.2\times$ length and $1.6\text{--}1.7\times$ malar space. In dorsal view of the head temple about $1/3$ eye length or slightly less. Mandibles with 4 teeth each (Fig. 2B). Antenna with scape in lateral view gradually widening distally and reaching above level of vertex; both anelli transverse; all funicular segments longer than wide (Fig. 2E). Scape length $4.8\text{--}5.0\times$ width. Pedicel length $1.2\text{--}1.4\times$ width in lateral view. F1 length $1.6\text{--}1.8\times$ width, length $1.1\text{--}1.6\times$ pedicel length; F6 length $1.3\text{--}1.4\times$ width; clava length $2.7\text{--}3.0\times$ width, slightly longer than F5+F6.

Mesosoma. Pronotal collar posterior to setal row smooth. Mesoscutum and scutellum strongly and uniformly reticulate (Fig. 2F). Axillae with fine reticulation. Anterior margin of mesoscutellum separated from posterior margin of mesoscutum by several deep pits (Fig. 2F). Metascutellum virtually smooth (Fig. 2F). Propodeum (Fig. 2F) with small round basal foveae and median carina vaguely indicated, the latter extending to distinct, almost smooth nucha; median area centrally reticulate and almost smooth laterally, adjacent to conspicuous postspiracular sulci, the latter strongly convergent towards nucha; callus mainly smooth except superficially reticulate above hind coxa, with few setae. Mesepisternum reticulate; upper mesepimeron smooth, lower mesepimeron reticulate (Fig. 2G). Metapleuron reticulate. Hind coxa with dorsal basal setae extremely long. Mesosoma length $1.10\text{--}1.15\times$ width, length $1.4\text{--}1.5\times$ height. Mesoscutum width $2.3\text{--}2.4\times$ length. Scutellum width $1.0\text{--}1.1\times$ length. Propodeum median length about $0.60\times$ scutellum length. Fore wing (Fig. 2D) with MV moderately thickened; parastigma with hyaline break; costal cell on dorsal side of the wing with single row of setae in distal half, on ventral side with several rows of setae in distal half and single anterior row extending to base; basal cell with several scattered setae

and delimited by completely setose basal and cubital folds; speculum moderate, not extending beyond parastigma and closed below. Fore wing length $1.9\text{--}2.0\times$ width. SM $3.0\text{--}3.9\times$ MV. MV $0.9\text{--}1.1\times$ SV. PV about $1.6\times$ MV.

Metasoma. Dorsally flat or convex (Figs 2A, 3A). Petiole (MT1) subtriangular, about as long as wide; GT1 (MT2) long and narrow, occupying $1/3\text{--}1/4$ of gaster length (Fig. 3A); GT2–4 trapezoidal, GT4 the largest; GT2 longer than GT3 and shorter than GT4; GT5–7 very short, partly to completely retracted; hypopygium extending to about $0.8\text{--}0.9\times$ of gaster length; ovipositor sheaths short and visible only on ventral side of gaster (Fig. 3A). Gaster length $1.4\text{--}2.0\times$ width.

Male (Fig. 3C, D). Similar to the female, it differs in having the fore wing entirely hyaline (see also the remarks below).

Remarks. *Acroclisoides sinicus*, together with *A. maculatus* Sureshan & Narendran, *A. megacephalus* Girault & Dodd and *A. spilopterus* (Masi) (Hymenoptera: Pteromalidae), belongs to a group normally having maculate fore wings in females. *Acroclisoides sinicus* can be separated from the other species cited above by the whitish color of F6, sometimes also of F5, the latter especially in males (color also slightly variable, but at least on the ventral side of the antenna the segment is slightly to distinctly lighter than other segments) and different shape, size and position of the brownish spot on the fore wing in females (usually at least slightly visible, of small to moderate size, behind the stigmal vein and not projecting beyond it). According to the original description of *A. solus* and the paratypes we examined, this species is extremely close to *A. sinicus* in most characters, including the color of the funicle and fore wing. According to Grissell and Smith (2006), both sexes of *A. solus* differ from *A. sinicus* only in having a longer PV as compared with the MV (over $1.6\times$ versus less than $1.4\times$ in *A. sinicus*); in addition, the female of *A. solus* has a longer F1 as compared with the pedicel ($2\times$ versus slightly longer than pedicel in *A. sinicus*), clava slightly longer than F5+F6 versus clava longer than F4+F6 in *A. sinicus*, and the flagellar setae are depressed versus outstanding in *A. sinicus*. In our specimens from Italy and South Korea (MICO), PV was about $1.6\times$ as long as MV; F1 was $1.1\text{--}1.6\times$ as long as the pedicel; clava was slightly longer than F5+F6; flagellar setae were moderately depressed. All examined specimens fit well with the original description of *A. sinicus* (Huang & Liao, 1988).

