

Hesperomyces harmoniae, a new name for a common ectoparasitic fungus on the invasive alien ladybird *Harmonia axyridis*

Danny Haelewaters^{1,2,3*}, Warre Van Caenegem¹ & André De Kesel⁴

¹ Research Group Mycology, Department of Biology, Ghent University, Ghent, Belgium

² Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic

³ Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

⁴ Meise Botanic Garden, Meise, Belgium

* e-mail: danny.haelewaters@gmail.com

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Fungi in the order Laboulbeniales (Ascomycota, Laboulbeniomycetes) are obligate, microscopic ectoparasites of arthropods. These fungi, unlike their close relatives, never form hyphae. Instead, they produce a three-dimensional thallus that consists of several hundred to a thousand vegetative cells derived from a two-celled ascospore by determinate mitotic divisions. Of 2,325 described species, 80 % are known from beetles (Coleoptera). *Hesperomyces* is a genus of 11 species associated with ladybirds (Coleoptera, Coccinellidae) and false skin beetles (Biphyllidae). One species, *Hesperomyces virescens*, is known from all continents except Australia and Antarctica, and has been reported on 30 ladybird hosts in 20 genera. Previous work, based on geometric morphometrics, molecular phylogeny, sequence-based species delimitation methods, and host information, pointed out that *He. virescens* is a complex of multiple species segregated by host. Here, we formally describe the most recorded species in the complex, *Hesperomyces harmoniae*—parasite of the harlequin ladybird *Harmonia axyridis*, a globally invasive species. Using DNA isolates of *Hesperomyces* from multiple host species, including the host on which *He. virescens* was originally described (*Chilocorus stigma*), we found that *He. harmoniae* forms a single clade in our phylogenetic reconstruction of a two-locus ribosomal dataset. *Hesperomyces harmoniae* is currently known from five continents and 31 countries: Canada, El Salvador, Mexico, the USA (North America); Argentina, Colombia, Ecuador (South America); Austria, Belgium, Bulgaria, Croatia, Czech Republic, France, Germany, Greece, Hungary, Italy, Luxembourg, Montenegro, The Netherlands, Poland, Romania, Russia, Serbia, Slovakia, Switzerland, the UK (Europe); South Africa (Africa); China, Japan, and Turkey (Asia).

Keywords: arthropod-associated fungi, Coccinellidae, integrative taxonomy, phylogeny, ribosomal DNA, species delimitation. – 1 new species.

The study of fungi in the order Laboulbeniales (Ascomycota), microfungi that are biotrophs of arthropods, has long been hampered by their minute size, minimal morphological characteristics, the inability of taxa to grow in pure culture, and the absence of comparative traits to place them among related fungi (Benjamin 1971, Blackwell et al. 2020). The field of Laboulbeniales research has recently caught up with other groups with the increased use of technological advances by several researchers, including state-of-the-art DNA isolation methods and the use of scanning electron microscopy and X-ray microtomography reconstructions (Tragust et al. 2016, Sundberg et al. 2018, Haelewaters et al. 2019b, Perreau et al. 2021, Reboleira et al. 2021, Lubbers et al. 2022). While alpha taxonomy is still important for, e.g., discovery of Laboulbeniales on insects preserved in en-

tomological collections, researchers lacking equipment or the funds to generate sequences should consider collaborating with molecular systematists (Haelewaters et al. 2021). In fact, species descriptions and taxonomic treatments of Laboulbeniales now often incorporate evidence from molecular phylogenetic analyses and ecology. Recent work has shown that morphology alone is insufficient to resolve species limits in the following genera: *Arthrorhynchus* Kolen. (Haelewaters et al. 2020), *Chitonomyces* Peyr. (Goldmann & Weir 2012), *Coreomyces* Thaxt. (Sundberg et al. 2021), *Gloeandromyces* Thaxt. (Haelewaters & Pfister 2019), *Hesperomyces* Thaxt. (Goldmann et al. 2013, Haelewaters et al. 2018a), *Laboulbenia* Mont. & C.P. Robin (Haelewaters et al. 2019a), and *Nycteromyces* Thaxt. (W. Van Caenegem & D. Haelewaters, unpublished data).

One of the contemporary challenges is that phylogenetic reconstructions have unearthed a species diversity that was previously unrecognized by researchers. Some taxa have been shown to be complexes of multiple species that are cryptic (that is, they are morphologically indistinguishable) or semicryptic (with at least some overlap in phenotypic characters with other evolutionary separated species). One of these is *Hesperomyces virescens* Thaxt., a taxon originally described on a *Chilocorus stigma* (Say, 1835) ladybird from California, USA (Thaxter 1891) and afterwards reported on many other ladybird genera (summarized in Haelewaters & De Kessel 2017). The combination of molecular phylogenetic data, sequence-based species delimitation analyses, morphometrics, and ecology (host associations) revealed that this taxon truly is a complex of species, segregated by host (Haelewaters et al. 2018a). Thus far, we have described two species within the complex, *He. halyziae* Haelew. & De Kessel from *Halyzia sedecimguttata* (Linnaeus, 1758) in Belgium and the Netherlands (Haelewaters & De Kessel 2020), as well as *He. parexochomi* Mironova & Haelew. from *Parexochomus nigripennis* (Erichson, 1843) and *P. quadriplagiatus* (Wollaston, 1864) in the Canary Islands (Crous et al. 2021). In this paper, we describe the species that is strictly associated with the globally invasive ladybird *Harmonia axyridis* (Pallas, 1773).

Materials and methods

Collection of material, morphological study

Harmonia axyridis ladybirds (Coleoptera, Coccinellidae) were collected and screened for the presence of Laboulbeniales under 40–50× magnification. Collections were made by the authors or collaborators in Canada, the USA (Georgia, Kentucky, Massachusetts, Wisconsin), Europe (Belgium, Czech Republic, The Netherlands, Switzerland), and Africa (South Africa). Additional *Hesperomyces*-infected ladybirds from multiple countries were sent to the first author by entomologists. Thalli were removed from the host at the point of attachment and mounted in permanent slides using a double-cover-slip mounting technique as described by Liu et al. (2020). Mounted thalli were viewed at 200–1000× magnification under an Olympus CX21 microscope (Olympus, Center Valley, PA). Photos were taken with a Nikon DS-Fi3 microscope camera mounted on an Eclipse Ni-U compound microscope (Nikon, Nelville, NY) equipped with differential interference contrast (DIC) optics, and processed using NIS-Elements BR 5.0.03 imaging software (Nikon).

Thalli, structures, and ascospores were measured using ImageJ 1.51h image processing and analysis software (Abràmoff et al. 2004). All measurements were taken as described and illustrated by Haelewaters et al. (2018a). The diagnosis is modeled after Sánchez-García et al. (2016) and Haelewaters & Pfister (2019), with an overview of unique molecular synapomorphies and motifs. Measurements in the morphological description are noted as (a)b–c–d(e), with a, e = extreme values; b, d = mean minus/plus standard deviation; and c = mean. The notation [x/y] stands for x = number of structures observed and measured, and y = number of hosts that observed structures originate from. Voucher host specimens are deposited at the Brabant Museum of Nature, Tilburg (NNKN); Florida State Collection of Arthropods, Gainesville, FL (FSCA); Meise Botanic Garden (BR); Museum of Comparative Zoology at Harvard University, Cambridge, MA (MCZ); and Purdue Entomological Research Collection, West Lafayette, IN (PERC). Microscope slides of Laboulbeniales are deposited at BR, FH, GENT, PUL (Thiers continuously updated), and the Zoology collection at the Natural History Museum of Denmark, Copenhagen (Z-NHMD).

DNA extraction, PCR amplification, and sequencing

DNA was extracted from 1–3 thalli using the REPLI-g Single Cell (Qiagen, Stanford, CA). A Minut Pin ((BioQuip, Rancho Dominguez, CA, #1208SA), inserted onto a wooden rod for holdfast, was coated in glycerin to avoid loss of thalli during transfer. Detached thalli were placed in a droplet of glycerin on a microscope slide. To ensure successful lysis, we cut each perithecium transversally once using a #10 surgical blade on disposable Bard-Parker handle (Aspen Surgical, Caledonia, MI). Thalli were then placed in 0.2-ml PCR tubes with 4 µl of phosphate-buffered saline. After addition of 3 ml of prepared D2 buffer, tubes were incubated at 65 °C for 30 min. Subsequent steps followed the manufacturer's instructions (Qiagen).

For the purpose of species delimitation, the internal transcribed spacer region (ITS) and large subunit (LSU) of the ribosomal RNA gene were amplified using the following primer pairs: ITS1f/ITS4 and ITShespL/ITShespR for ITS (White et al. 1990, Gardes & Bruns 1993, Haelewaters et al. 2019b), and LR0R/LR5 and LIC24R/LR3 for LSU (Vilgalys & Hester 1990, White et al. 1990, Hopple 1994, Miadlikowska & Lutzoni 2000). PCR reactions (20 µl total) consisted of 10 µl of RedExtract Taq polymer-

ase (Sigma-Aldrich, St. Louis, MO), 2 µl of each 10 µM primer, 7 µl of ddH₂O, and 1 µl of DNA extract. PCR conditions were as follows: for ITS: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 45 s, extension at 72 °C for 90 s; and final extension at 72 °C for 10 min (Haelewaters et al. 2018a). For LSU: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 7 min (Liu et al. 2020).

We also amplified the small subunit (SSU) of the ribosomal RNA gene and the single-copy gene *MCM7* that encodes a licensing factor required for DNA replication initiation and cell proliferation. Primer pairs were NS1/NS2, NSL1/NSL2, and SL344/NS6 for SSU (White et al. 1990, Landvik et al. 1997, Haelewaters et al. 2015) and MCM7-709for/MCM7-1384rev for *MCM7* (Schmitt et al. 2009). Cycling conditions were as follows: for SSU: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, and extension at 72 °C for 1 min/1 kb; and final extension at 72 °C for 10 min (Haelewaters et al. 2020). For *MCM7*: initial denaturation at 94 °C for 10 min; 38 cycles of denaturation at 94 °C for 45 s, annealing at 46–47 °C for 50 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min. We initially tried a gradient between 45 and 50 °C to find the most suitable annealing temperature for the amplification of *MCM7* using DNA extracts of different Laboulbeniomyces species in the genera *Herpomyces* Thaxt., *Hesperomyces* Thaxt., and *Stigmatomyces* H. Karst.

