



# Two new species of ectoparasitic microfungi within the *Hesperomyces virescens* complex from Algeria, Italy, and Malaysia

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## Abstract

Arthropods are hosts to a variety of parasitic fungi, including those within the order of *Laboulbeniales* (*Ascomycota*, *Laboulbeniomycetes*). Species in the genus *Hesperomyces* are specialized in growing on ladybirds (*Coccinellidae*). Recent studies have uncovered multiple cryptic species within the *Hesperomyces virescens* species complex. They found that these species are segregated not only by host, but also by a geographic component. Here, we describe two new species in this species complex, based on morphological, molecular phylogenetic, ecological, and geographical evidence: *Hesperomyces chilocori-bipustulati* sp. nov. on *Chilocorus bipustulatus* from the Mediterranean region, and *He. coccinellae-transversalis* sp. nov. on *Coccinella transversalis* from Peninsular Malaysia. *Hesperomyces chilocori-bipustulati* is the second species known to associate with a representative of the genus *Chilocorus*, after *He. virescens* sensu stricto which was described from *Ch. stigma* from the USA. The findings of this paper contribute to our understanding of the diversity, host specificity, and biogeographical distribution within the *Hesperomyces virescens* species complex and highlight the need for further integrative taxonomy approaches in *Laboulbeniales*.

**Keywords** 2 new species · *Coccinellidae* · *Hesperomyces* · *Laboulbeniales* · Ladybirds · Malaysia · Mediterranean

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## Introduction

Arthropods are attacked by a variety of parasitic and pathogenic fungi. These include fungi from the orders *Entomophthorales* (*Entomophthoromycota*: *Entomophthoromycetes*), *Hypocreales* (*Ascomycota*: *Sordariomycetes*), *Nosematida* (*Rozellomycota*: *Microsporidea*), and *Laboulbeniales* (*Ascomycota*: *Laboulbeniomycetes*) (Wang and Wang 2017; Wijayawardene et al. 2020). The latter, with over 2300 described species, consists of thallus-forming microfungi that are ectobionts and ectoparasites associated with a wide range of arthropods (Haelewaters et al. 2021, 2024). Species of *Laboulbeniales* that penetrate the integument of their host (with a rhizoidal structure called a haustorium) are ectoparasites, while non-haustorial species, which are superficially attached to their host, are referred to as ectobionts (Reboleira et al. 2021). Some haustorial species have been shown to negatively affect their hosts. However, the full extent of these interactions is uncertain—they are suggested to span a wide range on the spectrum of symbiosis (Haelewaters et al. 2022a).

The most common hosts of *Laboulbeniales* are beetles (*Coleoptera*). One of the beetle-specialized *Laboulbeniales*

genera is *Hesperomyces* Thaxt. Known members of this genus are parasites of ladybirds (*Coccinellidae*), with two exceptions; *Hesperomyces biphyllyi* K. Sugiy. & T. Majewski and *He. catopii* Thaxt. are reported from *Biphyllyidae* and *Mycetophagidae*, respectively (Thaxter 1931; Sugiyama and Majewski 1985). Both taxa are only known from the type locality and their status as species within the genus *Hesperomyces* needs to be confirmed. Other species of *Hesperomyces* are associated with ladybirds in the following tribes: *Azyini*, *Brachiacanthadini*, *Chilocorini*, *Coccidulini*, *Coccinellini*, *Diomini*, *Epilachnini*, *Hyperaspidini*, *Noviini*, *Scymnini*, *Stethorini*, and *Sticholotidini* (Smirnov 1953; Haelewaters and De Kesel 2017; Das et al. 2018; Haelewaters et al. 2018; Ceryngier et al. 2024).

Recent studies based on integrative taxonomy have shown that there are more species of *Hesperomyces* than previously thought, though many are yet to be formally described. Most of them belong to the *He. virescens* Thaxt. species complex or *He. virescens* sensu lato (s.l.). Species within this complex are semicryptic and host specific (Haelewaters et al. 2018, 2022b). Thus far, four species have been formally described within the complex: *He. halyziae* Haelew. & De Kesel, *He. harmoniae* Haelew. & De Kesel, *He. parexochomi* Mironova & Haelew., and *He. virescens* sensu stricto (s.s.) (Thaxter 1891; Haelewaters and De Kesel 2020; Crous et al. 2021; Haelewaters et al. 2022b). However, the known host spectrum of *He. virescens* s.l. includes members from around 20 ladybird genera (Haelewaters and De Kesel 2017). The formal description of additional species in the *He. virescens* complex is an ongoing process (D. Haelewaters et al., unpubl. data).

Van Caenegem et al. (2023b) revealed that species within the *He. virescens* species complex are not only segregated by host, but that there is a geographical component involved as well. This was illustrated by the occurrence of two phylogenetically distinct lineages, *He. virescens* s.s. occurring on *Chilocorus stigma* (Say, 1835) from the USA and an undescribed species on *Ch. bipustulatus* (Linnaeus, 1758) from Algeria. The authors refrained from formally describing this species, as only juvenile thalli were available. These patterns are in contrast to *Hesperomyces* growing on *Adalia* Mulsant, 1846 ladybirds; previous work revealed that thalli from *Adalia bipunctata* (Linnaeus, 1758) and *A. decempunctata* (Linnaeus, 1758) collected in different countries across Europe represent one species (Haelewaters et al. 2018). Also *Hesperomyces* on *Hippodamia tredecimpunctata* (Linnaeus, 1758) from Czechia and *Hi. variegata* (Goeze, 1777) from Chile represents a single species (Haelewaters et al. 2022a, b). This is likely because *Hi. variegata* established successfully in Chile following its deliberate introduction in the 1970s (Grez et al. 2012) and coexists

with *Hi. tredecimpunctata* in the native range (Segers 2015; Biranvand et al. 2021), allowing for spore transmission of *Hesperomyces*. It is fair to say that we do not have any molecular phylogenetic data supporting the notion of a species of *Laboulbeniales* with a global distribution, save species associated with invasive hosts (e.g., *He. harmoniae*, *Hesperomyces ex Hippodamia* spp.).

Here, we describe two new species within the *H. virescens* species complex: *H. chilocori-bipustulati* sp. nov. on *Chilocorus bipustulatus* from the Mediterranean region, and *H. coccinellae-transversalis* sp. nov. on *Coccinella transversalis* from Peninsular Malaysia.