Males and females of *A. sinicus* are very similar. The brownish infuscation of the fore wing is found only in females but is not always present. This character can thus be used to confirm that a specimen is female, but the absence of the infuscation cannot be used to reliably determine that a specimen is male. The presence of an ovipositor, ovipositor sheaths or a projecting aedeagus can be used to confirm the sex. In cases where the terminal gastral tergites are retracted and the wings are hyaline, unambiguous determination of sex may require dissection to expose the genitalia.

Distribution. China (Huang and Liao 1988; Xiao and Huang 2000), South Korea (Ko et al. 2018), Canada, USA, Italy, Switzerland; previously recorded for Italy, USA and Canada as *A. solus* (Grissell and Smith 2006; Garipey et al. 2014; Moraglio et al. 2019).

Specimens	Country	Site/State or Region or Province	Year of Collection, Name of Collector ^a	Pentatomid host (host plant)	GenBank Accession Number	Barcode length in bp	Haplotype	DNA collection code ^b	Voucher and Collection code ^c
<i>A. sinicus</i> (2 specimens)	USA	Frederick Co./ Maryland	2017, RW & LR	<i>Euschistus</i> sp. (<i>Cercis canadensis</i>)	MN395451–MN395452 (this study)	619	H3	Acro7-8, EBCL	Acro7-8, BIIR
<i>A. sinicus</i> (4 specimens)	USA	Frederick Co./ Maryland	2017, RW & LR	<i>Euschistus</i> sp. (<i>Cercis canadensis</i>)	MN395453–MN395456 (this study)	619	H4	Acro9-12, EBCL	Acro9-12, BIIR
<i>A. sinicus</i> (2 specimens)	USA	Allegany Co./Maryland	2017, RW & SF	<i>Brochymena</i> sp. (<i>Lonicera japonica</i>)	MN395457–MN395458 (this study)	652	H4	Acro13-14, EBCL	Acro13-14, BIIR
<i>A. sinicus</i> (<i>A. solus</i>)	Canada	Hamilton/Ontario	2012, TG (Garipey et al. 2014)	n.a.	MK188333	652	H4	Asco-0004, AAFc	Asco-0004, AAFc
		Hamilton/Ontario	2012, TG (Garipey et al. 2014)	n.a.	MK188332	652	H4	Asco-0003, AAFc	Asco-0003, AAFc
		Hamilton/Ontario	2012, TG (Garipey et al. 2014)	n.a.	MK188331	652	H4	Asco-0005, AAFc	Asco-0005, AAFc
<i>A. sinicus</i> (3 specimens)	China	Beijing, National Botanical Garden	2017, KH	<i>H. halys</i> (on apples)	MN395459–MN395461 (this study)	619	H5	Acro16-18, EBCL	Acro16-18, BIIR
<i>A. sinicus</i> (2 specimens)	China	Beijing, National Botanical Garden	2017, KH	<i>H. halys</i> (<i>Sophora japonica</i>)	MN395462–MN395463 (this study)	619	H6	Acro19-20, EBCL	Acro19-20, BIIR
<i>A. sinicus</i>	China	Nanjing, Zhongshan Botanical Garden	2014, KH	<i>Ertheina fulva</i>	MN395464 (this study)	619	H5	Acro21, EBCL	Acro21, BIIR
<i>A. sinicus</i> (2 specimens)	China	Nanjing, Zhongshan Botanical Garden	2014, KH	<i>Ertheina fulva</i>	MN395465–MN395466 (this study)	619	H7	Acro22-23, EBCL	Acro22-23, BIIR
<i>A. sinicus</i> (4 specimens)	South Korea	Suwon Arboretum (SNU)/ Gyeonggi	2014, KH	<i>Plautia stali</i> (<i>Magnolia</i> sp.)	MN395467–MN395470 (this study)	619	H4	Acro24-27, EBCL	Acro24-27, BIIR
<i>A. sinicus</i> (2 specimens)	South Korea	Anyang/ Gyeonggi	2014, KH	Undet. egg masses (<i>Acer palmatum</i>)	MN395471–MN395472 (this study)	619	H3	Acro29-30, EBCL	Acro29-30, BIIR
<i>A. sinicus</i>	South Korea	Gyeongsang-si/ Gyeongsangbuk-do	2016, LF	Sweep net	MN413503 (this study)	652	H3	AcKo0101, UAIC	AcKo0101, MICO
		Gyeongsang-si/ Gyeongsangbuk-do	2016, LF	Sweep net	MN413504 (this study)	652	H3	AcKo0102, UAIC	AcKo0102, MICO
		Gyeongsang-si/ Gyeongsangbuk-do	2016, LF	Sweep net	MN413505 (this study)	652	H3	AcKo0103, UAIC	AcKo0103, MICO

