

Butterfly–parasitoid–hostplant interactions in Western Palaearctic Hesperidae: a DNA barcoding reference library

ERIC TORO-DELGADO^{1,✉}, JUAN HERNÁNDEZ-ROLDÁN^{1,2,✉}, VLAD DINCĂ^{3,4,✉}, JUAN CARLOS VICENTE⁵, MARK R. SHAW⁶, DONALD L.J. QUICKE⁷, RALUCA VODĂ⁸, MARTIN ALBRECHT⁹, JOSÉ FERNÁNDEZ-TRIANA¹⁰, BLAI VIDIELLA^{11,✉}, SERGI VALVERDE^{1,12,✉}, LEONARDO DAPPORTO¹³, PAUL D. N. HEBERT¹⁴, GERARD TALAVERA^{15,✉} and ROGER VILA^{1,✉}

¹*Institut de Biologia Evolutiva (CSIC-UPF), 03008 Barcelona, Spain*

²*Departamento de Biología (Zoología), Facultad de Ciencias, Universidad Autónoma de Madrid, c/ Darwin, 2, ES-28049 Madrid, Spain*

³*Ecology and Genetics Research Unit, PO Box 3000, University of Oulu, 90014 Oulu, Finland*

⁴*Research Institute of the University of Bucharest (ICUB), University of Bucharest, Bucharest, Romania*

⁵*c/ Witerico, 9A, Bajo B, ES-28025 Madrid, Spain*

⁶*National Museums of Scotland, Edinburgh, UK*

⁷*Department of Biology, Faculty of Life Sciences, Chulalongkorn University, Bangkok, Thailand*

⁸*Via Barge 3, 10139 Torino, Italy*

⁹*Hühnerbühlrain 4, CH-3065 Bolligen, Switzerland*

¹⁰*Canadian National Collection of Insects, Ottawa, Canada*

¹¹*Centre de Recerca Matemàtica, Edifici C, Campus de Bellaterra, Barcelona, Spain*

¹²*European Centre for Living Technology, Venice, Italy*

¹³*Dipartimento di Biologia, University of Florence, 50019 Sesto Fiorentino, Italy*

¹⁴*Centre for Biodiversity Genomics, University of Guelph, Guelph, ON N1G 2W1, Canada*

¹⁵*Institut Botànic de Barcelona (IBB), CSIC-Ajuntament de Barcelona, Passeig del Migdia s/n, 08038 Barcelona, Spain*

Received 6 September 2021; revised 20 March 2022; accepted for publication 25 May 2022

The study of ecological interactions between plants, phytophagous insects and their natural enemies is an essential but challenging component for understanding ecosystem dynamics. Molecular methods such as DNA barcoding can help elucidate these interactions. In this study, we employed DNA barcoding to establish hostplant and parasitoid interactions with hesperiid butterflies, using a complete reference library for Hesperidae of continental Europe and north-western Africa (53 species, 100% of those recorded) based on 2934 sequences from 38 countries. A total of 233 hostplant and parasitoid interactions are presented, some recovered by DNA barcoding larval remains or parasitoid cocoons. Combining DNA barcode results with other lines of evidence allowed 94% species-level identification for Hesperidae, but success was lower for parasitoids, in part due to unresolved taxonomy. Potential cases of cryptic diversity, both in Hesperidae and Microgastrinae, are discussed. We briefly analyse the resulting interaction networks. Future DNA barcoding initiatives in this region should focus attention on north-western Africa and on parasitoids, because in these cases barcode reference libraries and taxonomy are less well developed.

*Corresponding author. Email: roger.vila@csic.es

ADDITIONAL KEYWORDS: Braconidae – coevolution – *COI* mtDNA – cryptic species – ecology – host–parasite systems – hostplants – Lepidoptera.

INTRODUCTION

Studying ecological interactions between plants, phytophagous insects and their natural enemies is essential for understanding ecosystem dynamics, because these interactions play an influential role in determining both the distributions of species and their abundances at sites where they occur (Hawkins, 1994). However, the size and complexity of such systems makes this a substantial challenge.

In this study, we focussed on clarifying ecological interactions between Western Palaearctic HesperIIDae

(Lepidoptera: Papilionoidea), their hostplants and their parasitoids. The larvae of parasitoid insects feed on a single host, ultimately killing it (Lafferty & Kuris, 2002; Shaw *et al.*, 2009). Many hesperiid larvae build silk shelters, which aid their detection (Greeney & Jones, 2003; Greeney, 2009) and thus facilitate the study of their hostplants and parasitoids. However, immature stages can be difficult or sometimes impossible to identify by morphology (Hernández-Roldán *et al.*, 2012, 2018). Many parasitoids are also difficult to identify even as adults, for instance, in the Ichneumonoidea (e.g. Quicke, 2015). An additional challenge is the