Purification of PCR products was performed with 1.5 µl of Exo-FAP (0.5 µl Exonuclease I, 1 µl FAST Alkaline Phosphatase) (Thermo Fisher Scientific, Waltham, MA) per 10 µl of PCR product, at 37 °C for 15 min followed by deactivation at 85 °C for 15 min. Finally, the purified PCR products were sequenced using an automated ABI 3730 XL capillary sequencer (Life Technology at Macrogen, Amsterdam, The Netherlands). Forward and reverse sequence reads were assembled and edited in Sequencher version 5.4.6 (Gene Codes Corporation, Ann Arbor, MI). Newly generated sequences were submitted to NCBI GenBank (accession numbers in Tab. 1).

Phylogenetic analyses

In addition to the newly generated sequences of *He. virescens* sensu lato (s.l.) (11 ITS, 15 LSU), we downloaded 93 sequences (54 ITS, 39 LSU) from

NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>). Our final dataset included sequence data of two loci for 72 isolates, including five isolates for *Hesperomyces* ex *Azya orbiger* Mulsant, 1850, *He. coccinelloides* (Thaxt.) Thaxt., and *He. coleomegillae* W. Rossi & A. Weir as outgroup taxa (Tab. 1). We aligned sequences by locus using MAFFT on XSEDE (version 7.471) (Kato & Toh 2010) and then combined the aligned sequences in MEGA version 7.0.26 (Kumar et al. 2016) to create a matrix of 3247 bp. Models of nucleotide substitution were selected for each partition with ModelFinder (Kalyaanamoorthy et al. 2017) according to the corrected Akaike Information Criterion (AICc). Our aligned, concatenated data matrix included four partitions: the ITS1 and ITS2 spacers, the highly conserved 5.8S component of the ITS, and LSU. Maximum likelihood (ML) was inferred using IQ-TREE (Nguyen et al. 2015) under partitioned models (Chernomor et al. 2016). Finally, ultrafast bootstrapping (BS) was performed with 1,000 replicates (Hoang et al. 2017).

We also performed Bayesian analyses using a Markov chain Monte Carlo (MCMC) coalescent approach with the help of the BEAST package version 1.8.4 (Drummond et al. 2012). First, we used jModelTest 2.0 (Darriba et al. 2012) on the CIPRES Science Gateway (Miller et al. 2010) to select for the substitution models under the AICc for ITS and LSU. The ITS was not further divided into three partitions as overpartitioning negatively affects MCMC convergence (Rannala 2002, Fenn et al. 2008). The following priors were entered into BEAUti to generate an XML file: the selected substitution models for ITS and LSU, strict molecular clock, a birth–death tree prior accommodated for incomplete sampling (Stadler 2009) to model the speciation of nodes in the topology, with a randomly generated starting tree, 40 million generations, and 4,000 as sampling frequency. Four independent runs were performed using BEAST on XSEDE (Drummond et al. 2012) in CIPRES (Miller et al. 2010). Resulting log files were imported in Tracer 1.6 (Rambaut et al. 2014) to assess their MCMC trace plots and effective sample sizes. A standard burn-in of 10 % was used for three runs. For the fourth run, the burn-in was increased to 15 % to achieve convergence. Trees files were combined in LogCombiner version 1.8.4 after removal of appropriate burn-in and the posterior probability (PP) density was summarized as a maximum clade credibility (MCC) tree in TreeAnnotator version 1.8.4. Final trees with bootstrap support values and posterior probabilities of inferred clades were visual-

Tab. 1. Details of *Hesperomyces* isolates included in the molecular analyses, with country of collection, host species, and GenBank accession numbers for ITS and LSU sequences.

Species	Isolate	Country	Host	ITS	LSU
<i>He. coccinelloides</i>	D. Haelew. 1428a	Spain	<i>Stethorus tenerifensis</i> Fürsch, 1987	OL335930	
<i>He. coccinelloides</i>	D. Haelew. 1428b	Spain	<i>Stethorus tenerifensis</i>	OL335931	OL335915
<i>He. coleomegillae</i>	D. Haelew. 1287b	Panama	<i>Coleomegilla maculata</i> (DeGeer, 1775)	OL335932	MG745334
<i>He. coleomegillae</i>	D. Haelew. 1291c	Panama	<i>Coleomegilla maculata</i>	OL335933	MG745335
<i>He. halyziae</i>	D. Haelew. 955b ^T	The Netherlands	<i>Halyzia sedecimguttata</i> (Linnaeus, 1758)	MG757813	
<i>He. harmoniae</i>	D. Haelew. 1005c	South Africa	<i>Harmonia axyridis</i> (Pallas, 1773)	MG757814	
<i>He. harmoniae</i>	D. Haelew. 1174a	The Netherlands	<i>Harmonia axyridis</i>	MG757815	MG745345
<i>He. harmoniae</i>	D. Haelew. 1188g	USA	<i>Harmonia axyridis</i>	MF458363	MF458364
<i>He. harmoniae</i>	D. Haelew. 1268b	Japan	<i>Harmonia axyridis</i>	MG757829	MG745357
<i>He. harmoniae</i>	D. Haelew. 1268d	Japan	<i>Harmonia axyridis</i>	MG757830	MG745358
<i>He. harmoniae</i>	D. Haelew. 1443a	USA	<i>Harmonia axyridis</i>	OL335934	OL335920
<i>He. harmoniae</i>	D. Haelew. 1551b	Czech Republic	<i>Harmonia axyridis</i>	OL335935	
<i>He. harmoniae</i>	D. Haelew. 1808b ^T	USA	<i>Harmonia axyridis</i>	OL335936	OL335921
<i>He. harmoniae</i>	D. Haelew. 316a	USA	<i>Harmonia axyridis</i>	MG438315	KJ842339
<i>He. harmoniae</i>	D. Haelew. 316d	USA	<i>Harmonia axyridis</i>	MG757801	
<i>He. harmoniae</i>	D. Haelew. 516a	USA	<i>Harmonia axyridis</i>	MG757802	MG745340
<i>He. harmoniae</i>	D. Haelew. 646c	Germany	<i>Harmonia axyridis</i>		KT800015
<i>He. harmoniae</i>	D. Haelew. 648b	South Africa	<i>Harmonia axyridis</i>		OL335919
<i>He. harmoniae</i>	D. Haelew. 648c	South Africa	<i>Harmonia axyridis</i>	KU574864	KU574865
<i>He. harmoniae</i>	D. Haelew. 669a	South Africa	<i>Harmonia axyridis</i>	MG757807	
<i>He. harmoniae</i>	D. Haelew. 943a	South Africa	<i>Harmonia axyridis</i>	MG757809	
<i>He. harmoniae</i>	D. Haelew. 943b	South Africa	<i>Harmonia axyridis</i>	MG757810	MG745344
<i>He. parexochomi</i>	D. Haelew. 1462a	Spain	<i>Parexochomus nigripennis</i> (Erichson, 1843)	MZ994855	
<i>He. parexochomi</i>	D. Haelew. 1463a	Spain	<i>Parexochomus nigripennis</i>	MZ994856	
<i>He. parexochomi</i>	D. Haelew. 1690b	Spain	<i>Parexochomus nigripennis</i>	MZ994861	MZ994872
<i>He. parexochomi</i>	D. Haelew. 1690c	Spain	<i>Parexochomus nigripennis</i>	MZ994862	MZ994873
<i>He. parexochomi</i>	D. Haelew. 1690d	Spain	<i>Parexochomus nigripennis</i>	MZ994863	MZ994874
<i>He. parexochomi</i>	D. Haelew. 1691c	Spain	<i>Parexochomus nigripennis</i>	MZ994864	MZ994875
<i>He. parexochomi</i>	D. Haelew. 1691d	Spain	<i>Parexochomus nigripennis</i>	MZ994865	MZ994876
<i>He. parexochomi</i>	D. Haelew. 1693a	Spain	<i>Parexochomus nigripennis</i>	MZ994866	MZ994877
<i>He. parexochomi</i>	D. Haelew. 1693b	Spain	<i>Parexochomus nigripennis</i>	MZ994867	MZ994878
<i>He. parexochomi</i>	D. Haelew. 1465a	Spain	<i>Parexochomus quadriplagiatus</i> (Wollaston, 1864)	MZ994860	MZ994871
<i>He. parexochomi</i>	D. Haelew. 1465b ^T	Spain	<i>Parexochomus quadriplagiatus</i>	MZ994868	MZ994879
<i>He. parexochomi</i>	D. Haelew. 1584a	Spain	<i>Parexochomus quadriplagiatus</i>	MZ994858	MZ994869
<i>He. parexochomi</i>	D. Haelew. 1584b	Spain	<i>Parexochomus quadriplagiatus</i>	MZ994859	MZ994870
<i>Hesperomyces</i> sp.	D. Haelew. 928g	Panama	<i>Azya orbigera</i> (Mulsant, 1850)	MG745343	MG745343
<i>He. virescens</i> s.l.	D. Haelew. 1193a	Denmark	<i>Adalia bipunctata</i> (Linnaeus, 1758)	MG757816	
<i>He. virescens</i> s.l.	D. Haelew. 1193g	Denmark	<i>Adalia bipunctata</i>	MG757817	MG745346
<i>He. virescens</i> s.l.	D. Haelew. 1199h	Sweden	<i>Adalia bipunctata</i>	MG757818	MG745347
<i>He. virescens</i> s.l.	D. Haelew. 1231a	Italy	<i>Adalia bipunctata</i>	MG757821	MG745350
<i>He. virescens</i> s.l.	D. Haelew. 1232a	Italy	<i>Adalia bipunctata</i>	MG757822	MG745351
<i>He. virescens</i> s.l.	D. Haelew. 1248b	Italy	<i>Adalia decempunctata</i> (Linnaeus, 1758)	MG757823	MG745353
<i>He. virescens</i> s.l.	D. Haelew. 1249a	Italy	<i>Adalia decempunctata</i>	MG757824	
<i>He. virescens</i> s.l.	D. Haelew. 1259a	South Africa	<i>Cheilomenes propinqua</i> (Mulsant, 1850)	MG757828	
<i>He. virescens</i> s.l.	D. Haelew. 653a	South Africa	<i>Cheilomenes propinqua</i>	MG757803	
<i>He. virescens</i> s.l.	D. Haelew. 655c	South Africa	<i>Cheilomenes propinqua</i>	MG757804	KU574867
<i>He. virescens</i> s.l.	D. Haelew. 659a	South Africa	<i>Cheilomenes propinqua</i>		OL335918
<i>He. virescens</i> s.l.	D. Haelew. 659b	South Africa	<i>Cheilomenes propinqua</i>	MG757805	MG745342
<i>He. virescens</i> s.l.	D. Haelew. 659d	South Africa	<i>Cheilomenes propinqua</i>	MG757806	