^aName of collectors: RB, Rammohan Balusu, LB, Luca Benvenuto, IB, Iris Bernardinelli, MF, Martina Falagiarola, SF, Sherry Frick, LF, Lucian Fusu, TG, Tara Garipey, EG, Emily Grove, TH, Tim Haye, KH, Kim Hoelmer, EL, Emily Lemke, GM, Giorgio Malosini, CR, Cindy Reilly, LR, Laura Robinson, DS, Davide Scaccini, DRS, David R. Smith, ET, Elijah Talamas, PT, Paola Trello, GT, Glynn Tillman, RW, Rebecca Waterworth.

^bDNA collections: AAFc, Agriculture and Agri-Food Canada, Canada; DISAFA, University of Turin, Torino, Italy; EBCL, European Biological Control Laboratory – USDA-ARS, France; FSCA, Florida Department of Agriculture and Consumer Service, Florida, USA; UAIC, University of Iasi, Romania.

^cVoucher collections: AAFc, Agriculture and Agri-Food Canada, Canada; BIIR, Beneficial Insects Introduction Research Unit-USDA-ARS, Newark, USA; DAFNAE, University of Padua, Padova, Italy; DISAFA, University of Turin, Torino, Italy; FSCA, Florida Department of Agriculture and Consumer Service, Florida, USA; Laimburg RC, Laimburg Research Center, Vadena, Italy; MICO, Mitroliu Collection, Iasi, Romania; NHMB, National History Museum of Bern, Switzerland.

Molecular analysis

The 5'-*COI* barcode fragment was successfully obtained from 45 individuals collected in numerous countries and from different hosts, plus four sequences from GenBank (Table 4). The lengths of these sequences varied from 432 bp for the paratype of *A. solus* to 652bp for three Italian, all Swiss, two American and three South Korean specimens. After edge trimming, the final data matrix of 49 sequences including the four GenBank accessions consisted of 429 characters with seven parsimony informative sites. As is typical for insect mitochondrial genes, the AT content in *Acroclisoides* was high (74.8%), similar to the AT content reported in Cynipidae (74.8%) at the higher end of the range observed for parasitic wasps (Rokas et al. 2002). A total of 7 haplotypes were recorded among the analyzed individuals. In Italy, we detected one unique haplotype (H1) which is shared with Switzerland. In Switzerland where H1 predominates (8 out of 9 specimens), we also found a second haplotype (H2) in one specimen. Although H1 and H2 did not match any of the 5 haplotypes found outside Europe, the network analysis showed that they are closely related as H1 differed only by one substitution from the haplotype H6 found in China and by two substitutions from the haplotype H3 found in South Korea and North America (Fig. 4). The two haplotypes found in North America, H3, which includes the *A. solus* paratype and H4, were shared with South Korea. Three haplotypes (H5, H6 and H7) were identified in China, H6