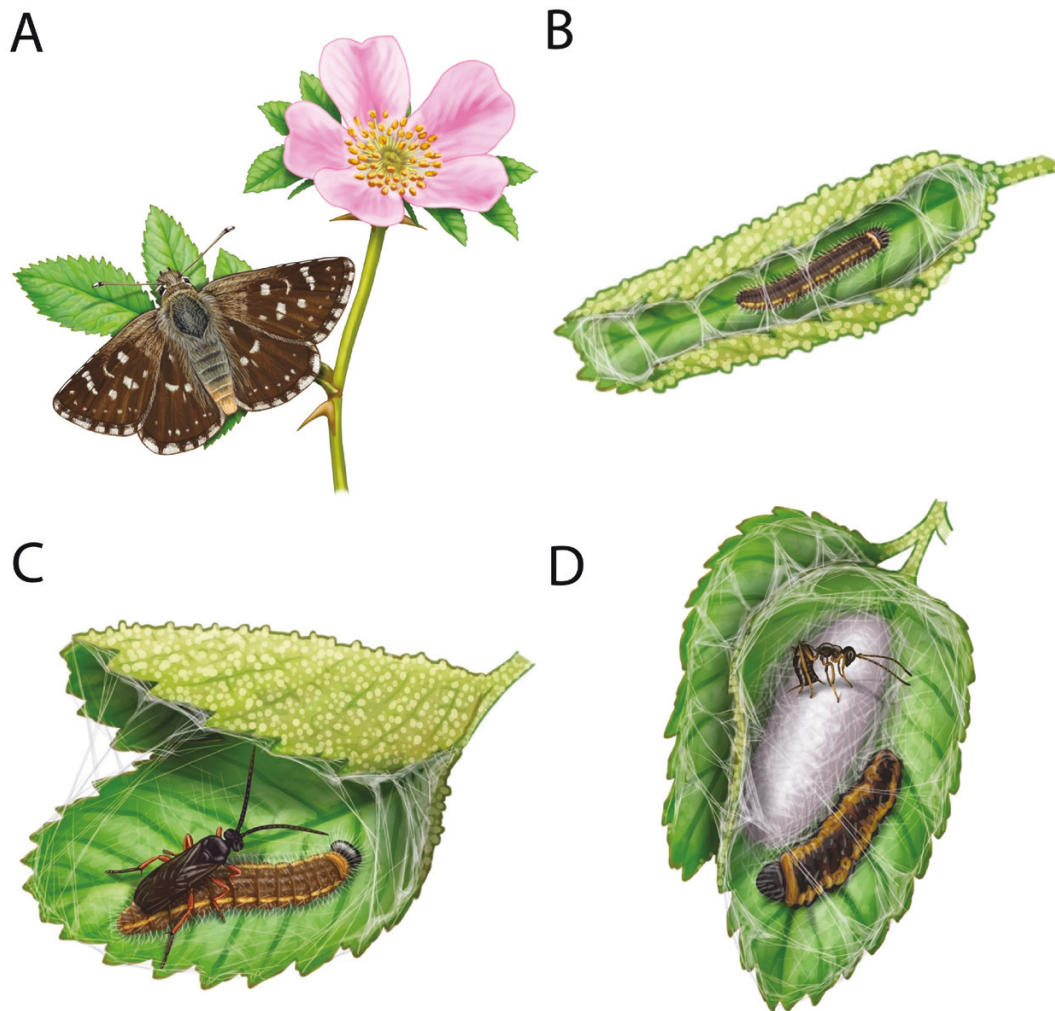


Figure 1. Representation of the study system. Hesperiid larvae feeding on their hostplants can be attacked by a number of parasitoids, which can in turn be attacked by various hyperparasitoids. A, *Spialia rosae* on its hostplant *Rosa sicula*. B, third instar larva of *Sp. rosae* on a silk shelter. C, *Microgaster australis* parasitizing an L3 *Sp. rosae* larva. D, *Gelis* sp. parasitizing *M. australis* on its cocoon after emerging from the *Sp. rosae* larva. Drawings by Martí Franch.

complexity of the parasitoid assemblage attacking any group of hosts (Fig. 1). In the case of butterflies, common parasitoids of larvae and pupae belong to three hymenopteran families (Braconidae, Ichneumonidae and Pteromalidae) and the dipteran family Tachinidae (Shaw *et al.*, 2009). Other parasitoids of these stages, albeit less common, belong to Bombyliidae (Diptera), Chalcididae and Eulophidae (Hymenoptera), while eggs are parasitized by several families of Chalcidoidea and Scelionidae (Hymenoptera). Life histories of many of these families are diverse, with some species being primary parasitoids, but others being secondary parasitoids (for a review, see: Shaw *et al.*, 2009).