Species	Isolate	Country	Host	ITS	LSU
<i>He. virescens</i> s.l.	D. Haelew. 1374a	Panama	<i>Cycloneda sanguinea</i> (Linnaeus, 1763)	MG757831	
<i>He. virescens</i> s.l.	D. Haelew. 924a	Panama	<i>Cycloneda sanguinea</i>	MG757808	
<i>He. virescens</i> s.l.	D. Haelew. 3187a	Czech Republic	<i>Hippodamia tredecimpunctata</i> Linnaeus, 1758	OL335937	OL335923
<i>He. virescens</i> s.l.	D. Haelew. 1809c	Chile	<i>Hippodamia variegata</i> (Goeze, 1777)		OL335922
<i>He. virescens</i> s.l.	D. Haelew. 1200h	USA	<i>Olla v-nigrum</i> (Mulsant, 1866)	MG757819	MG745348
<i>He. virescens</i> s.l.	D. Haelew. 1200i	USA	<i>Olla v-nigrum</i>	MG757820	MG745349
<i>He. virescens</i> s.l.	D. Haelew. 3202a	Mexico	<i>Olla v-nigrum</i>	OL335938	OL335925
<i>He. virescens</i> s.l.	D. Haelew. 3202b	Mexico	<i>Olla v-nigrum</i>	OL335939	OL335926
<i>He. virescens</i> s.l.	D. Haelew. 3202c	Mexico	<i>Olla v-nigrum</i>		OL335927
<i>He. virescens</i> s.l.	D. Haelew. 3203a	Mexico	<i>Olla v-nigrum</i>	OL335940	OL335928
<i>He. virescens</i> s.l.	D. Haelew. 3204c	Mexico	<i>Olla v-nigrum</i>		OL335929
<i>He. virescens</i> s.l.	D. Haelew. 954d	USA	<i>Olla v-nigrum</i>	MG757811	
<i>He. virescens</i> s.l.	D. Haelew. 954e	USA	<i>Olla v-nigrum</i>	MG757812	
<i>He. virescens</i> s.l.	JP352a	USA	<i>Olla v-nigrum</i>		OL335924
<i>He. virescens</i> s.l.	JP352b	USA	<i>Olla v-nigrum</i>	MG757798	MG745337
<i>He. virescens</i> s.l.	JP353a	USA	<i>Olla v-nigrum</i>	KT800043	KT800013
<i>He. virescens</i> s.l.	JP353b	USA	<i>Olla v-nigrum</i>	MG757799	MG745338
<i>He. virescens</i> s.l.	JP354b	USA	<i>Olla v-nigrum</i>	MG757800	MG745339
<i>He. virescens</i> s.l.	D. Haelew. 1250b	USA	<i>Psyllobora vigintimaculata</i> (Say, 1824)	MG757825	MG745354
<i>He. virescens</i> s.l.	D. Haelew. 1250c	USA	<i>Psyllobora vigintimaculata</i>	MG757826	MG745355
<i>He. virescens</i> s.l.	D. Haelew. 1251b	USA	<i>Psyllobora vigintimaculata</i>	MG757827	MG745356
<i>He. virescens</i> s.s.	D. Haelew. 1444a	USA	<i>Chilocorus stigma</i> (Say, 1835)	MT373697	OL335916
<i>He. virescens</i> s.s.	D. Haelew. 1444b	USA	<i>Chilocorus stigma</i>	MT373698	OL335917

ized in FigTree version 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>) and edited in Adobe Illustrator CC 2018 (San Jose, CA).

Species delimitation approaches

A total of thirteen species hypotheses, as identified from our ML and Bayesian analyses, were evaluated using four species delimitation methods. First, we used the Bayesian coalescent method in the software Bayesian Phylogenetics and Phylogeography (BPP) (Yang 2015). We performed analysis A11 (Yang & Rannala 2014) for unguided species delimitation using rjMCMC algorithm 0 (Yang & Rannala 2010). Because posterior probabilities are affected by prior distributions on the ancestral population size (θ) and root age (τ_0), we ran analyses with three different combinations of priors (sensu Leache & Fujita 2010, De Lange et al. 2021): $\theta \sim \text{IG}(3,0.002)$ and $\tau_0 \sim \text{IG}(3,0.002)$, $\theta \sim \text{IG}(3,0.02)$ and $\tau_0 \sim \text{IG}(3,0.02)$, and $\theta \sim \text{IG}(3,0.02)$ and $\tau_0 \sim \text{IG}(3,0.002)$, with IG being the inverse-gamma prior distribution. For each combination of priors, two analyses were run twice with a different seed to confirm consistency. The number of generations was 200,000 with a sampling interval of 2 and a burn-in of

50,000. All six analyses were run using the High Performance Computing (HPC) core facilities of the Flemish Supercomputing Centre.

Second, we applied the Automatic Barcode Gap Discovery (ABGD) method of Puillandre et al. (2012). ABGD partitions isolates into a maximum number of groups, equaling species hypotheses, based on nucleotide divergence among them. We used the following parameters in the online version of ABGD (<https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>): Pmin = 0.001, Pmax = 0.01, steps = 10, and Nb bins = 20. We ran analyses with twelve different combinations of distance metrics and gap width values: JC69 (Jukes & Cantor 1969), K80 (Kimura 1980), and simple distance metrics; and gap width values X = 0.1, 0.5, 1.0, and 1.5.

Third, we applied the General Mixed Yule Coalescent (GMYC) approach, which models processes at both the population level (coalescence) and species level (speciation) based on a fully resolved ultrametric tree (Pons et al. 2006). We transformed the MCC tree constructed above to a NEXUS file using BEAST 1.8.4 (Drummond et al. 2012) on a local computer with the following command: treeannotator -burnin 0 input.mcctree output.nex. Next, we conducted GMYC in R (R Core Team 2013) using the

Tab. 2. Gene regions included in phylogenetic analyses, with for every partition the number of sequences, total number of sites, informative sites, and invariable sites.

Partition	Locus	Sequences	Sites	Informative	Invariable
1	ITS1	63	539	177	337
2	5.8S	65	158	9	149
3	ITS2	65	394	128	239
4	LSU	54	2,156	83	2,014
Concatenated	ITS-LSU	72	3,247	397	2,739

packages *rmcl* (Michonneau et al. 2015) and *splits* (Ezard et al. 2009).

Finally, we used STACEY version 1.2.5 (Jones 2017) implemented in BEAST2 (Bouckaert et al. 2019). Parameters and priors were set in BEAUti version 2.6.6 to prepare an XML file as input for the BEAST runs. As with the Bayesian inference (BI) analyses above, we used two partitions: ITS and LSU. Both alignments were imported as NEXUS files into BEAUti and hypothesized species were assigned to each isolate (species A to M). We selected substitution models for ITS and LSU, a strict molecular clock, and a Yule species tree prior, with the Relative Death Rate parameter μ/λ set at 0.0 and not estimated. The Collapse Height parameter ε was set to be 10^{-5} . The Collapse Weight parameter ω was estimated at 0.5 and given a uniform prior, meaning every number of species between one and thirteen is regarded as equally likely a priori. We performed four independent MCMC runs for 1,000,000,000 generations each, with tree sampling frequency of 1,000. Six STACEY analyses were run on the Command Prompt of Windows, the CIPRES Science Gateway (Miller et al. 2010), and the HPC core facilities of the Flemish Supercomputing Centre. A burn-in of 20 % was discarded from each run. Remaining posterior samples were combined in LogCombiner version 2.6.6 and used to calculate the most likely number of clusters (= putative species) with SpeciesDelimitationAnalyzer (Jones et al. 2014).

Data availability

Voucher specimens of infected ladybirds are deposited at the Brabant Museum of Nature, Tilburg (NNKN); Florida State Collection of Arthropods, Gainesville, FL (FSCA); Meise Botanic Garden (BR); Museum of Comparative Zoology at Harvard University, Cambridge, MA (MCZ); and Purdue Entomological Research Collection, West Lafayette, IN (PERC). Voucher slides of *He. harmoniae* are deposited at Meise Botanic Garden (BR), Farlow Herbarium

at Harvard University (FH), Herbarium Universitatis Gandavensis (GENT), Kriebel Herbarium at Purdue University (PUL), and the Zoology collection at the Natural History Museum of Denmark, Copenhagen (Z-NHMD). All generated sequences have been uploaded to NCBI GenBank (accession numbers in Tab. 1 and Additional material sequenced section below). The following data are available from Open Science Framework <https://doi.org/10.17605/osf.io/mtgfn>: measurements of 207 thalli made for this study (in XLSX format), sequence alignments generated during this study (in FASTA and NEXUS format), input XML file for Bayesian analyses.

Results

Our concatenated ITS-LSU data matrix consisted of 3,247 characters for 72 isolates (Tab. 2). Selected models were TVM+F+G4 (ITS1, 539 bp, $-\ln L = 4528.398$), TNe (5.8S, 158 bp, $-\ln L = 570.735$), TPM2+F+G4 (ITS2, 394 bp, $-\ln L = 3572.606$), and TIM+F+I (LSU, 2,157 bp, $-\ln L = 8273.306$) using ModelFinder, and TVM+G (ITS, 1,091 bp, $-\ln L = 4342.0526$) and GTR+G (LSU, $-\ln L = 4083.8668$) using jModelTest 2.0. The name *He. virescens* s.l. is assigned to the node holding 10 clades, with maximum support from ML and BI (Fig. 1). All 10 clades within *He. virescens* s.l. have high to maximum support (MLBS = 99–100, BIPP = 1), except for the *He. halyziae* singleton clade, which has moderate support (MLBS = 70, BIPP = 0.89). The full set of 13 species hypotheses was recovered as the highest supported species model in the BPP analysis under all combinations of priors (Fig. 1). Posterior probabilities ranged between 0.89 and 0.96 (Tab. 3). The ABGD analyses of the concatenated dataset resulted in 1 to 11 species hypotheses, depending on the prior intraspecific divergence parameter and the substitution model (Tab. 4). As a result, we also performed ABGD analyses of the single-locus datasets. The analyses of the ITS dataset resulted in 13 spe-



Fig. 1. Phylogeny of *Hesperomyces*, reconstructed from an ITS–LSU dataset. The consensus tree topology ($-\ln L = 8427.038$) is the result of ML inference performed with IQ-TREE. For each node, the ML bootstrap support (≥ 70) and Bayesian posterior probability (≥ 0.7) are presented above or in front of the branch leading to that node. Clades within *Hesperomyces virescens* sensu lato color-coded by host (alternating light and dark green), *He. harmoniae* sp. nov. highlighted in boldface, [†] indicating ex-isotype sequences, countries shown for all isolates as two-letter codes as designated by the International Organization for Standardization country codes (ISO 3166, http://www.iso.org/iso/country_codes.htm). Species delimitation methods are summarized in the five columns at the right: BPP (column I), ABGD of the ITS dataset (II), ABGD of the LSU dataset (III), GMYC (IV), and STACEY (V). No coloration for a given isolate in columns II or III means it was absent in the corresponding dataset. Color schemes from <https://colorbrewer2.org> by C.A. Brewer, Geography, Pennsylvania State University.