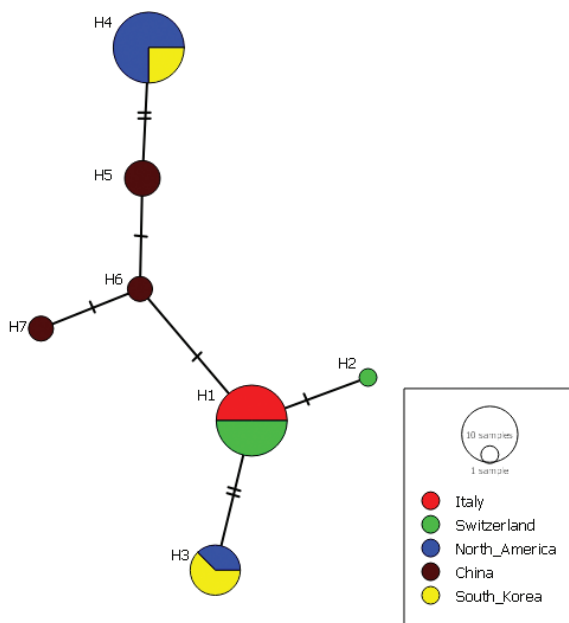


Figure 4. Haplotype network of the 49 *Acroclisoides sinicus* barcodes analyzed in this study. Each circle corresponds to one haplotype; circle size gives the proportion of individuals belonging to the haplotype. The color of the circles represents the geographical origin. Numbers correspond to the haplotype numbers. Hash marks symbolize the number of mutations between haplotypes.

being only one mutational step away from the other two. The degree of divergence was found to be very low at the sampling level, with a mean K2P distance of 0.64%, ranging from 1.41% between the two haplotypes H3 and H4 both detected in North America and South Korea to 0.23% between H1 from Europe and H6 found in China or H2 in Switzerland. Haplotype diversity (H_d) was 0.118 (SD of 0.101) and 0.343 (SD of 0.128) in Europe and North America respectively. Nucleotide diversity (π) was 0.00027 (SD of 0.00024) and 0.0048 (SD of 0.00179) in Europe and North America respectively. In Asia, H_d was 0.824 (SD of 0.047), and π was 0.00662 (SD of 0.0007). In order to confirm that the range of divergence is what is expected at the intraspecific level and given that there are no data available at the genus level, we compared these data with other species in the subfamily. A total of 432 Pteromalinae barcodes representing of a minimum of two species in four genera (*Lyrus* Walker, *Mesopolobus* Westwood, *Pachyneuron* Walker and *Pteromalus* Swederus) were downloaded and aligned. From this 283bp dataset (available upon request to authors), K2P distances for two classes (intraspecific and interspecific) were calculated. Figure 5 plots K2P values for the Pteromalinae, along with the divergence observed between *Acroclisoides* haplotypes, clearly confirming that divergence between *Acroclisoides* haplotypes is not significantly different from the Pteromalinae intraspecific divergence (Kruskal-Wallis test, $p=0.294$), but significantly different from the Pteromalinae congeneric divergence (Kruskal-Wallis test, $p<0.0001$). Species boundaries between *A. solus* and *A. sinicus* are not supported by the present mitochondrial data.

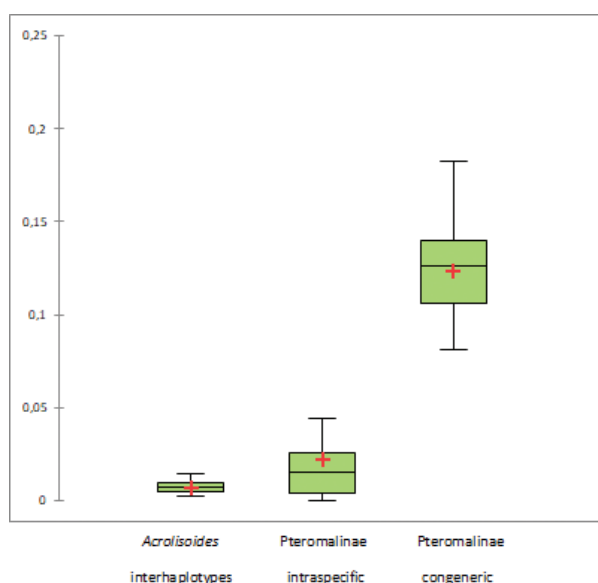


Figure 5. Boxplot of pair-wise molecular distances between (left) the *Acroclisoides* haplotypes evidenced in this study, (center) conspecific individuals in the Pteromalinae, (right) individuals from different species of the same genus in the Pteromalinae.