The preceding complications make molecular methods, such as DNA barcoding, promising tools to facilitate the study of these interactions. DNA barcoding relies on reference libraries of short DNA sequences to assign sequences of uncertain taxonomic origin to a species (Hebert *et al.*, 2003). This simple but powerful method has gained wide adoption, because of its multiple applications in fields of research beyond taxonomic identification. In addition to its value for mapping species distributions (e.g. Litman *et al.*, 2018), DNA barcoding is often used in phylogeography (e.g. Menchetti *et al.*, 2021) and can also reveal potential cryptic species (Wang & Qiao, 2009; Dincă *et al.*, 2015). DNA barcodes have also been incorporated into species descriptions (Goldstein & DeSalle, 2011; Sharkey *et al.*, 2021). Furthermore, it can improve the monitoring of endangered wildlife (Akrim *et al.*, 2018), inform species reintroductions (Dincă *et al.*, 2018), help combat illegal species trade (Bunholi *et al.*, 2018), aid higher taxonomy (Talavera *et al.*, 2022) and allow the identification of immature stages (Peoples *et al.*, 2017).

DNA metabarcoding further extends applications as it allows the determination of the species present in bulk samples. This technique has found diverse applications, such as resolving plant-pollinator interactions (Bell *et al.*, 2017), identifying ichthyoplankton (Nobile *et al.*, 2019) and determining insect migratory movements (Suchan *et al.*, 2019). Metabarcoding has also been widely applied to study microbial communities and microbiomes (Abdelfattah *et al.*, 2018; Burtseva *et al.*, 2021).

Both DNA barcoding and metabarcoding can be applied to the study of species interactions, obtaining information that would otherwise be inaccessible. For example, Moran *et al.* (2015) used DNA barcoding to identify prey items from the stomach contents of catfish, revealing 23% more items than by morphology alone. Jurado-Rivera *et al.* (2009) reconstructed host-plant relationships among chrysomelid beetles by extracting DNA from whole insects, while González-Varo *et al.* (2014) recovered avian DNA from plant seeds to identify their main dispersers. Kaartinen *et al.* (2010) applied DNA barcoding to reveal a food

web of gall-forming wasps and their natural enemies, and found that species designation changed for 31% of the sequenced specimens relative to their initial morphological identification. Rougerie *et al.* (2011) successfully sequenced host DNA from the gut contents of adult parasitoids, while Wirta *et al.* (2014) also recovered parasitoid DNA from the tissue of their hosts.

Nevertheless, the capacity of DNA barcoding to generate reliable identifications depends on access to complete reference libraries for the taxonomic group(s) under study across the geographical range being investigated. For instance, Bergsten *et al.* (2012) found a decrease in identification success with increasing spatial scale for beetles of the tribe Agabini, whereas Lukhtanov *et al.* (2009) found consistent species identification at large scales in Central Asian butterflies. Finally, the performance of the method differs among taxa as species in groups with rapid, recent diversification can be difficult to distinguish by DNA barcoding (Wiemers & Fiedler, 2007).

The DNA barcode library for Western Palaearctic butterflies is relatively advanced due to several barcoding projects implemented at both regional (Dincă *et al.*, 2011, 2015; Hausmann *et al.*, 2011; Litman *et al.*, 2018; Dapporto *et al.*, 2019) and continental (Dincă *et al.*, 2021) scales. Moreover, the taxonomy of this group seems to be approaching a consensus (e.g. Wiemers *et al.*, 2018), although debate still exists, as there have been recent rearrangements (Zhang *et al.*, 2020) and cryptic species continue to be discovered (Hinojosa *et al.*, 2021). Most work has focused on Europe, so the taxonomic framework and barcode reference libraries for North Africa are much less developed, although this region shares a Mediterranean biome and many species with Europe. This is especially true for HesperIIDae, which are among the least-studied families of butterflies, owing to their generally small size and inconspicuous wing patterns.

In contrast to the situation for butterflies, the DNA barcoding libraries for Ichneumonoidea, one of the biggest superfamilies of hymenopteran parasitoids, are still far from complete. This may, in part, be due to their still largely uncertain taxonomy (Quicke *et al.*, 2012). The subfamily Microgastrinae (Braconidae), for example, which are exclusively parasitoids of Lepidoptera (Fernandez-Triana *et al.*, 2020), are one of the best represented subfamilies in the Barcode of Life Data System (BOLD; Ratnasingham & Hebert, 2007) with sequences available for almost 80% of the 81 genera, but most sequences originate from Canada and Costa Rica (Fernandez-Triana *et al.*, 2020). For the Western Palaearctic, just 1829 sequences of Microgastrinae are publicly available (consulted on 7 June 2021) with most of these records deriving from continental Europe and few records from the Mediterranean Basin. Quicke *et al.* (2012) assessed the

utility of DNA barcoding for the global ichneumonoid fauna, releasing *c.* 1800 sequences. Their results suggested that for relatively well-sampled groups, such as Microgastrinae, DNA barcoding provided robust results (Smith *et al.*, 2013), but many other subfamilies lack sufficient sampling to achieve this.

In this study, we present the first compilation of hostplant and parasitoid interactions for the entire HesperIIDae fauna of Europe and North Africa. We (1) compiled a DNA barcode reference library for HesperIIDae, achieving complete coverage for the fauna of Europe and north-western Africa (53 species) based on 2934 sequences; (2) compiled records of parasitoid and hostplant interactions of HesperIIDae (established in part by barcoding hesperiid larvae and their parasitoids); (3) pinpointed possible cases of cryptic diversity in both the butterflies and the parasitoids; and (4) discuss the effectiveness of this approach for the study of host–parasitoid interactions.