Tab. 3. Results of the Bayesian Phylogenetics and Phylogeography (BPP) analyses. Posterior probabilities (PP) are shown for hypothesized number of species [1 to 13] for each of the two replicates (r1, r2) of three different combinations (c1, c2, c3) of priors for ancestral population size (θ) and root age (τ_0).

# species	PP c1,r1	PP c1,r 2	PP c2,r1	PP c2,r2	PP c3,r1	PP c3,r2
[1]	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
[2]	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
[3]	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
[4]	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
[5]	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
[6]	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
[7]	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
[8]	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
[9]	0.000000	0.000000	0.000000	0.000010	0.000000	0.000000
[10]	0.000005	0.000000	0.000055	0.000160	0.000125	0.000115
[11]	0.001465	0.000880	0.003800	0.004765	0.003920	0.005005
[12]	0.047680	0.035770	0.092780	0.103120	0.092780	0.102915
[13]	0.950850	0.963350	0.903365	0.891945	0.903175	0.891965

Tab. 4. Results of the Automatic Barcode Gap Discovery (ABGD) analyses for the concatenated ITS–LSU dataset, for relative gap widths of 0.1, 0.5, 1.0, and 1.5, and prior intraspecific divergence values between 0.001 and 0.01. JC69, Jukes–Cantor substitution model (Jukes & Cantor 1969); K80, Kimura 2-parameter substitution model (Kimura 1980).

ITS–LSU	0.001	0.001292	0.001668	0.002154	0.002783	0.003594	0.004642	0.005995	0.007743	0.01
JC69										
X 0.1	5	5	5	5	5	5	5	5	5	5
X 0.5	5	5	5	5	5	5	5	5	5	5
X 1.0	5	5	5	5	5	5	5	5	5	5
X 1.5	5	5	5	5	5	5	5	5	5	5
K80										
X 0.1	4	4	4	4	4	4	4	1		
X 0.5	4	4	4	4	4	4	4	1		
X 1.0	4	4	4	4	4	4	1			
X 1.5	4	4	4	4	4	4	1			
Simple distance										
X 0.1	11	11	11	11	11	11	11	11	11	11
X 0.5	11	11	11	11	11	11	11	11	11	11
X 1.0	11	11	11	11	11	11	11	11	11	11
X 1.5	11	11	11	11	11	11	11	11	11	11

cies hypotheses for the majority of parameter combinations. Only when the prior intraspecific divergence was 0.001 and 0.001292, the number of species hypotheses was 16 and 14, respectively, for both JC69 and K80 (Tab. 5). The ABGD analyses of the LSU dataset resulted in 11 species hypotheses, except when the prior intraspecific divergence was 0.001 for both JC69 and K80 (15 species hypotheses) (Tab. 6). Coalescent analyses with GMYC resulted in 13 species hypotheses. And finally, the STACEY analysis resulted in the highest likelihood (posterior probability of 0.9997) for the model with 13 clusters (= species).

Taxonomy

Hesperomyces harmoniae Haelew. & De Kesel, sp. nov. – Figs. 2, 3

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Holotypus. – USA. Kentucky, Whitley County, Williamsburg, Cumberland Falls State Resort Park, DuPont Lodge, men's room first floor, 36.838907 N, 84.338376 W, hand collection, leg. D. Haelewaters, 27 October 2019, on female *Harmonia axyridis* (Pallas, 1773) (Coleoptera, Coccinellidae), slide D. Haelew. 1808a (holotype at GENT, 3 mature thalli, right protarsus between claws). Sequences ex-holotype: isolate D. Haelew. 1808b (2 mature thalli, ITS = OL335936, LSU = OL335921).

Diagnosis. – Morphologically very similar to *Hesperomyces virescens* sensu stricto (ex *Chilocorus stigma*), but

Tab. 5. Results of the Automatic Barcode Gap Discovery (ABGD) analyses for the ITS dataset, for relative gap widths of 0.1, 0.5, 1.0, and 1.5, and prior intraspecific divergence values between 0.001 and 0.01. JC69, Jukes–Cantor substitution model (Jukes & Cantor 1969); K80, Kimura 2-parameter substitution model (Kimura 1980).

ITS	0.001	0.001292	0.001668	0.002154	0.002783	0.003594	0.004642	0.005995	0.007743	0.01
JC69										
X 0.1	16	14	13	13	13	13	13	13	13	13
X 0.5	16	14	13	13	13	13	13	13	13	13
X 1.0	16	14	13	13	13	13	13	13	13	13
X 1.5	16	14	13	13	13	13	13	13	13	13
K80										
X 0.1	16	14	13	13	13	13	13	13	13	13
X 0.5	16	14	13	13	13	13	13	13	13	13
X 1.0	16	14	13	13	13	13	13	13	13	13
X 1.5	16	14	13	13	13	13	13	13	13	13
Simple distance										
X 0.1	13	13	13	13	13	13	13	13	13	13
X 0.5	13	13	13	13	13	13	13	13	13	13
X 1.0	13	13	13	13	13	13	13	13	13	13
X 1.5	13	13	13	13	13	13	13	13	13	13

Tab. 6. Results of the Automatic Barcode Gap Discovery (ABGD) analyses for the LSU dataset, for relative gap widths of 0.1, 0.5, 1.0, and 1.5, and prior intraspecific divergence values between 0.001 and 0.01. JC69, Jukes–Cantor substitution model (Jukes & Cantor 1969); K80, Kimura 2-parameter substitution model (Kimura 1980).

LSU	0.001	0.001292	0.001668	0.002154	0.002783	0.003594	0.004642	0.005995	0.007743	0.01
JC69										
X 0.1	15	11	11	11	11	11	11	11	11	11
X 0.5	15	11	11	11	11	11	11	11	11	11
X 1.0	15	11	11	11	11	11	11	11	11	11
X 1.5	15	11	11	11	11	11	11	11	11	11
K80										
X 0.1	15	11	11	11	11	11	11	11	11	11
X 0.5	15	11	11	11	11	11	11	11	11	11
X 1.0	15	11	11	11	11	11	11	11	11	11
X 1.5	15	11	11	11	11	11	11	11	11	11
Simple distance										
X 0.1	11	11	11	11	11	11	11	11	11	11
X 0.5	11	11	11	11	11	11	11	11	11	11
X 1.0	11	11	11	11	11	11	11	11	11	11
X 1.5	11	11	11	11	11	11	11	11	11	11

forming a distinct species-level clade supported by ITS and LSU data. Unique molecular synapomorphies and motifs in the ITS at positions 203–209 (5′-GGGCTAA-3′), 316 (T), 515 (A), 827 (A), 851 (T). Unique molecular synapomorphies and motifs in the LSU at positions 183–186 (5′-CACA-3′), 198 (G), 603–608 (5′-TTACAG-3′), 644 (G), 754 (A), 798 (C), 800 (A), 808 (A), 813 (C), 817 (A).

Description. – **Thallus** (290)372–431–490(653) µm long from foot to perithecial apex; colored hyaline to yellowish-green [207/32]. – **Receptacle** (61)73–81–89(118) µm long [207/32]. – **Cell I** (55)60–67–74(92) × (17)20–24–28(38) µm, triangular to rhomboidal, longer than broad

[207/32]. – **Cell II** (22)29–33–37(47) × (12)18–21–24(33) µm, slightly longer than broad, rhomboidal to trapezoidal [206/32]. – **Cell III** (10)13–16–19(25) × (9)15–19–23(28) µm, shorter than cell II, almost isodiametric, dorsally convex [207/32]. – **Primary appendage** (58)68–74–80(94) µm long, consisting of 4–5 superposed cells; basal cell (15)19–21–23(30) µm long, longer than any of the other cells of the appendage; remaining cells each bearing one antheridium directed outwardly, the uppermost cell bearing one to two antheridia as well as a terminal spinous process [202/32]. – **Antheridia** (18)23–26–29(35) µm long; with outwardly curved efferent



Fig. 2. In-situ photos of *Hesperomyces harmoniae* sp. nov. on its invasive alien ladybird host, *Harmonia axyridis*. **a.** Photo record from T. Moon, 18 August 2010, New York, USA. **b.** iNaturalist observation #117449007 by V. Charny, 16 May 2022, Alabama, USA. **c.** iNaturalist #43924977 by G. Okatov, 27 April 2020, Russia. **d.** iNaturalist #82437352 by D. Foster, 10 June 2021, Colombia. **e.** Photo record from G. San Martin, 13 May 2012, Belgium.