## Material and methods

### Collection and identification of hosts

Specimens for this study were collected in lemon (*Citrus × limon*) and orange (*Citrus × sinensis*) groves in Algeria, on coccid-infested *Laurus nobilis* plants in Italy, and in sweet potato (*Ipomoea batatas*) and long bean (*Vigna unguiculata* subsp. *sesquipedalis*) farms in Peninsular Malaysia between 2019 and 2022. Sampling took place by hand or through shaking bushes and branches onto a 1 m × 1 m white beating sheet. Collected ladybirds were preserved in 96% ethanol. Screening for the presence of *Laboulbeniales* thalli was done in the laboratory using an RZB-PL 65.500 dissecting microscope (Novex, Arnhem, The Netherlands) at 10–40 ×. Infected specimens were separated and assigned a unique identifier.

### Morphological study of *Laboulbeniales*

Thalli were removed from ladybird hosts under an RZB-PL 65.500 dissecting microscope (Novex) using a hypodermic needle. Permanent slides were made using the double-cover-slip technique from Liu et al. (2020). We used a 1:1 Hoyer's medium:glycerin mixture instead of pure Hoyer's medium to reduce the chance that the medium would solidify during micro-manipulation (Van Caenegem et al. 2023a, b). Mounted thalli were viewed at 400 × to 1000 × using a BH-2 microscope (Olympus, Center Valley, PA, USA). Images of thalli were made with a DS-Fi3 microscope camera mounted on an Eclipse Ni-U compound microscope (Nikon, Melville, NY, USA), equipped with differential interference contrast optics, and processed using NIS-Elements BR 5.0.03 imaging software (Nikon). Photos were enhanced and the background was removed using cutout.pro (<https://www.cutout.pro/>). Composite plates were assembled in PowerPoint version 2409 (Microsoft, Redmont, WA, USA).

Thalli, cells, structures, and ascospores were measured using ImageJ 1.51 h image processing and analysis software (Abramoff et al. 2004). All measurements were taken as described and illustrated by Haelewaters et al. (2018). Measurements in the morphological descriptions are noted as (a–)b–c–d(–e) [n], with a, e = extreme values; b, d = mean minus/plus standard deviation; c = mean; and n = number of structures measured. Ladybirds are preserved in the Taxon Expeditions collection, Leiden, The Netherlands (TXEX), while permanent slides of *Laboulbeniales* are deposited in the Herbarium Universitatis Gandavensis, Ghent, Belgium (GENT).

### DNA extraction, PCR amplification, and sequencing of *Laboulbeniales*

DNA extractions were done using the REPLI-g Single Cell Kit (Qiagen, Stanford, CA) (Van Caenegem et al. 2023b; Van Caenegem and Haelewaters 2024). The internal transcribed region (ITS) and the large subunit (28S) of the ribosomal RNA gene (rDNA) were amplified using the following primer pairs: ITSshesPL/ITS2 and ITS3/ITSshesPR (White et al. 1990; Haelewaters et al. 2019) for ITS, and LR0R/LR5 (Vilgalys and Hester 1990; Hopple 1994), NL1/NL4 (Kurtzman and Robnett 1997), and LIC24R/LR3 (Vilgalys and Hester 1990; Miadlikowska and Lutzoni 2000) for 28S. The minichromosome maintenance complex component 7 (*MCM7*) protein-coding gene was amplified using the primer pair Mcm7-709for/Mcm7-1384rev (Schmitt et al. 2009). All 25- $\mu$ l PCR reactions consisted of 13.3  $\mu$ l of RedExtract Taq polymerase (Sigma-Aldrich, St. Louis, MO, USA), 2.5  $\mu$ l of each 10  $\mu$ M primer, 5.45  $\mu$ l of ddH<sub>2</sub>O, and 1  $\mu$ l of DNA extract. The COMBSIL PCR cycling program of Van Caenegem and Haelewaters (2024) was used for the amplification of both ITS and 28S: initial denaturation at 94 °C for 5 min; 39 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s; and final extension at 72 °C for 10 min. PCR conditions for *MCM7* were as follows: initial denaturation at 94 °C for 5 min; 10 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C (–1 °C/cycle) for 50 s, and extension at 72 °C for 1 min; 24 cycles of denaturation at 94 °C for 45 s, annealing at 47 °C for 50 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min.

Purification of PCR products was done using 1.5  $\mu$ l of Exo-FAP (0.5  $\mu$ l Exonuclease I, 1  $\mu$ l FAST Alkaline Phosphatase) (Thermo Fisher Scientific, Waltham, MA, USA) per 10  $\mu$ l of PCR product, at 37 °C for 15 min followed by deactivation at 85 °C for 15 min. The purified PCR products were sequenced using an automated ABI 3730 XL capillary sequencer (Life Technology at Macro-gen, 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 (<http://www.ncbi.nlm.nih.gov/>; accession numbers in Table 1).

### Alignments and phylogenetic reconstruction

Newly generated sequences were supplemented by sequences downloaded from NCBI GenBank, resulting in 45 ITS, 39 28S, and 19 *MCM7* sequences (Table 1). We used *Hesperomyces* ex *Azya orbiger* Mulsant, 1850, *H. coccinelloides* (Thaxt.) Thaxt., and *H. coleomegillae* W. Rossi & A. Weir as outgroup, following Haelewaters et al. (2022b). Given the unequally large number of sequences for *H. harmoniae*, we only used a selection of publicly available sequences as per Van Caenegem et al. (2023b). Sequences were aligned by locus with the E-INS-i strategy using the online version 7 of MAFFT (Kuraku et al. 2013; Katoh et al. 2019). Aligned sequences were then manually trimmed using BioEdit Sequence Alignment Editor version 7.2.6 (Hall 1999) and concatenated in SequenceMatrix 1.9 (Vaidya et al. 2011).

Our concatenated dataset included 5 partitions: the ITS1 and ITS2 spacer regions, the 5.8S gene, 28S, and *MCM7*. Models for nucleotide substitution were selected for each partition with ModelFinder (Kalyaanamoorthy et al. 2017) according to the corrected Akaike Information Criterion (AICc). Maximum likelihood (ML) analyses were conducted using IQ-TREE (Nguyen et al. 2015) under partitioned models (Chernomor et al. 2016). Ultrafast bootstrapping was performed with 1000 replicates (Hoang et al. 2018). Phylogenetic trees were visualized in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) and edited using Inkscape (<https://www.inkscape.org>).