MATERIAL AND METHODS

SAMPLING AND DATA COLLECTION

For this study, we limited the area of interest to Europe [as defined in Wiemers *et al.* (2018)] and north-western Africa (defined as Morocco, Algeria and Tunisia). To recover ecological interactions, records of HesperIIDae that were parasitized from the study area and/or included information on their hostplant, accumulated by the authors during the course of their respective research, were extracted from collection databases of the authors. The search for caterpillars was done by tracking butterfly females displaying oviposition behaviour, as well as by manually inspecting potential hostplants. Unless already known, larvae were reared to confirm that they successfully feed on the plant the eggs were laid on. This review yielded 233 records linked to specimens collected during the last 16 years (from 2004 to 2020) in 11 countries of the study region (Supporting Information, Table S1). For all records with tissue available, DNA was extracted, amplified and sequenced employing the protocols below. In most cases, hesperiiids were collected as larvae, but some were collected as eggs, pupae or pupal exuviae. Similarly, in cases of parasitism, some larvae were reared and thus the adult parasitoids were preserved, but in other instances only remains of the dead host and parasitoid cocoons were collected.

To assemble the DNA barcode reference library for HesperIIDae, we gathered all publicly available sequences from BOLD and added additional sequences from the study region that were generated for this study. In all cases, specimens were determined independently based on external morphology and genitalia or, when

this was insufficient (i.e. in synmorphic species), based on life-history traits, known distribution or nuclear genetic markers (for details see: Dincă *et al.*, 2021; Hinojosa *et al.*, 2021).

DNA EXTRACTION, PCR AND SEQUENCING

Only hesperiiids and their parasitoids were barcoded. The following protocol was carried out at the Institute of Evolutionary Biology (Barcelona, Spain) for both hesperiiids and parasitoids: DNA was extracted using Chelex 100 Chelating Resin (Bio-Rad). A piece of tissue from each specimen was ground in 100 µL of 10% Chelex suspension and 5 µL of proteinase K (20mg/mL) were added afterwards. For hesperiid larvae, the head was used for larger instars, while the whole sample was used for the first instars or eggs. For adult parasitoids, a hind or midleg was used, except when multiple specimens of the same brood were available, in which case a whole specimen was used. For samples with potentially low DNA content (e.g. empty cocoons, exuviae and dead larval remains) the whole sample was used, except in the case of gregarious parasitoids with many cocoons available. Extracts were left overnight at 55 °C in continuous agitation and incubated at 100 °C for 15 min the day after, and then the barcode region of the cytochrome *c* oxidase subunit 1 (*COI*) gene was amplified using the polymerase chain reaction (PCR). Each 25 µL reaction mix included 0.1 µL of GoTaq Flexi DNA Polymerase (Promega), 5 µL of 5X GoTaq Green Flexi Buffer (Promega), 2 µL of MgCl₂ 25mmol/L, 0.5 µL of dNTPs 10 mmol/L and 0.5 µL of each primer (forward and reverse) 10 µmol/L. The PCR program for all reactions involved initial denaturation at 92 °C for 1 min; five cycles with 15 s at 92 °C, 45 s at 49 °C and 150 s at 62 °C, followed by 30 cycles with 15 s at 92 °C, 45 s at 52 °C, and 150 s at 62 °C, with a final extension at 62 °C for 7 min.

To discriminate between hosts and parasitoids, the forward primers LepF1b and LCOpar were designed using a variety of butterfly and hymenopteran sequences, respectively. Different primer pairs were used depending on the organism (Table 1). Except for LCOpar and HCO, all primers were tailed (5′ TAATACGACTCACTATAGGG 3′ for forward, 5′ ATTAACCCTCACTAAAG 3′ for reverse). Sanger sequencing of PCR products was performed by MacroGen Inc. Europe (Amsterdam, The Netherlands).

DNA sequencing of the remaining parasitoids was carried out following standard protocols (Ivanova *et al.*, 2006; deWaard *et al.*, 2008; Hebert *et al.*, 2013) at the Centre for Biodiversity Genomics, University of Guelph, Canada. All new sequences have been deposited in BOLD in the datasets DS-HESPPAR (HesperIIDae) and DS-HESPPARB (parasitoids), which are publicly

Table 1. Primers used for amplification of the barcode region of the cytochrome *c* oxidase subunit 1 gene (*COI*). Except for LCOpar and HCO, all forward primers were tailed with 5' TAATACGACTCACTATAGGG 3' and all reverse primers with 5' ATTAACCCTCACTAAAG 3'. *no DNA was amplified from these groups