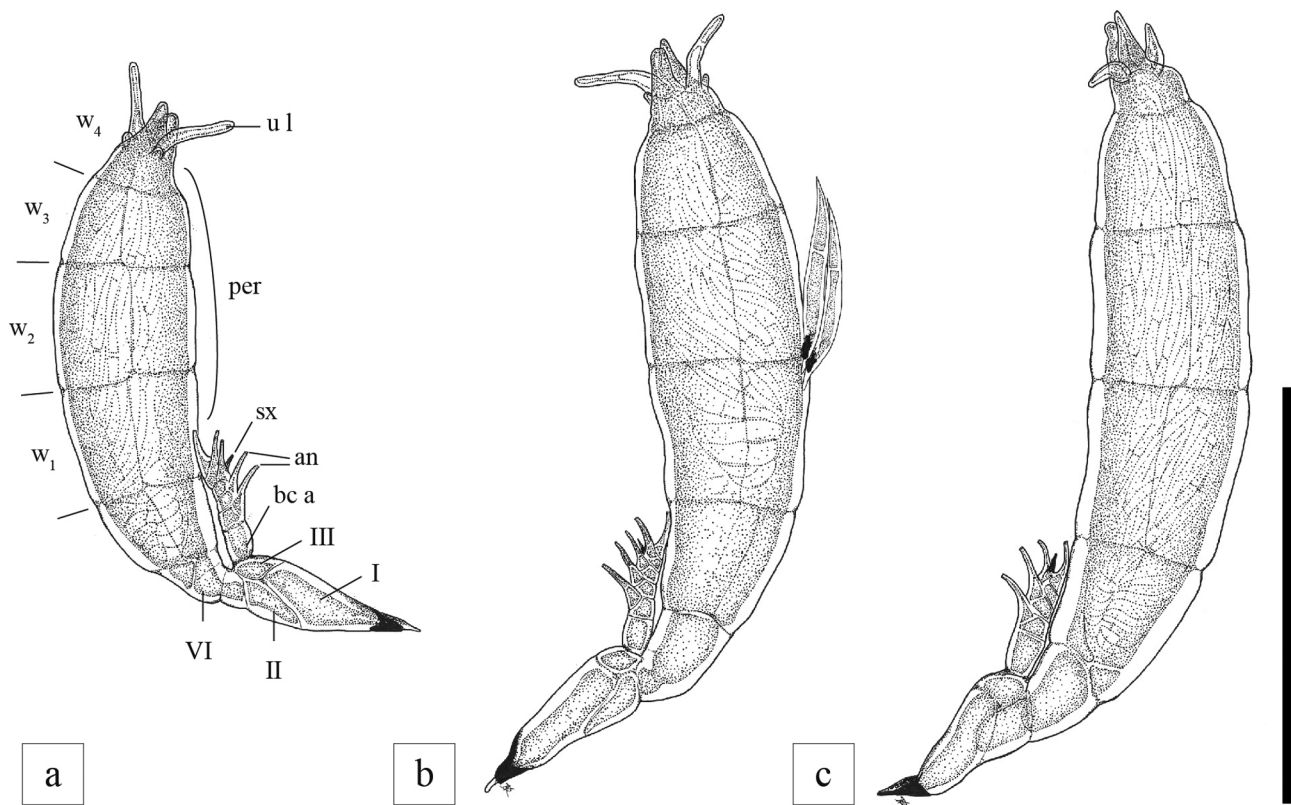


Fig. 3. *Hesperomyces harmoniae* sp. nov. **a.** Mature thallus from elytra, slide D. Haelew. 326c (FH 00313613). Byron, Peach County, Georgia, USA. Indicated are cells I, II, and III of the receptacle, the basal cell of the appendage (bc a), two antheridia (an), the spinous process or remains of the original spore apex (sx), cell VI, the perithecium (per) with four tiers of outer wall cells (w_1 through w_4), and one of the upper lobes (u l). **b.** Mature thallus from left elytron, slide D. Haelew. 1255b (FH 00313644). Stellenbosch, South Africa. Two ascospores are attached to the ventral margin of the perithecium. **c.** Mature thallus from right elytron, slide D. Haelew. 601c (FH 00313617). Byron, Peach County, Georgia, USA. Note that the upper lobes do not exceed the perithecial apex. Scale bar = 200 μm , del. A. De Kesel.

necks, (12)14–16–18(21) μm [202/32]. – Cell VI (27)38–56–74(171) $\times$ (20)27–34–41(54) μm , elongated, broadening distally [207/32]. – Perithecium (201)260–303–346(412) $\times$ (50)67–77–87(106) μm , on average 4 $\times$ longer than broad, asymmetric, fusiform, broadest near the middle, and then gradually tapering towards a short, broad, indistinct neck, and a subacute, lobulated, asymmetrical apex; septa between the horizontal tiers of wall cells marked by constrictions; perithecial tip with two lower lobes, two upper lobes, and two prominent lips surrounding the ostiole; lower lobes minute; upper lobes (34)45–50–55(65) μm long, unicellular, finger-like, usually curved outwards, their tips exceeding the perithecial apex; ostiole with two lips, one lip triangular, the other slightly shorter, blunt or rounded [207/32]. – Ascospores two-celled, (66)78–86–94(106) $\times$ (4)5–6–7(8) μm , with gelatinous sheath covering the larger cell [103/11].

Etymology. – Referring to the host genus, *Harmonia*.

Hosts and distribution. – Known on *Harmonia axyridis* (Coleoptera, Coccinellidae) from all continents except Australia and Antarctica. Thus far reported in the following countries (Tab. 7): In North America: Canada, El Salvador, Mexico, and the USA; in South America: Argentina, Colombia, and Ecuador; in Europe: Austria, Belgium, Bulgaria, Croatia, Czech Republic, France, Germany, Greece, Hungary, Italy, Luxembourg, Montenegro, The Netherlands, Poland, Romania, Russia, Serbia, Slovakia, Switzerland, the United Kingdom; in Africa: South Africa; in Asia: China, Japan, and Turkey.

Additional material examined. – **NORTH AMERICA.** – CANADA. British Columbia Province, Vancouver, Main Mall Greenway, 49.256900 N, 123.245983 W, hand collection in community garden, leg. D. Haelewaters, 31 July

Tab. 7. Overview of worldwide records of *He. harmoniae* to date.

Country	Region	Year	Reference(s)
China	Sichuan Province	1930–1939	Haelewaters et al. (2014)
USA	Pocahontas State Park, Virginia	2002 (May)	Haelewaters et al. (2017)
USA	Great Smoky Mountains National Park, Tennessee	2002 (Jul)	Haelewaters et al. (2017)
USA	Wayne County, Ohio	2002 (Jul–Nov)	Garcés & Williams (2004)
USA	Lebanon County, Pennsylvania	2002–2003	Riddick & Schaefer (2005)
USA	Wake County, North Carolina	2003–2004	Nalepa & Weir (2007)
USA	Oktibbeha County, Mississippi	2003–2004	Riddick (2006)
USA	Harrison County, Kentucky	2004	Harwood et al. (2006b)
USA	Stephens County, Georgia	2004	This paper: Additional material examined
USA	Brookings County, South Dakota	2005	Harwood et al. (2006a)
USA	Centre County, Pennsylvania	2005	Harwood et al. (2006a)
USA	Kentucky	2005	Harwood et al. (2006a)
USA	Payne County, Oklahoma	2005	Harwood et al. (2006a)
USA	Washington County, Arkansas	2005	Harwood et al. (2006a)
USA	Jefferson County, West Virginia	2005	Harwood et al. (2006a)
USA	Boston Harbor Islands, Massachusetts	2005–2007	Haelewaters et al. (2015)
Belgium	Meise, Flemish Region	2006–2007	De Kesel (2011)
USA	Peach County, Georgia	2007	Riddick & Cottrell (2010)
USA	Mississippi	2007–2009	Riddick (2010)
The Netherlands	De Kaaistoep nature reserve, North Brabant Province	2008	Haelewaters & De Kesel (2011)
Germany	Hesse	2008–2009	Herz & Kleespiel (2012)
The Netherlands	De Kaaistoep nature reserve, North Brabant Province	2008–2011	Raak-van den Berg et al. (2014)
The Netherlands	De Kaaistoep nature reserve, North Brabant Province	2008–2015	van Wielink (2017)
Germany	Hohenstein, Hesse	2009	Steenberg & Harding (2010)
The Netherlands	De Kaaistoep nature reserve, North Brabant Province	2009	Haelewaters et al. (2012b)
The Netherlands	Zundert, North Brabant Province	2009	Raak-van den Berg et al. (2014)
USA	Peach County, Georgia	2009	Cottrell & Riddick (2012)
USA	Saint Lucie County, Florida	2009	Haelewaters et al. (2017)
Belgium	Ukkel, Brussels-Capital Region	2010	De Kesel (2011)
Canada	Elgin County, Ontario Province	2010	Haelewaters et al. (2017)
Germany	Giessen, Hesse	2010	Ceryngier & Twardowska (2013)
The Netherlands	Alkmaar, North Holland Province	2010	Raak-van den Berg et al. (2014)
The Netherlands	Alphen-aan-de-Rijn, South Holland Province	2010	Raak-van den Berg et al. (2014)
The Netherlands	Deelen, Gelderland Province	2010	Raak-van den Berg et al. (2014)
The Netherlands	Doesburg, Gelderland Province	2010	Raak-van den Berg et al. (2014)
The Netherlands	Kootwijk, Gelderland Province	2010	Raak-van den Berg et al. (2014)
The Netherlands	Tilburg, North Brabant Province	2010	Raak-van den Berg et al. (2014)
The Netherlands	Wageningen, Gelderland Province	2010	Raak-van den Berg et al. (2014)
The Netherlands	Winterswijk, Gelderland Province	2010	Raak-van den Berg et al. (2014)
USA	Monroe County, New York	2010	Flickr*
Belgium	Flemish Region	2011	De Kesel (2011)
Belgium	Meise, Flemish Region	2011–2016	Haelewaters et al. (2017)
The Netherlands	De Kaaistoep nature reserve, North Brabant Province	2011	Haelewaters et al. (2012a)
The Netherlands	Deelen, Gelderland Province	2011	Raak-van den Berg et al. (2014)
The Netherlands	Tilburg, North Brabant Province	2011	Haelewaters et al. (2014)
The Netherlands	Utrecht, Utrecht Province	2011	Haelewaters et al. (2012a)
UK	Oxfordshire County, South East England	2011	Haelewaters et al. (2014)
USA	Montgomery County, Maryland	2011	This paper: Additional material examined
Belgium	Watermael-Boitsfort, Brussels-Capital Region	2012	Flickr*
USA	Baltimore, Maryland	2012	Haelewaters et al. (2017)
USA	Delaware County, Ohio	2012	This paper: Additional material examined
Belgium	Destelbergen, Flemish Region	2013	Haelewaters et al. (2014)
Croatia	Pelješac Peninsula, Dubrovnik-Neretva	2013	Ceryngier et al. (2013)