### Results

We generated 25 new sequences (9 ITS, 10 28S, and 5 *MCM7*) for this study (Table 1). The concatenated ITS–28S–*MCM7* dataset consisted of 2361 characters for 47 isolates. Selected models for each partition in our concatenated dataset were TPM2 + F + G4 for ITS1 (415 bp, -lnL = 4358.959), TNe for 5.8S (158 bp, -lnL = 575.243), GTR + F + G4 for ITS2 (272 bp, -lnL = 3412.669), TIM + F + G4 for 28S (894 bp, -lnL = 4560.723), and TIM3e + G4 for *MCM7* (622 bp, -lnL = 2896.484). The reconstructed phylogeny of *H. virescens* s.l. is shown in Fig. 1 (concatenated ITS–28S–*MCM7* dataset). Here, the *Hesperomyces virescens* species complex consists of 14 lineages, each with near-maximum to maximum support (UFBoot2 support 99–100%) except for the singleton branch with isolate D. Haelew. 4049a (UFBoot2 support 93%).

**Table 1** All isolates used in the study, including species name, country of collection, host species, and the GenBank accession numbers of the internal transcribed spacer (ITS), large subunit (28S), and minichromosome maintenance complex component 7 (*MCM7*) sequences

Species	Isolate	Country	Host	ITS	28S	<i>MCM7</i>
<i>He. aff. coleomegillae</i>	D. Haelew. 1287b	Panama	<i>Coleomegilla maculata</i> (De Geer, 1775)	OL335932	MG745334	
<i>He. aff. coleomegillae</i>	D. Haelew. 1291c	Panama	<i>Coleomegilla maculata</i>	OL335933	MG745335	
<i>He. chilocori-bipustulati</i>	D. Haelew. 4693a	Italy	<i>Chilocorus bipustulatus</i> (Linnaeus, 1758)	PQ370002	PQ370011	PQ374038
<i>He. chilocori-bipustulati</i>	D. Haelew. 4695b	Italy	<i>Chilocorus bipustulatus</i>	PQ370003	PQ370012	PQ374041
<i>He. chilocori-bipustulati</i>	D. Haelew. 4696a	Italy	<i>Chilocorus bipustulatus</i>	PQ370004	PQ370013	PQ374040
<i>He. chilocori-bipustulati</i>	D. Haelew. 4698a	Italy	<i>Chilocorus bipustulatus</i>	PQ370005	PQ370014	PQ374039
<i>He. chilocori-bipustulati</i>	D. Haelew. 4699b	Italy	<i>Chilocorus bipustulatus</i>		PQ370015	
<i>He. chilocori-bipustulati</i>	D. Haelew. 4879a	Algeria	<i>Chilocorus bipustulatus</i>	PQ370006	PQ370016	
<i>He. chilocori-bipustulati</i>	D. Haelew. 4880a	Algeria	<i>Chilocorus bipustulatus</i>	PQ370007	PQ370017	
<i>He. coccinellae-transversalis</i>	D. Haelew. 4343b	Malaysia	<i>Coccinella transversalis</i> Fabricius, 1781	PQ369999	PQ370008	
<i>He. coccinellae-transversalis</i>	D. Haelew. 4344a	Malaysia	<i>Coccinella transversalis</i>	PQ370000	PQ370009	
<i>He. coccinellae-transversalis</i>	D. Haelew. 4345b	Malaysia	<i>Coccinella transversalis</i>	PQ370001	PQ370010	PQ374037
<i>He. coccinelloides</i>	D. Haelew. 1428a	Spain (Canary Islands)	<i>Stethorus tenerifensis</i> Fürsch, 1987	OL335930	PP091221	OP947141
<i>He. coccinelloides</i>	D. Haelew. 1428b	Spain (Canary Islands)	<i>Stethorus tenerifensis</i>	OL335931		OP947140
<i>He. halyziae</i>	D. Haelew. 955b	The Netherlands	<i>Halyzia sedecimguttata</i> (Linnaeus, 1758)	MG757813		
<i>He. halyziae</i>	D. Haelew. 4209a	The Netherlands	<i>Halyzia sedecimguttata</i>	OP933656	OP933659	
<i>He. harmoniae</i>	D. Haelew. 1174a	The Netherlands	<i>Harmonia axyridis</i> (Pallas, 1773)	MG757815	MG745345	
<i>He. harmoniae</i>	D. Haelew. 1268b	Japan	<i>Harmonia axyridis</i>	MG757829	MG745357	OP037811
<i>He. harmoniae</i>	D. Haelew. 1551b	Czech Republic	<i>Harmonia axyridis</i>	OL335935		
<i>He. parexochomi</i>	D. Haelew. 1462a	Spain (Canary Islands)	<i>Parexochomus nigripennis</i> (Erichson, 1843)	MZ994855		
<i>He. parexochomi</i>	D. Haelew. 1690d	Spain (Canary Islands)	<i>Parexochomus nigripennis</i>	MZ994863	MZ994874	OP947154
<i>He. parexochomi</i>	D. Haelew. 1691c	Spain (Canary Islands)	<i>Parexochomus nigripennis</i>	MZ994864	MZ994875	OP947156
<i>He. parexochomi</i>	D. Haelew. 1465a	Spain (Canary Islands)	<i>Parexochomus quadriplagiatus</i> (Wollaston, 1864)	MZ994860	MZ994871	OP947156
<i>He. parexochomi</i>	D. Haelew. 1465b	Spain (Canary Islands)	<i>Parexochomus quadriplagiatus</i>	MZ994868	MZ994879	OP947155
<i>He. parexochomi</i>	D. Haelew. 1584a	Spain (Canary Islands)	<i>Parexochomus quadriplagiatus</i>	MZ994858	MZ994869	
<i>He. virescens</i> s.l.	D. Haelew. 1193g	Denmark	<i>Adalia bipunctata</i> (Linnaeus, 1758)	MG757817	MG745346	OP947147
<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	OP947146
<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. 655c	South Africa	<i>Cheilomenes propinqua</i> (Mulsant, 1850)	MG757804	KU574867	
<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. 1259a	South Africa	<i>Cheilomenes propinqua</i>	MG757828		
<i>He. virescens</i> s.l.	D. Haelew. 4049a	Algeria	<i>Chilocorus bipustulatus</i>	OP933655	OP933658	
<i>He. virescens</i> s.l.	D. Haelew. 924a	Panama	<i>Cycloneda sanguinea</i> (Linnaeus, 1763)	MG757808		
<i>He. virescens</i> s.l.	D. Haelew. 1374a	Panama	<i>Cycloneda sanguinea</i>	MG757831		
<i>He. virescens</i> s.l.	D. Haelew. 3187a	Czech Republic	<i>Hippodamia tredecimpunctata</i> (Linnaeus, 1758)	OL335937	OL335923	