Groups sequenced	Primer pair (forward and reverse)	Primer sequences (forward and reverse)
Lepidoptera	LepF1b LepR1	5' ATTCAACCAATCATAAAGATATTGGAAC 3' 5' TAAACTTCTGGATGTCCAAAAAATCA 3'
Lepidoptera, Hymenoptera	LepF1 LepR1	5' ATTCAACCAATCATAAAGATATTGG 3' 5' TAAACTTCTGGATGTCCAAAAAATCA 3'
Hymenoptera	LCOpar Nancy	5' GGTCAACAAAATCATAAAGATATTGGKAT 3' 5' CCCGGTAAAAATAAAAATATAAACTTC 3'
Diptera*, Nematomorpha*	LCO1490 HCO	5' GGTCAACAAAATCATAAAGATATTGG 3' 5' TAAACTTCAGGGTGACCAAAAAAATCA 3'

available ([dx.doi.org/10.5883/DS-HESPPAR](https://doi.org/10.5883/DS-HESPPAR) and [dx.doi.org/10.5883/DS-HESPPARB](https://doi.org/10.5883/DS-HESPPARB)).

SEQUENCE ANALYSES, SPECIMEN IDENTIFICATION AND NETWORK ANALYSIS

The new sequences were edited using GENEIOUS PRIME v.2021.1.3 and molecular identifications were provided using the Basic Local Alignment Search Tool (BLAST; [Altschul et al., 1990](#)). In the case of hesperiids, we also included them in a neighbour-joining tree built using the reference library (the specimens of which were not identified by their barcodes, but independently). Morphological identifications for both HesperIIDae and their parasitoids were also provided to the lowest possible taxonomic level and compared to those obtained through molecular analysis. The taxonomy of HesperIIDae followed [Wiemers et al. \(2018\)](#) with modifications from [Zhang et al. \(2020\)](#) and [Hinojosa et al. \(2021\)](#) for European species, and [Tshikolovets \(2011\)](#) for species restricted to Africa. Hostplants were identified morphologically in the field or from pressed samples; identifications were verified by botanists Llorenç Sáez (Autonomous University of Barcelona, Barcelona) and Modesto Luceño (Pablo de Olavide University, Seville), and the taxonomy follows the Plants of the World Online initiative ([POWO, 2022](#)).

Uncorrected *p*-distances were calculated separately for all parasitoid and for all congeneric HesperIIDae sequences using MEGA-X ([Kumar et al., 2018](#)), and maximum intraspecific and minimum interspecific distances were calculated using R statistical software (v4.1.2; [R Core Team 2021](#)). For HesperIIDae reference library we also conducted a barcode index number (BIN) analysis ([Ratnasingham & Hebert, 2013](#)).

In order to assess the clustering of barcodes, neighbour-joining trees based on *p*-distance were constructed separately for HesperIIDae and their parasitoids using MEGA-X with 350 bootstrap pseudoreplicates. A plant cladogram (including only

the plants involved in the interactions presented here) was manually edited based on current knowledge ([Potter et al., 2007](#); [Dobeš & Paule, 2010](#); [Bendiksy et al., 2011](#); [Mathiesen et al., 2011](#); [Li et al., 2016](#); [Zeng et al., 2017](#); [Byng et al., 2018](#); [Persson et al., 2020](#)). The three trees were then combined into an inwards circular cladogram with the *ggtree* package in R ([Yu et al., 2017, 2018](#); [Yu, 2020](#)) to which we added the ecological interactions to visualize host relationships together with genetic data. The HesperIIDae cladogram in this figure was constrained according to the established subfamily relationships.

We also calculated different summary statistics to analyse patterns among the recovered interactions. We calculated the number of host species and host genera used by each parasitoid, as well as the number of parasitoid species and genera parasitizing each hesperiid species. Modularity and nestedness were also calculated for the HesperIIDae–hostplant, HesperIIDae–parasitoid and parasitoid–hostplant interaction networks ([Flores et al., 2011](#)) and compared against modularity and nestedness distributions drawn from randomized networks.

RESULTS

AMPLIFICATION AND IDENTIFICATION SUCCESS FROM DIFFERENT TYPES OF SAMPLES

A total of 233 individual-level interactions were recovered, which involved 26 hesperiid species in eight genera, nearly all in subfamily Pyrginae ([Supporting Information, Table S1](#)). Among these, 93 had both hostplant and parasitoid data, 116 had hostplant data only and 24 had parasitoid data only. These records encompassed 43 different hesperiid–parasitoid interactions (i.e. involving different species pairs) and 45 different hesperiid–plant interactions. For 168 of these records, hesperiid tissue was available for amplification. For the total of 118 records involving

parasitoids, all but four had tissue available for amplification (either parasitoid tissue or their host larvae). Photographs for the majority of samples are available in the [Supporting Information, Fig. S1A–I](#).

A total of 128 barcode sequences of Hesperidae were recovered from the 168 samples with tissue (76.2%). Among these, four were adults reared from eggs, 14 were eggs, 63 were larvae, three were pupae, nine were pupal exuviae, two were head capsules and 33 were parasitized larvae ([Supporting Information, Table S1](#)). On one occasion, the primer pair LepF1b/LepR1 amplified the parasitoid instead of the host larva.