Country	Region	Year	Reference(s)
Czech Republic	České Budějovice, South Bohemian Region	2013	Ceryngier & Twardowska (2013)
France	Paris, île-de-France	2013	Haelewaters et al. (2017)
Germany	Gereuth, Bavaria Free State	2013	Haelewaters et al. (2018a)
Poland	Warsaw, Mazovia	2013	Haelewaters et al. (2017)
USA	Washington County, Ohio	2013	This paper: Additional material examined
South Africa	Stellenbosch, Western Cape Province	2013–2015	Haelewaters et al. (2016)
Czech Republic	České Budějovice, South Bohemian Region	2014	Fiedler & Nedvěd (2019), Haelewaters et al. (2017)
Ecuador	N/A	2014	Cornejo & González (2015)
Hungary	Debrecen, Hajdú-Bihar County	2014	Pfliegler (2014), Haelewaters et al. (2017)
The Netherlands	Ijsselstein, Utrecht Province	2014	Haelewaters et al. (2018a)
USA	Bristol County, Massachusetts	2014	This paper: Additional material examined
USA	Cheshire County, New Hampshire	2014	Haelewaters et al. (2017)
USA	Middlesex County, Massachusetts	2014	This paper: Additional material sequenced
USA	Peach County, Georgia	2014	Haelewaters et al. (2016, 2018a)
USA	Suffolk County, Massachusetts	2014	This paper: Additional material sequenced
Poland	Warsaw, Masovia	2014–2015	Gorczak et al. (2016)
Canada	Vancouver, British Columbia Province	2015	Haelewaters et al. (2017)
Croatia	Pelješac Peninsula, Dubrovnik-Neretva	2015	Haelewaters et al. (2017)
Hungary	Szentmártonkáta, Pest County	2015	Haelewaters et al. (2017)
Slovakia	Bratislava Region	2015	Haelewaters et al. (2017)
USA	Baltimore, Connecticut	2015	Haelewaters et al. (2019a)
USA	Middlesex County, New Jersey	2015	Haelewaters et al. (2017)
USA	Peach County, Georgia	2015	This paper: Additional material examined
USA	Washtenaw County, Michigan	2015	Haelewaters et al. (2017)
Argentina	Agua Verde, Buenos Aires	2016	Haelewaters et al. (2017)
Austria	Vienna, Vienna State	2016	Haelewaters et al. (2017)
Hungary	Debrecen, Hajdú-Bihar County	2016	Haelewaters et al. (2017)
The Netherlands	Tilburg, North Brabant Province	2016	Haelewaters et al. (2018a)
USA	Benton County, Oregon	2016	Haelewaters et al. (2017)
USA	Chittenden County, Vermont	2016	Haelewaters et al. (2017)
USA	Middlesex County, Massachusetts	2016	Haelewaters et al. (2018b)
USA	Monongalia County, West Virginia	2016	This paper: Additional material sequenced
USA	Tuscaloosa County, Alabama	2016	Haelewaters et al. (2017)
Bulgaria	South-Western Region	2017	Ceryngier & Romanowski (2017)
Greece	Central Macedonian Region	2017	Ceryngier & Romanowski (2017)
Japan	Kyoto, Kansai Region	2017	Haelewaters et al. (2018a)
Montenegro	Bistrica, Podgorica	2017	Ceryngier & Romanowski (2017)
USA	Dane County, Wisconsin	2017	This paper: Additional material examined
USA	Middlesex County, Massachusetts	2017	This paper: Additional material examined
USA	Windham County, Vermont	2017	Crous et al. (2021)
Czech Republic	České Budějovice, South Bohemian Region	2018	This paper: Additional material examined
Czech Republic	Konětopy, Central Bohemian Region	2018	This paper: Additional material examined
Czech Republic	Myšnice, South Bohemian Region	2018	Knapp et al. (2022)
Czech Republic	Přílepy, Central Bohemian Region	2018	This paper: Additional material examined
Czech Republic	Skalsko, Central Bohemian Region	2018	Crous et al. (2021)
Mexico	Morelos, Nuevo León	2018	López-Arroyo et al. (2018)
Russia	Kholmskaya, Krasnodar Krai	2018	Orlova-Bienkowskaja et al. (2018)

Country	Region	Year	Reference(s)
Switzerland	Delémont, Canton of Jura	2018	Haelewaters et al. (2022)
Italy	Rodigo, Mantua Province	2019	iNaturalist*
Romania	Bacău County, Nord-Est Development Region	2019	This paper: Additional material examined
Romania	Hunedoara County, Vest Development Region	2019	This paper: Additional material examined
USA	Whitley County, Kentucky	2019	This paper: Holotypus
Belgium	Herzele, Flemish Region	2020	This paper: Additional material examined
Serbia	Valjevo, Kolubarski District	2020	iNaturalist*
Canada	Elin County, Ontario Province	2020	This paper: Additional material examined
El Salvador	Ciudad Real, San Salvador Department	2020	iNaturalist*
USA	Alachua County, Florida	2020	This paper: Additional material examined
Belgium	Oudenaarde, Flemish Region	2021	This paper: Additional material examined
Czech Republic	České Budějovice, South Bohemian Region	2021	Lubbers et al. (2022)
Italy	Moncalieri	2021	iNaturalist*
Luxembourg	Grevenmacher	2021	iNaturalist*
Turkey	Çankaya, Eskişehir Province	2021	iNaturalist*
Russia	Kholmskaya, Krasnodar Krai	2021	iNaturalist*
Colombia	Medellín, Antioquia Department	2021	iNaturalist*
USA	Dauphin County, Pennsylvania	2022	iNaturalist*
USA	Jackson County, Mississippi	2022	iNaturalist*
USA	Jefferson County, Alabama	2022	iNaturalist*
USA	Washington County, Rhode Island	2022	iNaturalist*

* Details of unpublished Flickr and iNaturalist records. – **NORTH AMERICA.** – EL SALVADOR. San Salvador Department, Ciudad Real, 13.994303 N, 89.640413 W, iNaturalist observation #42963099 by F.E. Vásquez Pacheco, 23 April 2020. – USA. Alabama, Jefferson County, Hoover, Old Rocky Ridge Road, 33.392497 N, 86.773159 W, iNaturalist #117449007 by V. Charny, 16 May 2022 (Fig. 2b); Mississippi, Jackson County, Ocean Springs, Washington Avenue, 30.41131 N, 88.827806 W, iNaturalist #118577236 by C. Stempien, 22 May 2022; New York, Monroe County, Rochester, Portsmouth Terrace, 43.153669 N, 77.581126 W, photo record by T. Moon (<https://flickr.com/photos/elusivebyte/4905619381/>), 18 August 2010 (Fig. 2a); Pennsylvania, Dauphin County, Harrisburg, Capital Area Greenbelt, 40.318676 N, 76.889232 W, iNaturalist #118291290 by C. Meyers, 22 May 2022; Rhode Island, Washington County, South Kingstown, Shelldrake Road, 41.381537 N, 71.550121 W, iNaturalist #120452687 by S. Thoms, 4 June 2022. – **SOUTH AMERICA.** COLOMBIA. Medellín, Castropol neighborhood, 6.216389 N, 75.565 W, iNaturalist #82437352 by D. Foster, 10 June 2021 (Fig. 2d). – **EUROPE.** – BELGIUM. Brussels-Capital Region, Watermael-Boitsfort, photo record from G. San Martin (<https://flickr.com/photos/sanmartin/7211922934/in/album-72157600404369181/>), 13 May 2012 (Fig. 2e). – ITALY. Lombardy Region, Mantua Province, Rodigo, 45.185964 N, 10.678411 E, iNaturalist #73107164 by M. Munari, 28 April 2019; Piedmont Region, Moncalieri, Parco Fluviale del Po, 44.954602 N, 7.703298 E, iNaturalist #78194299 by E. Castello, 9 May 2021. – LUXEMBOURG. Canton Grevenmacher, Grevenmacher, 49.665192 N, 6.423672 E, iNaturalist #87744891 by A. Weigel, 19 July 2021. – RUSSIA. Southern Federal District, Krasnodar Krai, Abinsky District, Kholmskaya, 44.828552 N, 38.380828 E, iNaturalist #43924977 by G. Okatov, 27 April 2020 (Fig. 2c); Southern Federal District, Krasnodar Krai, Gelendzhik, 44.580878 N, 37.979694 E, iNaturalist #120151781 by V.V. Krylenko, 4 June 2022. – SERBIA. Šumadija and Western Serbia Region, Valjevo, Stubo, iNaturalist #41318175 by M. Jovanovic, 3 April 2020. – **ASIA.** – TURKEY. Central Anatolia Region, Eskişehir Province, Odunpazari District, Çankaya neighborhood, 39.750689 N, 30.536545 E, iNaturalist #79255275 by M.A. Suna, 14 May 2021.

2015, on male *Ha. axyridis* forma *novemdecimsignata* (PERC 0151963), slide D. Haelew. 3527a (GENT, 9 mature thalli, second sternite); Ontario Province, Elin County, Bayham Municipality, Port Burwell, cottage at 57755 Lakeshore Line, 10 meters from the edge of a cliff overlooking Lake Erie, hand collection, *leg.* B. Bolin (iNaturalist observation #78471051), 7 November 2020, on male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151960), slide D. Haelew. 3393b (GENT, 4 mature thalli, left elytral tip). – USA. Alabama, Tuscaloosa County, Tulip Tree Springs, off Echola Road, 33.33480 N, 87.79355 W, *leg.* J.C. Abbott, 1 July 2016, on three specimens of female *Ha. axyridis* f. *novemdecimsignata* (PERC 0151955), slides D. Haelew. 3286b

(GENT, 1 mature thallus, right elytral tip), D. Haelew. 3289b (GENT, 3 mature thalli, left elytral tip), and D. Haelew. 3291b (GENT, 5 mature thalli, left elytral tip); *ibid.*, on two specimens of male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151956), slides D. Haelew. 3287b (GENT, 4 mature thalli, left metafemur), D. Haelew. 3287c (GENT, 6 mature thalli, left metafemur), and D. Haelew. 3288b (GENT, 8 mature thalli, fourth sternite); Florida, Alachua County, Gainesville, hand collection, *leg.* C.N. Keiser, 10 April 2020, on *Ha. axyridis*, D. Haelew. 3090 (no slides); Georgia, Peach County, Byron, 480-ha land of the USDA Southeastern Fruit and Tree Nut Research Laboratory located at 32.657792 N, 83.7383580 W, Tedders pyramidal trap