**Table 1** (continued)

Species	Isolate	Country	Host	ITS	28S	MCM7
<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. 3939b	Spain (Canary Islands)	<i>Hyperaspis vinciguerrae</i> Capra, 1929		OP933649	OP947144
<i>He. virescens</i> s.l.	D. Haelew. 3939c	Spain (Canary Islands)	<i>Hyperaspis vinciguerrae</i>	OP933657	OP933650	OP947145
<i>He. virescens</i> s.l.	D. Haelew. 1200h	USA	<i>Olla v-nigrum</i> (Mulsant, 1866)	MG757819	MG745348	OP947150
<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. 954e	USA	<i>Olla v-nigrum</i>	MG757798		
<i>He. virescens</i> s.l.	D. Haelew. 1250c	USA	<i>Psyllobora vigintimaculata</i> (Say, 1824)	MG757826	MG745355	OP947151
<i>He. virescens</i> s.l.	D. Haelew. 1251b	USA	<i>Psyllobora vigintimaculata</i>	MG757827	MG745356	OP947152
<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	
<i>Hesperomyces</i> sp.	D. Haelew. 928g	Panama	<i>Azya orbiger</i> (Mulsant, 1850)	MG745343	MG745343	

## Taxonomy

***Hesperomyces chilocori-bipustulati*** Van Caenegem & Haelew., sp. nov., Fig. 2A–F.

*Index Fungorum* number: IF902823.

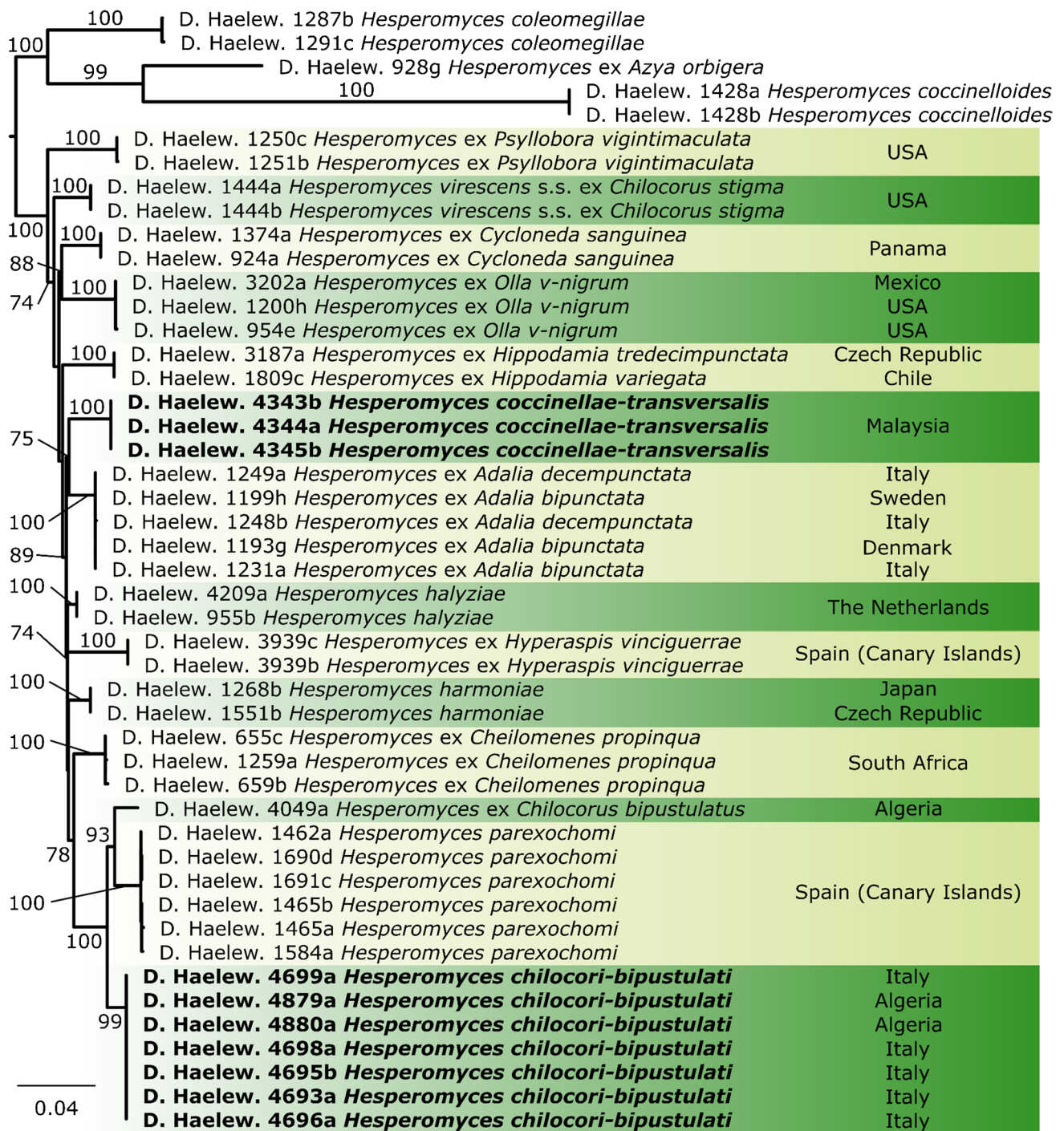
*Type*: ALGERIA, MOSTAGANEM PROVINCE: Hassi Mamèche District, Mazagran, Abdelhamid Ibn Badis University of Mostaganem's experimental farm, 35.8930556 N 0.0788889 E, plot 2, from *Citrus × limon* leaves, 149 m a.s.l., 24 Aug 2019, on *Chilocorus bipustulatus* (Linnaeus, 1758) (*Coleoptera*, *Coccinellidae*, *Chilocorini*), leg. A. Merzoug, in coll. TXEX (TXEX.COL.03593), slide D. Haelew. 4880c (**holotype** GENT:GENTFL01607, 40 adult thalli from abdominal sternites), isolate D. Haelew. 4880a (3 adult thalli from abdominal sternites), GenBank accession nos. PQ370007 (ITS) and PQ370017 (28S).

*Etymology*: Referring to the host species, *Chilocorus bipustulatus*.