A total of 89 barcode sequences were obtained from the 118 parasitoids (75.4%). Among these, six were obtained from cocoons (all empty except one case with parasitoids still inside), while two were sequenced from the host larva ([Supporting Information, Table S1](#)). This represents a 28.6% amplification success from 21 cocoon samples and a success rate of 22% when attempting to amplify parasitoid DNA from nine hosts.

Identification to species level for Hesperidae increased from 91.8% (214/233), based on morphology and ecology alone, to 94.8% (221/233) when DNA barcodes were included. In the case of parasitoids, many specimens in the genera *Cotesia* Cameron, 1981 and *Dolichogenidea* Viereck, 1911 have an unresolved taxonomic status, potentially belonging to undescribed species morphologically similar to *Co. glabrata* Telenga, 1955 and *Do. sicaria* Marshall, 1885, respectively. When barcodes were obtained for these cases, closest matches in BLAST were identified at a genus level or as *Cotesia* sp. near *glabrata* and *Dolichogenidea* sp. near *sicaria*. Therefore, success in genus-level identification increased from 93.2% (110/118), based on morphology alone, to 96.6% (114/118) when DNA barcodes were included, while species-level identification rose from 36.4% (43/118), for morphology alone, to 44.9% (53/118), for morphology and barcodes. However, a few parasitoids belonged to groups outside the taxonomic expertise of the authors (e.g. Nematomorpha) and it is likely that additional species would be identifiable to genus or species level.

INTERACTION RECORDS RECOVERED

Most parasitoids (106/118 = 87.6%) belonged to the braconid subfamily Microgastrinae ([Figs 2, 3A–D](#)), but six were Ichneumonidae, two were Tachinidae (*Sturmia bella* Meigen, 1824), two were Nematomorpha and two were Chalcidoidea. All were primary parasitoids, except the pteromalid *Catolaccus ater* Ratzeburg, 1852 and the ichneumonid *Gelis* sp., which are pseudohyperparasitoids.

Among the Microgastrinae, *Cotesia* spp. comprised 58 records (54.6%). Among these, 15 records ex *Carcharodus alceae* Esper, 1780 or *Carcharodus*

tripolinus Verity, 1925 were *Cotesia glabrata*, while the rest appear to be undescribed species from other hosts. In particular, 42 specimens that were morphologically similar to *Co. glabrata* formed three distinct clades, each with different Pyrginae hosts. One clade comprised specimens ex *Pyrgus* Hübner, 1819, a second ex *Muschampia stauderi* Reverdin, 1913, *Mu. proteides* Wagner, 1929 and *Muschampia* sp., and the third ex *Spialia sertorius* Hoffmannsegg, 1804, *Mu. baeticus* Rambur, 1840 and *Mu. orientalis* Reverdin, 1913 ([Fig. 2; Supporting Information, Fig. S2](#)). Minimum pairwise distances between clades (including *Co. glabrata*) ranged from 2.2% to 4.2% ([Table S2](#)). Another, morphologically distinct *Cotesia* species was reared once from *Carterocephalus palaemon* Pallas, 1771. The second most frequent genus was *Microgaster* Latreille, 1804 with 36 records (six *Mi. nobilis* Reinhard, 1880; 27 *Mi. australis* Thomson, 1895; and three unidentified). Lastly, 12 records corresponded to a species of *Dolichogenidea* close to *Do. sicaria*.

On average, these parasitoid species used three hesperiid species as hosts ([Supporting Information, Table S3](#)). However, there was a large variation in this value. The least frequent parasitoids had only one host, whereas the average for the Microgastrinae was 4.4. *Microgaster australis* showed an unusually wide host range as it was recovered from 13 host species, including taxa in four genera. *Microgaster nobilis* was recovered from three genera, while all other parasitoids used one or two genera. Hesperiid species were parasitized, on average, by two species and two genera of parasitoids ([Supporting Information, Table S4](#)), with both values ranging from one to five. Most hesperiids were parasitized by one or two species, with the highest counts (four to five) reflecting also parasitoids poorly represented in our dataset (e.g. Nematomorpha, *Sturmia bella*). Hosts were parasitized by only one species per genus, except the case of *Pyrgus onopordi* Rambur, 1839 and *Muschampia baeticus*, which were parasitized by both *Microgaster australis* and *Microgaster nobilis*. It must be kept in mind that these values are based on current data and many species of Hesperidae have been reared infrequently or not at all, so these values may change with additional sampling.

Hostplant interactions corresponded with known relationships, with *Pyrgus* using mostly *Potentilla* L. spp., *Spialia* Swinhoe, 1912 using *Sanguisorba minor* Scop. agg. (except for *Sp. rosae* Hernández-Roldán *et al.*, 2016 on *Rosa* L. spp.), *Muschampia* Tutt, 1906 on *Phlomis* L. and other Lamiaceae, *Carcharodus* Hübner, 1819 on *Malva* L. and Hesperinae on Poaceae ([Fig. 2](#)). Of special interest is the use of *Potentilla asturica* Rothm., an Iberian endemic, as hostplant of the localized *Pyrgus cinarae* (Rambur, 1839).