Discussion

Grissell and Smith (2006) stated that *A. solus* was very similar to *A. sinicus* and gave a few morphological differences that they considered enough to separate the two species. They compared *A. solus* with two Chinese specimens of *A. sinicus* as mentioned in their acknowledgments section. No further information was provided and we could not locate those specimens. After examining the specimens mentioned in the above sections and finding intermediate features between *A. solus* and *A. sinicus*, we conclude that they comprise a single species.

More samples of *A. sinicus* and other *Acroclisoides* species are required to more accurately assess the intraspecific and interspecific variability, respectively, necessary to compute the “barcoding gap” (Meyer and Paulay 2005). However, our analysis of the barcode region showed that the level of divergence was less than 1% among specimens from Italy, Switzerland, South Korea, China, USA, Canada and the paratypes of *A. solus*. This level of divergence is consistent with that observed at the intraspecies level within the subfamily Pteromalinae, and hence supports the conspecificity of *A. solus* and *A. sinicus*. This study highlights the value of DNA barcoding in the identification and delineation of species in Hymenoptera (Stahlhut et al. 2013). Overall, this research expands the barcoding sequence database for parasitoids and hyperparasitoids and provides a foundation for additional molecular studies aimed to a better understanding of the biocontrol services provided by parasitoids (Gariépy et al. 2007; Ye et al. 2017; Halim et al. 2018).

There were no prior reports in the literature on the associations of *A. sinicus* and its hosts, with the exception of Xiao and Huang (2000) who reported that this species was reared from pentatomid eggs (Huang and Liao 1988; Xiao and Huang 2000; Grissell and Smith 2006; Ko et al. 2018). In our study, *A. sinicus* was found to be associated with *H. halys* in Italy and *P. prasina* and *H. halys* in Switzerland; in the USA it was associated with *Euschistus* sp., *Brochymena* sp., *C. hilaris*, and *H. halys*; and in Asia it is associated with *H. halys*, *Plautia stali* Scott and *Erthesina fullo* (Thunberg) (Hemiptera: Pentatomidae). In addition, in Italy *A. sinicus* (formerly identified as *A. solus*) was recently found associated with *Arma custos* (Fabricius) (Hemiptera: Pentatomidae) eggs (Moraglio et al. 2019).

We consider it likely that *A. sinicus* is a hyperparasitoid of both *Anastatus* and *Trissolcus*. In the Friuli Venezia Giulia region *A. sinicus* only emerged from egg masses associated with the egg parasitoid *An. bifasciatus*, even though egg masses parasitized by *T. mitsukurii* were present in the vicinity (Sabbatini Peverieri et al. 2018). However, in the Veneto region, *A. sinicus* emerged from two egg masses parasitized by *T. mitsukurii* and in Switzerland from two egg masses parasitized by *T. japonicus*, suggesting hyperparasitism might occur on species in both genera as previously reported by Clarke and Seymour (1992). In the southeastern USA, one egg mass of *C. hilaris* from Georgia produced specimens of *A. sinicus* and *T. edessae*; another egg mass produced specimens of both these parasitoid species as well as *An. reduvii*. Molecular analysis of remnants of parasitized host eggs [*Acrosternum hilare* (Say) (Hemiptera: Pentatomidae)] led to the suspicion that *A. sinicus* (formerly identified as *A. solus*) was associated with *Trissolcus*

sp. (Garipey et al. 2014). Preliminary laboratory no-choice experiments have found that *A. sinicus* does not accept unparasitized *H. halys* egg masses as a host (Sabbatini Peverieri unpublished data). Similar observations were made by Clarke and Seymour (1992), who exposed two undetermined *Acroclisoides* species collected in Australia to *Nezara viridula* L. (Hemiptera: Pentatomidae) eggs, showing that only eggs parasitized previously by *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) were accepted. James (1990) also found that the Australian species *Acroclisoides tectacori* (Girault) (Hymenoptera: Pteromalidae) is “difficult to rear” in laboratory conditions on eggs of *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae). To date, there are no other published studies that test the host range and preference of *A. sinicus* in a laboratory setting.