*Diagnosis*: Morphologically very similar to other members of the *Hesperomyces virescens* species complex but forming a distinct species-level clade supported by ITS, 28S, and MCM7 data. Strictly associated with *Chilocorus bipustulatus*. Unique molecular synapomorphies and motifs in the ITS at positions 127 (C), 128 (T), 139 (T), 147 (T), 154 (A), 179 (A), 371 (T), 392 (C), 683 (T), 725 (T), 804 (A), 826 (T), 833–835 (5'–GAC–3') (insertion). Unique molecular synapomorphy in the 28S at position 184 (T). Unique molecular synapomorphies in the MCM7 at positions 129 (C) and 561 (G).

*Description*: *Thallus* (232.4–)279.7–340.6–401.5(–484.1) µm long from foot to perithecial apex, colored hyaline to yellowish, with blackened foot cell [75]. *Receptacle*

(62.8–)72.8–80.0–87.2(–101.4) µm long [77]. *Cell I* (47.3–)57.1–63.4–69.7(–82.1) × (15.2–)17.7–20.1–22.6(–27.8) µm, triangular to quadrangular, longer than broad [77]. *Cell II* (18.1–)21.4–24.1–26.8(–32.8) × (8.6–)12.1–13.9–15.7(–21.2) µm, longer than broad, subrhomboid to irregular kite-shaped [77]. *Cell III* (12.7–)14.9–16.6–18.3(–20.9) × (8.6–)9.8–11.3–12.8(–15.2) µm, shorter than cell II, obliquely positioned [77]. *Primary appendage* (62.8–)72.8–80.0–87.2(–101.4) µm long, consisting of 5–7 superposed cells, with a slightly constricted septum between cell III and the basal cell; *basal cell* (12.3–)13.6–15.4–17.2(–22.6) µm long, longer than any of the other cells of the appendage; remaining cells each bearing one antheridium directed outwardly, the uppermost cell bearing two antheridia [73]. *Antheridia* (16.6–)18.3–19.5–20.7(–22.5) µm long [52]; with outwardly slightly curved efferent necks, (9.7–)11.1–12.3–13.5(–15.1) µm [52]. *Cell VI* (26.1–)32.4–66.8–101.2(–159.3) × (13.4–)18.6–22.1–25.5(–28.8) µm, stout (thalli growing dorsally) or elongated (thalli growing ventrally), slightly broadening distally [77]. *Perithecium* (134.2–)178.4–207.8–237.2(–269.7) × (32.5–)43.2–50.6–57.9(–68.6) µm [77], on average 4.1 × longer than broad, asymmetric, fusiform, broadest near the middle, and then gradually tapering towards a short, broad, indistinct neck, and an 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* either small (thalli growing dorsally) (13.4–)15.6–17.7–19.8(–21.1) µm [24] or long (thalli growing ventrally) (34.6–)39.5–42.8–46.1(–50.6) µm [64], unicellular, usually curved outwards; ostiole with two lips, one lip triangular, the other slightly shorter and rounded. *Ascospores*



**Fig. 1** Phylogeny of *Hesperomyces* reconstructed from a three-locus (ITS–28S–MCM7) dataset. The topology is the result of maximum-likelihood (ML) inference performed with IQ-TREE. For every node,

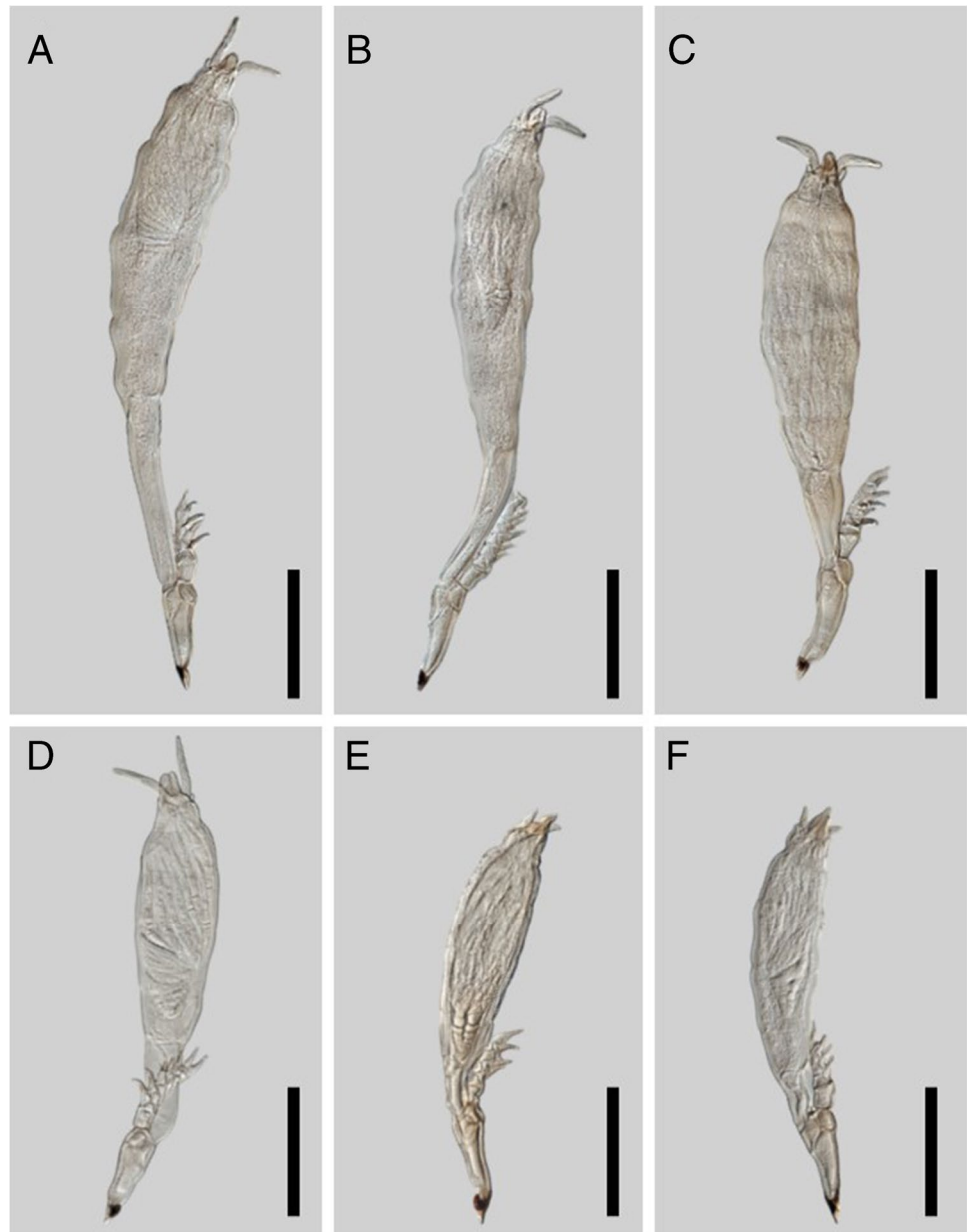
the ML UFBoot2 support value ( $\geq 70$ ) is given above, below, or next to the branch leading to that node. Newly described species are highlighted in bold

two-celled, (62.0–)61.1–64.0–66.9(–69.0)  $\times$  5.6–6.5–7.4(–7.8)  $\mu\text{m}$ , with gelatinous sheath covering the larger cell [5].