The Hesperidae–hostplant network showed a clearly modular structure ([Fig. 4A](#)), with a cluster

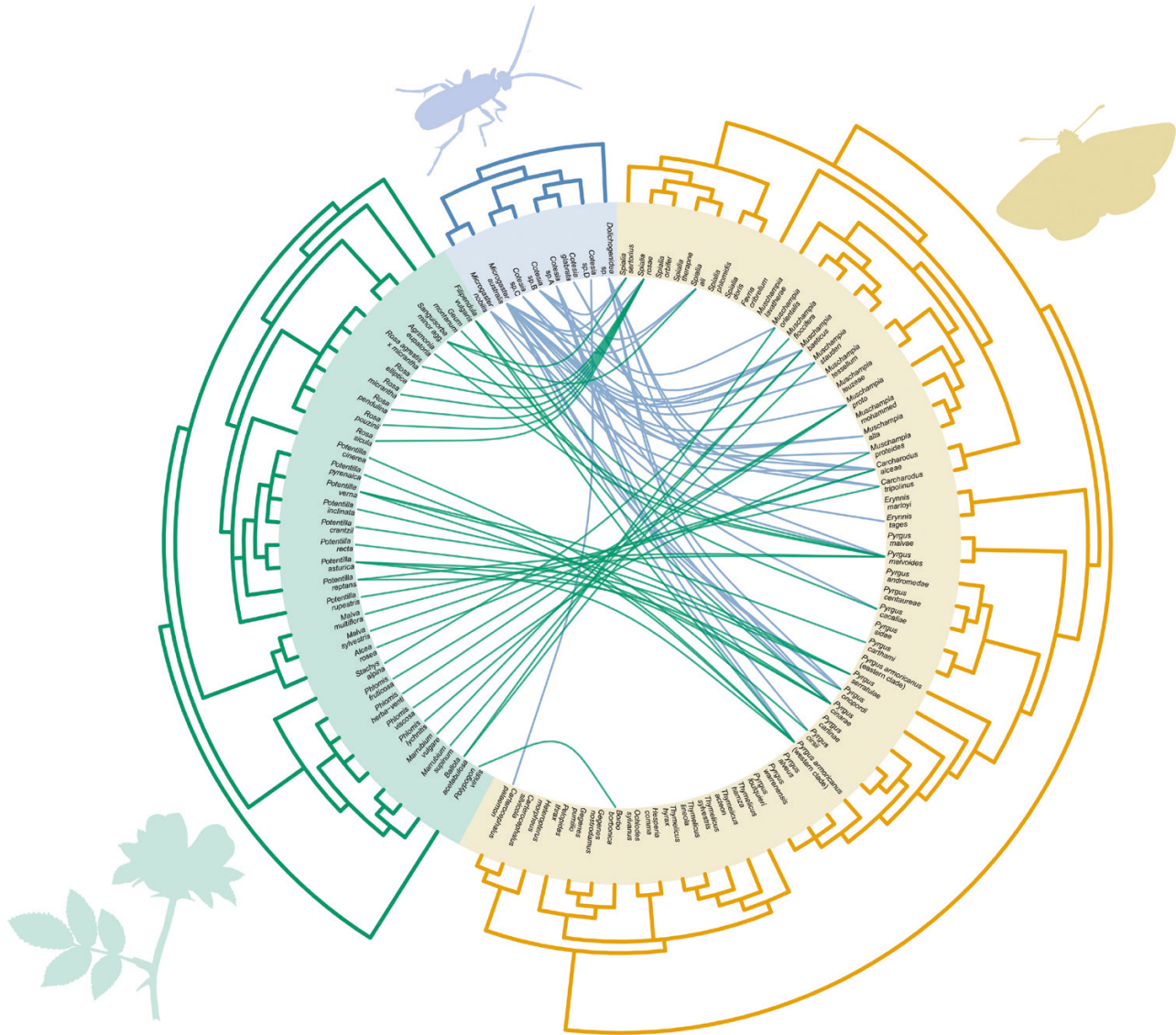


Figure 2. Circular cladogram showing ecological interactions among European and North African HesperIIDae, their hostplants, and their microgastrine parasitoids, recovered through DNA barcoding for HesperIIDae and/or parasitoids. Hesperiid, parasitoid and plant cladograms are coloured in orange, blue and green, respectively. Lines representing interactions with parasitoids are coloured in blue, while lines involving hostplant interactions are coloured in green.

of interactions involving *Pyrgus-Spialia* and *Potentilla-Sanguisorba*, and a trail of smaller clusters involving single hesperiid species on different plants. Visually, the HesperIIDae-parasitoid matrix had a nested structure (Fig. 4B), but this was not significant against a random distribution (Supporting Information, Fig. S3). This was caused by the low number of interactions in the first row of the matrix (corresponding to *Carcharodus alceae*) and the first column (corresponding to *Mi. australis*), as well as by the low number of parasitoids compared to hesperIIDae. Lastly, the parasitoid-hostplant matrix showed a

tendency towards nestedness (Fig. 4C), but this was not significant (Supporting Information, Fig. S3). This is likely due to the small size of the matrix and again due to a low number of interactions in the first row (*Mi. australis*) and column (*Malva sylvestris* L.).