The ability to identify emerged parasitoid species from host eggs collected in the field is a valuable tool for research. For example, the three parasitoid species that emerged in Italy from *H. halys* eggs produce distinct and diagnostic exit holes that can be used to assess parasitism if only empty parasitized egg masses are found. The distinct shape of the margin of the exit hole correlates well with the distinct shape of the mandibles in the three species of wasps. *Anastatus bifasciatus* has bidentate mandibles characteristic for the genus (Gibson 1995), with one small and one large tooth and the exit hole has serrated margins (Fig. 1A, B). *Trissolcus mitsukurii* and *T. japonicus* have tridentate mandibles with teeth of similar size (Talamas et al. 2017) and the exit hole has smaller serrations compared to *An. bifasciatus* (Fig. 1E, F). *Acroclisoides sinicus* has large mandibles with four long sharp teeth (Fig. 2B), and they were directly observed to cut and tear the egg chorion (Fig. 1C, D) while pushing it outward. Molecular analysis will be another useful tool to evaluate the impact of *Acroclisoides* on the parasitoid guild (Garipey et al. 2014; Garipey et al. 2018).

It is unknown how *A. sinicus* arrived in Europe or in the USA, but this could have happened via the same pathways as with adventive *T. japonicus* and *T. mitsukurii*. Another possibility is that *A. sinicus* is a Holarctic species, and its presence was only detected recently due to increased interest in field collection of pentatomid eggs as a result of *H. halys* biocontrol research. The latter hypothesis can be tested by thorough examination of insect collections that may contain specimens collected prior to the arrival of *H. halys*. It is noteworthy that we currently have no records of *A. sinicus* from North America or Europe that predate the arrival of *H. halys*, with the earliest detection in the USA in 2002 (Grissell and Smith 2006) and the earliest European record in 2016 (Moraglio et al. 2019). It should also be noted that one of us (MDM) has studied Pteromalidae in Europe for decades without detecting any specimens of *A. sinicus* in entomological collections. Moreover, Dieckhoff et al. (2017) have extensively surveyed parasitoids of *H. halys* and native pentatomids in Delaware, USA, since 2007 without any recoveries of *Acroclisoides* from natural or sentinel egg masses. This apparent absence is congruent with the hypothesis that the populations of *A. sinicus* in North America and Europe are adventive and recent.

Analysis of *COI* sequences revealed that all samples collected in Italy and all samples except one collected in Switzerland belong to the same *A. sinicus* haplotype (H1), while in the USA two haplotypes (H3 and H4) are present, both of which are shared with South Korea. The lower haplotype and nucleotide diversities observed in North

America and Europe compared to Asia are congruent with a mild population genetic bottleneck subsequent to an introduction (Hufbauer et al. 2004). Also, values of Hd (0.118 and 0.343) and π (0.00027 and 0.0048) in Europe and North America respectively are low for the scenario of a species with a long-established Holarctic distribution. For example *Aedes caspius* (Pallas) (Diptera: Culicidae), which persisted through the last glacial period across the Mediterranean Basin, has Hd and π of 0.971 and 0.0067 respectively (Porretta et al. 2011). The genetic similarity between North American and South Korean specimens suggests that either the North American specimens originated from South Korea or populations of *A. sinicus* in both countries originated from the same area. All specimens from Europe except one have the same haplotype (H1) but are different from those in the USA or Asia. This suggests that the European *Acroclisoides* did not originate in the USA but from an unknown region. In any case, *A. sinicus* appears to be a species that is not commonly found in the field but could become more prevalent in the future as a result of invasive *H. halys* populations.

Hyperparasitoids have traditionally been regarded as detrimental to biological control, although some authors have suggested they may provide stabilizing influences on populations depending on the model assumptions (Kidd and Jervis 2007; Snyder and Ives 2008). The effect that *A. sinicus* might have on the eventual biological control of *H. halys*, as well as on non-target species of Pentatomidae and their parasitoids in the environment, is the subject of ongoing studies.

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