**Additional specimens examined:** ALGERIA, MOSTAGANEM PROVINCE: Hassi Mamèche District, Mazagran,

Abdelhamid Ibn Badis University of Mostaganem's experimental farm, 35.8930556 N 0.0788889 E, plot 2, from *Citrus*  $\times$  *limon* leaves, 149 m a.s.l., 24 Aug 2019, on *Ch. bipustulatus*, leg. A. Merzoug, in coll. TXEX (TXEX.COL.03593),

**Fig. 2** Mature thalli of *Hesperomyces chilocori-bipustulati*. **A–D.** Thalli with long perithecial upper lobes and elongated cell VI. **E–F.** Thalli with short perithecial upper lobes and short cell VI. **A–C.** D. Haelew. 4880c (holotype). **D.** D. Haelew. 4969b. **E.** D. Haelew. 4879b. **F.** D. Haelew. 4880b (isotype). Scale bars = 100  $\mu$ m



slide D. Haelew. 4880b (isotype GENT:GENTFL01606, 9 adult thalli from right elytron); *ibid.*, plot 1, from *Citrus × sinensis* leaves, 26 Apr 2019, on *Ch. bipustulatus*, leg. A. Merzoug, in coll. TXEX (TXEX.COL.03592), slides D. Haelew. 4879b (GENT:GENTFL01604, 14 adult thalli from left elytron) and D. Haelew. 4879c (GENT:GENTFL01605, 9 adult thalli from right metafemur), isolate D. Haelew. 4879a (4 adult thalli from right metafemur), GenBank accession nos. PQ370006 (ITS) and PQ370016 (28S); *ibid.*, 06 Mar 2019, on *Ch. bipustulatus*, leg. A. Merzoug, in coll. TXEX (TXEX.COL.03594), slide D. Haelew. 4884a (GENT:GENTFL01608, 7 adult thalli from left elytron). ITALY, VENETO: Cavallinotreporti, Punta Sabbioni, 45.436 N 12.436 E, 18 Oct 2022, on *Ch. bipustulatus*, leg. P. Ceryngier, in coll. TXEX (TXEX.

COL.03595), isolate D. Haelew. 4693a (4 adult thalli from right elytron), GenBank accession nos. PQ370002 (ITS), PQ370011 (28S), and PQ374038 (*MCM7*); *ibid.*, in coll. TXEX (TXEX.COL.03596), isolate D. Haelew. 4695b (2 juvenile and 2 adult thalli from right elytron), GenBank accession nos. PQ370003 (ITS), PQ370012 (28S), and PQ374041 (*MCM7*); *ibid.*, in coll. TXEX (TXEX.COL.03597), slide D. Haelew. 4696b (GENT:GENTFL01603, 2 subadult and 9 adult thalli from left mesofemur), isolate D. Haelew. 4696a (5 adult thalli from left mesofemur), GenBank accession nos. PQ370004 (ITS), PQ370013 (28S), and PQ374040 (*MCM7*); *ibid.*, in coll. TXEX (TXEX.COL.03598), isolate D. Haelew. 4698b (4 adult thalli from right elytron), GenBank accession nos. PQ370005 (ITS), PQ370014 (28S), and PQ374039

(MCM7); *ibid.*, in coll. TXEX (TXEX.COL.03599), isolate D. Haelew. 4699b (1 juvenile, 1 subadult, and 3 adult thalli from right elytron), GenBank accession no. PQ370015 (28S).

**Hosts and distribution:** On *Chilocorus bipustulatus*. Thus far recorded in Algeria (this paper), Israel (Hecht 1936; Kamburov et al. 1967; Applebaum et al. 1971; Benjamin 1989), Italy (this paper), and Morocco (Maire and Werner 1937).

**Notes:** *Hesperomyces chilocori-bipustulati* is described based on 75 adult and two subadult thalli from four different host specimens. Thalli growing on the elytra are generally shorter compared to thalli that grow on the abdominal sternites and legs, in large part due to the shorter cell VI. The upper lobes also differ in length depending on the position of growth: they are short when thalli grow on the elytra and significantly longer when thalli grow on the abdominal sternites and legs.

One additional *Ch. bipustulatus* ladybird from Algeria was used in this study. Host specimen D. Haelew. 4049 (TXEX.COL.03600) was collected at the same location as the other Algerian specimens hosting *He. chilocori-bipustulati* from Algeria, although on a different date. Isolate D. Haelew. 4049a, first reported by Van Caenegem et al. (2023b), only shares 95.69% identity in its ITS and 98.66% in its 28S with all other sequences of *He. chilocori-bipustulati*, despite coming from the same host species. We re-sequenced both ITS and 28S to rule out errors during sequencing and assembly of the contigs, but no differences were found between sequencing rounds. This host only carried five juvenile thalli, which were all used for DNA extraction and so no morphological comparison can be made with the above description for *He. chilocori-bipustulati*. Several explanations can be given for the differing sequences of this isolate compared to others from the same host species. Firstly, intraspecific or intragenomic variation has been described within the ITS and 28S regions within different fungal groups (Smith et al. 2007; Nilsson et al. 2008; Simon and Weiß 2008), but these patterns are currently not studied in *Laboulbeniales*. Secondly, it is a possibility that a second, undescribed species of *Hesperomyces* occurs on *Ch. bipustulatus*. This may seem unlikely given that this has not yet been observed in any other host species infected by *He. virescens* s.l. However, infections by multiple species of the same genus have been observed in *Hesperomyces* before (Goldmann et al. 2013), as well as in *Gloeandromyces* on bat flies (Van Caenegem et al. 2023a) and *Herpomyces* on cockroaches (Wang et al. 2016). Finally, accidental infections have been described for several species of *Laboulbeniales* (Scheloske 1969; Huldén 1983; Santamaria 1989). It is possible that *Ch. bipustulatus* co-occurs with another ladybird species that hosts an undescribed species of *Hesperomyces*, offering options for spore transfer to *Ch. bipustulatus* as an accidental host (or “Zufallswirt”; Scheloske 1969). We did not include isolate D. Haelew. 4049a as part of the type series but report it here to encourage further

research into *Hesperomyces* ex *Ch. bipustulatus* and other ladybirds interacting with this host.