with insecticide-impregnated ear tag as killing agent, *leg.* E. Brooks Thompson, 10 April 2014, on two specimens of *Ha. axyridis*, slides D. Haelew. 1047a (FH 00313623, 2 mature thalli, right elytron) and D. Haelew. 1051a (FH 00313627, 11 mature thalli, left elytron) [reported as *He. virescens* in Haelewaters et al. (2018a)]; *ibid.*, 10 April 2014, on two specimens of *Ha. axyridis* (MCZ), slides D. Haelew. 1048a (FH 00313624, 13 mature thalli, right elytron), D. Haelew. 1048b (FH 00313625, 12 mature thalli, anterior edge of right elytron), and D. Haelew. 1050a (FH 00313626, 7 mature thalli, posterior tip of left elytron) [reported as *He. virescens* in Haelewaters et al. (2018a)]; *ibid.*, 15 April 2014, on *Ha. axyridis*, slide D. Haelew. 316c (FH 00313772, 4 mature thalli, sternites); *ibid.*, 15 April 2014, *Ha. axyridis* (MCZ), slide D. Haelew. 326c (FH 00313613, 6 mature thalli, elytra); *ibid.*, 17 April 2014, on *Ha. axyridis* (MCZ), slide D. Haelew. 600a (FH 00313614, 7 mature thalli, left elytron) [reported as *He. virescens* in Haelewaters et al. (2018a)]; *ibid.*, 17 April 2014, on three specimens of *Ha. axyridis*, slides D. Haelew. 601a (FH 00313615, 9 mature thalli, right-hand side of pronotum), D. Haelew. 601b (FH 00313616, 10 mature thalli, head), D. Haelew. 601c (FH 00313617, 15 mature thalli, right elytron) [reported as *He. virescens* in Haelewaters et al. (2018a)]; D. Haelew. 650a (FH 00313620, 6 mature thalli, right elytral tip), D. Haelew. 652a (FH 00313621, 2 mature thalli, right mesofemur), and D. Haelew. 652b (FH 00313622, 8 mature thalli, left elytron) [reported as *He. virescens* in Haelewaters et al. (2018a)]; *ibid.*, *leg.* T.E. Cottrell, 27 August 2015, on *Ha. axyridis*, D. Haelew. 1421 (no slides); Georgia, Stephens County, Toccoa Falls College, *leg.* R. Turnbow, 1 August 2004, on *Ha. axyridis* (FSCA), slide D. Haelew. 1156a (GENT, 6 mature thalli, right elytral tip); Kentucky, Witley County, Williamsburg, Cumberland Falls State Resort Park, DuPont Lodge, men's room first floor, 36.838907 N, 84.338376 W, hand collection, *leg.* D. Haelewaters, 27 October 2019, on male *Ha. axyridis*, slide D. Haelew. 1806a (GENT, 11 mature thalli, left elytral tip); *ibid.*, on male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151950), slides D. Haelew. 1807a (PUL F29115, 3 mature thalli, right protarsus) and D. Haelew. 1807b (GENT, 7 mature thalli, left profemur); Maryland, Montgomery County, North Potomac, 15703 Quince Orchard Road, 39.1130556 N, 77.2527778 W, 126.5 m a.s.l., *leg.* J.E. Wappes, 7 May 2011, on *Ha. axyridis* (FSCA), slide D. Haelew. 1154a (GENT, 2 mature thalli, left elytron); Massachusetts, Bristol County, Easton, Stonehill College, Shields Science Center, hallway southeast corner, 42.056326 N, 71.077517 W, hand collection, *leg.* D. Haelewaters & S.J.C. Verhaeghen, 6 November 2014, on male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151937), slide D. Haelew. 509a (GENT, 1 mature thallus, right elytral tip); Massachusetts, Middlesex County, Cambridge, Farlow Herbarium, Room 4, hand collection, *leg.* K. LoBuglio, 27 Jan. 2017, on *Ha. axyridis*, D. Haelew. 1270 (no slides); Massachusetts, Plymouth County, Boston Harbor Islands National Recreation Area, World's End peninsula, 42.264417 N, 70.877167 W, mercury vapor light, *leg.* J. Rykken, 16 August 2006, on female *Ha. axyridis* (MCZ ENT 00602410), slide D. Haelew. 486a (FH 00313516, 8 mature thalli, left elytron) [reported as *He. virescens* in Haelewaters et al. (2015)]; Ohio, Delaware County, Powell area, Bear Woods, *leg.* J.E. Wappes, 31 May 2012, on *Ha. axyridis* (FSCA), slide D. Haelew. 1155a (GENT, 4 mature thalli, right elytron); Ohio, Washington County, Little Hocking, hand collection from a *Catalpa* leaf, *leg.* D. Hennen, 9 June 2013, on female *Ha. axyridis*, slides D. Haelew. 3913d (FH, 5 juvenile and 10 mature thalli, right elytron) and D. Haelew. 3913e (FH, 4 mature thalli, left elipleuron); Wisconsin, Dane County, Madison, University of Wisconsin–Madison

Arboretum, Curtis Prairie, 43.037702 N, 89.431448 W, *leg.* A.C. Dirks, 1–30 June 2017, on *Ha. axyridis* (MCZ), slide D. Haelew. 1440a (FH, 1 juvenile and 6 mature thalli, lateral margin of left elytron); *ibid.*, slide D. Haelew. 1441c (FH, 9 mature thalli, sternites). – **EUROPE.** – **BELGIUM.** Antwerp Province, Branschaaat, Hemelakkers, 51.288599 N, 4.491232 E, hand collection, *leg.* A. De Kesel, 26 February 2011, on male *Ha. axyridis* f. *novemdecimsignata* (BR), slide A. De Kesel 4877 (BR5020195037648V, 5 mature thalli, right elytron); East Flanders Province, Herzele, Zonneveld 15, 50.8551 N, 3.8873 E, hand collection indoors, *leg.* W. Van Caenegem, 27 Dec. 2020, on female *Ha. axyridis* f. *novemdecimsignata* (PERC 0151961), slide D. Haelew. 3448d (GENT, 10 mature thalli, left elytral tip); East Flanders Province, Oudenaarde, Volkegembos, 50.8368 N, 3.6443 E, hand collection, *leg.* W. Van Caenegem, 9 July 2021, on male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151962), slide D. Haelew. 3526a (GENT, 11 mature thalli, left elytral tip); East Flanders Province, Ghent, in a green area between N424 and residential area, 51.0831 N, 3.7556 E, hand collection from *Lapsana communis*, *leg.* P. Mironova, 21 June 2021, on male *Ha. axyridis* f. *spectabilis* (PERC 0151967), slide D. Haelew. 3693a (PUL F29117, 6 mature thalli, left elytron). – **CZECH REPUBLIC.** Central Bohemian Region, Konětopy, 50.2738075 N, 14.6580831 E, *leg.* M. Seidl, 6 October 2018, on female *Ha. axyridis* f. *spectabilis*, D. Haelew. 1552 (no slides); *ibid.*, on male *Ha. axyridis* f. *spectabilis*, D. Haelew. 1553 (no slides); Central Bohemian Region, Rakovník District, Přílepy, 50.1215861 N, 13.6439547 E, *leg.* M. Řeřicha, 9 October 2018, on female *Ha. axyridis* f. *conspicua*, D. Haelew. 1555 (no slides); South Bohemian Region, České Budějovice, University of South Bohemia campus, southern walls of dormitories K1–K3, 48.977628 N, 14.451506 E, hand collection, *leg.* O. Nedvěď, 16 October 2018, on male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151951), slide D. Haelew. 3092a (GENT, 13 mature thalli, mesosternum); South Bohemian Region, České Budějovice, Branišovská, on field next to road, hand collection, *leg.* O. Nedvěď, 27 August 2021, on female *Ha. axyridis* f. *novemdecimsignata* (PERC 0151966), slide D. Haelew. 3691a (PUL F29116, 5 mature thalli, left elytron); South Bohemian Region, České Budějovice, 48.9865633 N, 14.4436756 E, hand collection from *Philadelphus coronarius* (Cornales, Hydrangeaceae), *leg.* O. Nedvěď, 8 June 2021, on *Ha. axyridis*, D. Haelew. 3654 (no slides) [reported as *He. virescens* in Lubbers et al. (2022)]; South Bohemian Region, Myšnice, 49.4540217 N, 13.9681197 E, *leg.* H. Šípková, 6 October 2018, on female *Ha. axyridis* f. *axyridis*, D. Haelew. 1554 (no slides). – **ROMANIA.** Nord-Est Development Region, Bacău County, near Comănești, 46.428275 N, 26.438577 E, hand collection, *leg.* A. Pintilioaie, 24 July 2019, on male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151964), slide D. Haelew. 3528a (GENT, 7 mature thalli, right elytron); Vest Development Region, Hunedoara County, near Leșnic, 45.924657 N, 22.741963 E, hand collection, *leg.* A. Pintilioaie, 21 August 2019, on female *Ha. axyridis* f. *novemdecimsignata* (PERC 0151965), slide D. Haelew. 3529a (GENT, 10 mature thalli, right elytral tip). – **RUSSIA.** Southern Federal District, Krasnodar Krai, Sochi, ex *Oleander*, *leg.* M. Orlova-Bienkowskaja & A. Bienkowski, 1 May 2018, on male *Ha. axyridis* f. *novemdecimsignata*, slide D. Haelew. 3951a (GENT, 3 mature thalli, pronotum). – **SWITZERLAND.** Canton Jura, Delémont, office building at Rue des Grillons 1, hand collection, *leg.* R. Eschen, November 2018, on male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151952), slide D. Haelew. 3093a (GENT, 3 mature thalli, right elytral tip). – **THE NETHERLANDS.** Noord Brabant Province, Tilburg, nature reserve De Kaaistoep, 51.53976 N, 5.00955 E, illuminated white screen,

leg. P. van Wielink, 31 August 2008, on *Ha. axyridis* (NNKN), slide D. Haelew. 011c (GENT, 7 mature thalli, left elytron) [reported as *He. virescens* in Haelewaters et al. (2012a, 2012b)]; Gelderland Province, Wageningen, leg. C.L. Raak-van den Berg, 14 October 2010, on *Ha. axyridis* (NNKN), slides D. Haelew. 091a (FH 00313220, 2 mature thalli, right elytron), D. Haelew. 091b (FH 00313221, 2 mature thalli, fourth sternite), and D. Haelew. 091c (FH 00313222, 8 mature thalli, first sternite). – **AFRICA**. – SOUTH AFRICA. Western Cape Province, Stellenbosch, 33.9330167 S, 18.8601528 E, hand collection, leg. I.A. Minnaar, 11–14 February 2015, on five specimens of *Ha. axyridis*, slides D. Haelew. 669f (FH 00313640, 8 mature thalli, right elytron), D. Haelew. 943c (FH 00313641, 9 mature thalli, right elytron), D. Haelew. 945d (FH 00313642, 1 juvenile and 7 mature thalli, left elytron), D. Haelew. 1005d (FH 00313643, 1 submature and 3 mature thalli, right elytron), and D. Haelew. 1255b (FH 00313644, 7 mature thalli, left elytron) [reported as *He. virescens* in Haelewaters et al. (2018a)]. – **ASIA**. – JAPAN. Kansai Region, Kyoto, hand collection, leg. T. Steenberg, 17 April 2017, on *Ha. axyridis* (NNKN), slides D. Haelew. 1268e (z-NHMD, 1 juvenile and 8 mature thalli, right profemur) and D. Haelew. 1268f (z-NHMD, 4 thalli, right profemur).