***Hesperomyces* sp. ALGERIA, MOSTAGANEM PROVINCE:** Hassi Mamèche District, Mazagran, Abdelhamid Ibn Badis University of Mostaganem’s experimental farm, 35.8930556 N 0.0788889 E, plot 2, from *Citrus × limon* leaves, 149 m a.s.l., 4 Feb 2019, on *Ch. bipustulatus*, leg. A. Merzoug & H. Faouzia, in coll. TXEX (TXEX.COL.03600), isolate D. Haelew. 4049a (5 juvenile thalli from right epipleuron), GenBank accession nos. OP933655 (ITS) and OP933658 (28S).

***Hesperomyces coccinellae-transversalis* Van Caenegem & Haelew., sp. nov., Fig. 3A–C**

**Index Fungorum number:** IF902824.

**Type:** MALAYSIA. SELANGOR STATE: Sepang, *Ipomoea batatas* farm, 2.695389 N 101.674139 E, 6 m a.s.l., 27 May 2021, on *Coccinella transversalis* Fabricius, 1781 (*Coleoptera, Coccinellidae, Coccinellini*), leg. N.N. Musa, in coll. TXEX (TXEX.COL.03589), slide D. Haelew. 4343c (**holotype** GENT:GENTFL01601, 11 adult thalli from right elytron), isolate D. Haelew. 4343b (5 adult thalli from left elytron), GenBank accession nos. PQ369999 (ITS) and PQ370008 (28S).

**Etymology:** Referring to the host species, *Coccinella transversalis*.

**Diagnosis:** Morphologically very similar to other members of the *Hesperomyces virescens* species complex but forming a distinct species-level clade supported by ITS, 28S, and MCM7 data. Strictly associated with *Coccinella transversalis*. Unique molecular synapomorphies in the ITS at positions 116 (T) (insertion), 137 (C), 188 (G), 273 (A), 316 (T), 318 (C), 323 (A), 334 (T), 370 (T), 601 (G), 653 (C), 665 (C), 711 (A), 755 (A), 770 (G), 785 (T), 786 (T), 819 (G). Unique molecular synapomorphies in the 28S at positions 188 (C) and 521 (T). Unique molecular synapomorphies in the MCM7 at positions 156 (G), 345 (C), 582 (C), 609 (A).

**Description:** *Thallus* (263.8–)263.9–302.9–341.9(–375.4) µm long from foot to perithecial apex, colored hyaline to yellowish, with blackened foot cell [13]. *Receptacle* (67.0–)69.7–76.2–82.6(–86.5) µm long [14]. *Cell I* (50.6–)52.1–57.1–62.2(–66.5) × (14.7–)17.3–20.4–23.6(–24.4) µm, triangular to quadrangular, longer than broad [14]. *Cell II* (18.9–)21.3–25.6–30.0(–32.4) × (11.0–)12.5–15.6–18.8(–20.3) µm, longer than broad, subtrapezoidal [15]. *Cell III* (16.2–)17.1–19.2–21.2(–22.8) × (9.3–)9.8–11.5–13.2(–15.4) µm, shorter than cell II, [15]. *Primary appendage* (43.2–)57.2–64.1–71.0 µm long, consisting of 3–4 superposed cells, with a distinct constricted septum between cell III and the basal cell; *basal cell* (10.3–)14.1–17.0–19.9(–21.5) µm long, longer than any of the other cells of the appendage; remaining cells each bearing one antheridium directed outwardly, the uppermost cell



**Fig. 3** Mature thalli of *Hesperomyces coccinellae-transversalis*. **A–B**, D. Haelew. 4343c (holotype). **C**, D. Haelew. 4343d, damaged thallus. Scale bar = 100  $\mu\text{m}$



bearing two antheridia, the uppermost antheridium bearing a short spinous process, which is the original ascospore apex [14]. *Antheridia* (19.8–)20.2–22.0–23.8(–25.3)  $\mu\text{m}$  long; with outwardly straight to curved efferent necks, (11.5–)12.1–13.2–14.3(–14.8)  $\mu\text{m}$  [15]. *Cell VI* (25.7–)28.7–35.7–42.7(–47.4)  $\times$  (21.9–)22.2–26.9–31.7(–37.8)  $\mu\text{m}$ , rather stout, broadening distally [15]. *Perithecium* 180.0–208.8–238.7(–266.9)  $\times$  (41.0–)42.5–51.2–59.9(–65.5)  $\mu\text{m}$ , on average 4.1  $\times$  longer than broad, asymmetric, fusiform, broadest near the middle, and then gradually tapering towards a short, broad, indistinct neck, and an 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* (12.9–)14.2–16.4–18.5(–19.9)  $\mu\text{m}$  long, unicellular, and their tips not exceeding the perithecial apex; ostiole with two lips, one lip triangular, the other slightly shorter, rounded [14]. *Ascospores* two-celled, (40.5–)41.9–46.7–51.4(–51.6)  $\times$  4.8–5.5–6.1(–6.4)  $\mu\text{m}$ , with gelatinous sheath covering the larger cell [4].

*Additional specimens examined*: MALAYSIA, SELANGOR STATE: Sepang, *Ipomoea batatas* farm, 2.695389 N 101.674139 E, 27 May 2021, on *Co. transversalis*, leg. N.N. Musa, in coll. TXEX (TXEX.COL.03589), slide D. Haelew. 4343d (isotype GENT:GENTFL01602, 5 adult thalli from left elytron); *ibid.*, in coll. TXEX (TXEX.COL.03590), isolate D. Haelew. 4344a (5 adult thalli from left elytron),

GenBank accession nos. PQ370000 (ITS) and PQ370009 (28S); MALACCA STATE: Alor Gajah, *Vigna unguiculata* subsp. *Sesquipedalis* farm, 2.402056 N 102.151361 E, 28 m a.s.l., 30 May 2021, on *Co. transversalis*, leg. N.N. Musa, in coll. TXEX (TXEX.COL.03591), isolate D. Haelew. 4345b (3 adult thalli from right elytron), GenBank accession nos. PQ370001 (ITS), PQ370010 (28S), and PQ374037 (MCM7).

*Hosts and distribution*: On *Coccinella transversalis* in the states of Malacca and Selangor, Malaysia.

*Notes*: *Hesperomyces coccinellae-transversalis* is described based on 14 adult thalli and one subadult thallus from a single host specimen. The two other available host specimens carried a small number of thalli (8 in total), which were all used for DNA extraction.

## Discussion

*Hesperomyces virescens* s.s. was described by Thaxter (1891) based on specimens parasitizing a member of the genus *Chilocorus* Leach, 1815, the North American species *Ch. stigma*. Here we formally described a second species associated with *Chilocorus* ladybirds, the Palearctic *Ch. bipustulatus*, with records from Algeria and Italy. All records of *Hesperomyces* associated with *Ch. bipustulatus* likely belong to this species, including those from Israel (Hecht 1936; Kamburov et al. 1967; Applebaum et al. 1971;

Benjamin 1989) and Morocco (Maire and Werner 1937). Thus, the parasite of *Ch. bipustulatus*, which is common in the Mediterranean region and was previously referred to as *He. virescens*, appears to be a different species: *He. chilocori-bipustulati*. This finding illustrates that different ladybird host species within a single genus can be infected by multiple *Hesperomyces* species, when these hosts do not co-occur in the same biogeographic region. On the other hand, the systematic status of *Chilocorus* is still under discussion. A recent phylogenetic analysis based on five loci and 86 morphological characteristics found that species traditionally included in this genus do not form a monophyletic clade (Li et al. 2020). To keep *Chilocorus* monophyletic, four other genera were synonymized with it.

In the Mediterranean region, parasitism of ladybirds by *Hesperomyces* has been recorded relatively often. Moreover, virtually all of the Palearctic reports by the 1990s were restricted to this region (Smirnoff 1953; Ceryngier and Twardowska 2013). The Mediterranean records of *Hesperomyces* were assigned to two species, *He. coccinelloides* Thaxt. and *He. virescens*. *Hesperomyces coccinelloides* was reported on *Pharoscyrnus* spp. in North Africa (Smirnoff 1953) and on *Scymnus* sp. in Catalonia, Spain (Santamaria 1995). More host species and locations are linked to the once presumed cosmopolitan species *He. virescens*. These include *Adalia bipunctata* and *A. decempunctata* in Italy (Webberley et al. 2006), *Chilocorus bipustulatus* in Israel and Morocco (here described as *He. chilocori-bipustulati*) (Hecht 1936; Maire and Werner 1937; Kamburov et al. 1967; Applebaum et al. 1971; Benjamin 1989), *Coccinula quatuordecimpustulata* (Linnaeus, 1758) in Greece (Castaldo et al. 2004), *Harmonia axyridis* (Pallas, 1773) in Croatia, France, Greece, Italy, Montenegro, and Turkey (previously described as *He. harmoniae*) (Ceryngier et al. 2013; Ceryngier and Romanowski 2017; Haelewaters et al. 2017, 2022b; de Groot et al. 2024), *Propylea quatuordecimpunctata* (Linnaeus, 1758) in Catalonia, Spain (Santamaria 1989), *Psyllobora vigintiduopunctata* (Linnaeus, 1758) in southern France (Balazuc 1974), and *Tytthaspis sedecimpunctata* (L.) in Greece (Castaldo et al. 2004). Only two of these species are described: *He. chilocori-bipustulati* on *Ch. bipustulatus* and *He. harmoniae* on *Ha. axyridis*. Records associated with other ladybird species likely represent undescribed species within the *He. virescens* species complex. We encourage the collection of infected ladybirds, proper storage (sensu Van Caenegem and Haelewaters 2024), and generating sequences of these microfungi, which will help to elucidate the species diversity within the complex.

Most available molecular data of *Hesperomyces virescens* s.l. comes from temperate regions in the Northern Hemisphere (Haelewaters et al. 2022a, b; Van Caenegem et al. 2023b). Other regions, particularly (sub-)tropical ones, remain severely underexplored for *Laboulbeniales* diversity

(Haelewaters et al. 2024). The newly described *He. chilocori-bipustulati* is the first record of a *Hesperomyces* species from Algeria. Thus far, 67 species of *Laboulbeniales* have been reported from Algeria (Amrani and Abdel-Azeem 2019). Additionally, the newly described *He. coccinellae-transversalis* is the second record of a *Hesperomyces* species from Malaysia (after *He. coccinelloides*). This brings the total to 93 species reported from Malaysia (Haelewaters et al. 2024). There is no doubt that future field campaigns in these underexplored regions will reveal many new and undescribed species within the *He. virescens* species complex, as well as *Laboulbeniales* in general.

## Conclusion

The description of two new species, *Hesperomyces chilocori-bipustulati* on *Chilocorus bipustulatus* from the Mediterranean region and *He. coccinellae-transversalis* on *Coccinella transversalis* from Peninsular Malaysia, underscores the importance of integrative taxonomy in clarifying species boundaries and host associations within the *Hesperomyces virescens* species complex. Both *He. chilocori-bipustulati* in the Mediterranean and *He. virescens* s.s. in the USA are associated with host species in the genus *Chilocorus*, demonstrating that a single host genus can harbor multiple semicryptic species of *Hesperomyces*. Additionally, the underexplored diversity of *Laboulbeniales* fungi in tropical regions highlights a significant gap in our understanding of the diversity of these fungi. Future research, including extensive fieldwork and molecular phylogenetic analyses, is crucial for uncovering the species diversity within the *He. virescens* species complex and for understanding the host–parasite interactions of these unique biotrophic relationships.

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**Author contribution** Conceptualization: W.V.C. and D.H. Methodology, Formal analysis, and Visualization: W.V.C. Investigation: W.V.C., A.M., P.C., A.B., M.B., N.N.M., and S.Y. Writing—Original Draft: W.V.C., P.C., and D.H. Writing—Review & Editing: W.V.C., P.C., A.V., and D.H. Supervision and Project administration: D.H.

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**Data availability** All final alignments and unedited trees are available through GitHub: [https://github.com/dannyhaelewaters/teamlaboul/tree/main/Two\\_new\\_Hesperomyces\\_species\\_Algeria\\_Italy\\_Malaysia\\_paper](https://github.com/dannyhaelewaters/teamlaboul/tree/main/Two_new_Hesperomyces_species_Algeria_Italy_Malaysia_paper). Newly generated sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>), under the following accession numbers: PQ369999–PQ370007 (ITS), PQ370008–PQ370017 (28S), PQ374037–PQ374041 (MCM7).

## Declarations

**Ethics approval** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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