Additional material sequenced. – **NORTH AMERICA**. – USA. Connecticut, Baltimore, 441 Opening Hill Road, hand collection, leg. C. Jander, 7 November–15 Dec. 2015, on *Ha. axyridis*, isolate D. Haelew. 1439a (4 mature thalli, right elytron, ITS = MN397128, LSU = MN397128, MCM7 = OP037812) [reported as *H. virescens* in Haelewaters et al. (2019a)]; Massachusetts, Middlesex County, Cambridge, Fresh Pond, hand collection, leg. J. Park and T.W. Wang, 13 September 2014, on *Ha. axyridis*, isolate D. Haelew. 519b (12 mature thalli, sternites, SSU = OP087653); Massachusetts, Suffolk County, Boston, Arnold Arboretum, Hunnewell building, hand collection, leg. M. Tyrrell and Environmental Botany students, 28 2014, on *Ha. axyridis* (MCZ), isolate D. Haelew. 502b (10 mature thalli, left elytral tip, SSU = OP087652, LSU = OP088711); West Virginia, Monongalia County, Morgantown, hand collection from bark of maple, leg. A.M. Metheny, 10 April 2016, on female *Ha. axyridis* f. *novemdecimsignata*, isolates D. Haelew. 1561a (6 mature thalli, left elytron, SSU = OP087654) and D. Haelew. 1561b (3 mature thalli, left elytron, SSU = OP087655). – **EUROPE**. – BELGIUM. East Flanders Province, Oudenaarde, Volkegembos, 50.8368N, 3.6443 E, hand collection, leg. W. Van Caenegem, 9 July 2021, on male *Ha. axyridis* f. *novemdecimsignata* (PERC 0151962), isolates D. Haelew. 3526b (1 mature thallus, left elytral tip, SSU = OP087656), D. Haelew. 3526c (2 mature thalli, left elytral tip, SSU = OP087657, LSU = OP088713), and D. Haelew. 3526d (3 mature thalli, left elytral tip, SSU = OP087658, LSU = OP088714). CZECH REPUBLIC. Central Bohemian Region, Skalsko, 49.8921119 N, 14.5384600 E, leg. M. Knapp, 7 Oct. 2018, on male *Ha. axyridis* f. *conspicua*, isolate D. Haelew. 1551b (1 mature thallus, right elytral tip, ITS = OL335935, LSU = OP088712). – **ASIA**. – JAPAN. Kansai Region, Kyoto, hand collection, leg. T. Steenberg, 17 April 2017, on *Ha. axyridis* (NNKN), isolates D. Haelew. 1268b (3 mature thalli, right profemur, SSU = MG760610, ITS = MG757829, LSU = MG745357, MCM7 = OP037811) and D. Haelew. 1268d (3 mature thalli, right profemur, SSU = MG760611, ITS = MG757830, LSU = MG745358).

Discussion

Hesperomyces virescens is a complex of semicryptic species

The concept of cryptic species in fungi, species that have been traditionally treated as a single taxon because of apparent identical morphologies, was discussed by Hawksworth & Rossman (1997) in their quest for lost and hidden fungi. Bickford et al. (2007) put forward the Kingdom Fungi as one of four “key targets” for studies in cryptic diversity. Indeed, many traditionally single taxa in both Ascomycota and Basidiomycota harbor multiple species (e.g., Pringle et al. 2005, Grünig et al. 2008, Runge et al. 2011, Piątek et al. 2013, Stefani et al. 2014, Udayanga et al. 2014, Hagen et al. 2015, Olariaga et al. 2017, Singh et al. 2015, Li et al. 2017, Accioly et al. 2019). Thus far, based on molecular phylogenetic data, cryptic diversity has also been revealed in three taxa of Laboulbeniales: *Arthrorhynchus eucampsipodae* Thaxt. (Haelewaters et al. 2020), *Laboulbenia flagellata* Peyr. (De Weggheleire 2019, Haelewaters et al. 2019a), and *He. virescens* (Haelewaters et al. 2018a).

Based on combined morphometric, molecular phylogenetic, and ecological data, *He. virescens* was shown to be a complex of multiple species strictly segregated by host (Haelewaters et al. 2018a). Since that study lacked material from *Chilocorus stigma*, the original host species from which *He. virescens* was described (Thaxter 1891), no taxonomic decisions and species descriptions were proposed. Haelewaters & De Kesel (2020) included *He. virescens* isolates removed from *C. stigma* (collected in Florida, USA) in their phylogenetic reconstruction of an ITS dataset and retrieved them in a single clade, which they designated as *He. virescens* sensu stricto (s.s.). This finding enabled us to unravel the species complex—or *He. virescens* sensu lato (s.l.)—resulting in the introduction of *He. halyziae* (Haelewaters & De Kesel 2020), *He. parexochomi* (Crous et al. 2021), and *He. harmoniae* (this paper).

Following the definitions of cryptic, semicryptic, and pseudocryptic taxa (Lajus et al. 2015, Loizides et al. 2022), species within *He. virescens* s.l. are semicryptic showing “subtle or unstable discriminating traits and considerable phenotypic overlap with other evolutionary isolated taxa.” The diagnosis of these semicryptic species can be resolved with characteristics other than morphology, including ecology (host association) and geographical distributions (e.g., Vondrák et al. 2009, Hodkinson & Lendemer 2011, Molina et al. 2011, Accioly et al. 2019, Cao et al. 2022, Loizides et al. 2022). In the case of *He. har-*

moniae, species recognition (sensu Lücking et al. 2020) is achieved through unique molecular synapomorphies in barcode regions and the obligate association with *Ha. axyridis*. Geographic distribution is not a diagnostic characteristic of *He. harmoniae* due to the invasive nature of its host.

Hesperomyces harmoniae is strictly associated with *Harmonia* hosts

Currently, *He. harmoniae* is only known from *Ha. axyridis*. However, we think that the specificity of *He. harmoniae* relates to host at the genus-level. Evidence points in that direction. We know that *Hesperomyces* isolates from *Adalia bipunctata* (Linnaeus, 1758) and *A. decempunctata* (Linnaeus, 1758) represent a single species that awaits formal description (Haelewaters et al. 2018a, Fig. 1). Also, the *Hippodamia* clade consists of two isolates originating from different host species in the same genus (Fig. 1): *Hi. tredecimpunctata* Linnaeus, 1758 and *Hi. variegata* (Goeze, 1777). Finally, *He. parexochomi* is known from both *Parexochomus nigripennis* and *P. quadriplagiatus* (Crous et al. 2021). Thus far, two records are known of *Hesperomyces*-infected *Ha. quadripunctata* (Pontoppidan, 1763), from 2012 in Belgium (San Martin 2022) and from 2020 in the Czech Republic (O. Nedvěd, pers. comm.). Molecular data are necessary to confirm the identity of the thalli on this host.

Hesperomyces harmoniae is probably native to eastern Asia

Harmonia axyridis, the only known host of *He. harmoniae*, is native to eastern Asia but has been introduced in numerous countries, both intentionally as a biological control agent as well as unintentionally, through secondary spread. This resulted in its establishment in North and South America, Europe, limited parts of Africa, western Asia, and New Zealand (Roy et al. 2016, Camacho-Cervantes et al. 2017, Ceryngier et al. 2018, Hiller & Haelewaters 2019, GBIF Secretariat 2021). Based on currently available data, apart from two historical records, *He. harmoniae* was absent in the native range of *Ha. axyridis* until 2017, when an infected *Ha. axyridis* f. *conspicua* specimen was collected in Kyoto, Japan (Haelewaters et al. 2014, 2017, 2018a). We hypothesize that (native) infection was lost when *Ha. axyridis* was introduced in North America. This is supported by the fact that *Ha. axyridis* collections made in the native range used for introduction in the USA were in USDA quarantine followed by transfer of eggs to partnering agencies for circula-

tion (Krafsur et al. 1997). Only after a time lag since establishment of *Ha. axyridis* in the USA (1988, in Louisiana; Chapin & Brou 1991) was *He. harmoniae* observed on its host—the first US records were made in May 2002 in Virginia, July 2002 in Tennessee (Haelewaters et al. 2017), and July–August 2002 in Ohio (Garcés & Williams 2004).

This time lag between establishment of an invasive alien species and establishment of specialized parasites can help to explain the explosive population growth of *Ha. axyridis* in new habitats, as posited by the so-called enemy release hypothesis (Keane & Crawley 2002, Roy et al. 2011). Also, data from laboratory bioassays support enemy release, revealing reduced regulatory effects of natural enemies on *Ha. axyridis*. Differential susceptibility is reported between *Ha. axyridis* and the American-native *Olla v-nigrum* (Mulsant, 1866) to infection with an entomopathogenic fungus (Cottrell & Shapiro-Ilan 2003) and entomopathogenic nematodes (Shapiro-Ilan & Cottrell 2005), and to co-infection with *He. virescens* s.l. and either *Beauveria bassiana* (Bals.-Criv.) Vuill. or *Metarhizium brunneum* Petch (Haelewaters et al. 2020). While many invasive species lose parasites due to host population bottlenecks or lack of the multiple hosts required for the parasites to complete all life stages (Torchin et al. 2003), the case of *He. harmoniae* parasitizing *Ha. axyridis* might be unique with specimens of the invasive host having been artificially “clean” due to quarantining before introduction.

We have long thought that *He. harmoniae* may have been transmitted from an American-native ladybird species to *Ha. axyridis*. Due to a number of behavioral and life history traits that render *Ha. axyridis* an excellent host (Ceryngier & Twardowska 2013, Haelewaters et al. 2016), it then became the main host and caused *He. harmoniae* to become a near-globally distributed exotic species, spreading around the world in pursuit of its invasive alien ladybird host. However, this hypothesis cannot explain where the historical Chinese records of *He. harmoniae* – long before the introduction of *Ha. axyridis* in the USA – came from. Instead, and given its strict specificity, we now think it is more plausible that *He. harmoniae* somehow spread from the native range of its host to North America, mediated through the trade or travel industry. It is strange that between 1930–1939 (Chinese records of *He. harmoniae*) and 2002 (first record of *He. harmoniae* in the USA), no other observations of the fungus are known. Museum studies may help in this regard (sensu Haelewaters et al. 2017: Fig. 2 & Supporting Material S2). We propose screening dried, pinned

collections of *Ha. axyridis* to provide observational insights in the spreading of *He. harmoniae*. Especially collections from Europe and Asia merit screening, but also continued screening of American material remains of value. In addition, population genetics will shed light on distributional pathways of *He. harmoniae* through time, though only when samples from *Ha. axyridis*' native range are included in the analyses.

Conclusion

In this paper, we formally described *Hesperomyces harmoniae* that is strictly associated with *Harmonia axyridis*. Even though records of this fungus have been known since 2002 in *Ha. axyridis*' invasive range, they were inaccurately identified because *He. virescens* was not recognized as a species complex until 2018. *Hesperomyces harmoniae* has a near-global distribution with numerous records across five continents. Supported by morphological, ecological, and molecular phylogenetic data, *He. harmoniae* is now one of the best documented species of Laboulbeniales. We stress the need for integrative taxonomy for the delimitation and description of new species in the order, as per best practices guidelines by the *International Commission on the Taxonomy of Fungi* (Aime et al. 2021).

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