DNA BARCODE LIBRARY FOR THE WESTERN PALAEARCTIC HESPERIIDAE

The final HesperIIDae dataset included 2934 sequences representing 53 species from 38 countries. All species of HesperIIDae known from Europe [as defined by

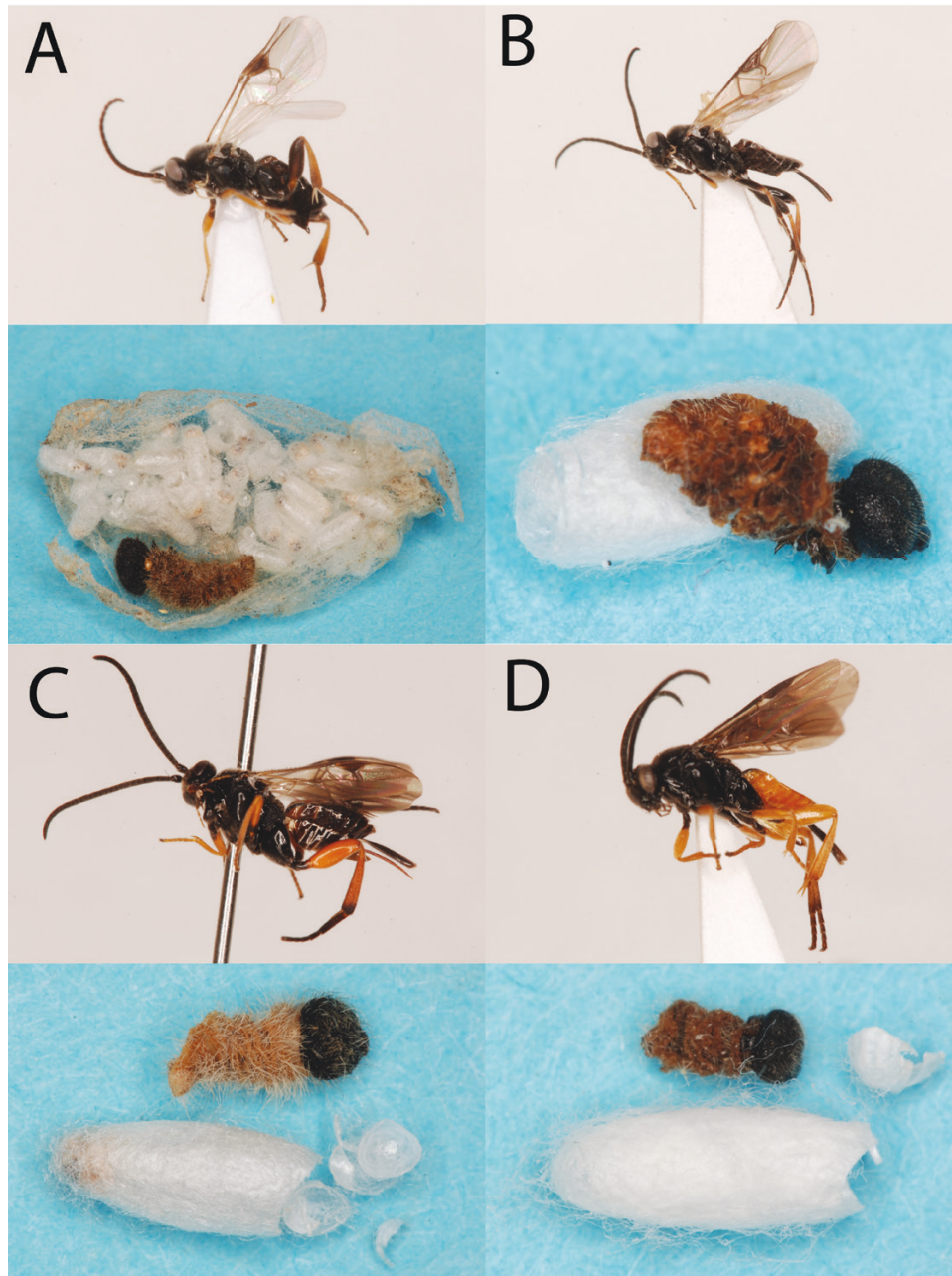


Figure 3. Mounted specimens illustrating the species of Microgastrinae recovered in this study. A, *Cotesia glabrata* Telenga ex *Carcharodus alceae*, Italy. Adult plus cocoons. Gregarious parasitoid; brood sizes vary considerably, host usually well grown or prepupal when killed. The other *Cotesia* species (near *glabrata*) look similar and behave in the same way. B, *Dolichogenidea* sp. near *sicaria* Marshall, ex *Carcharodus alceae*, Spain. Adult plus cocoon. Solitary parasitoid, killing the host while still quite young. C, *Microgaster australis* Thomson, ex *Muschampia stauderi*, Greece. Adult plus cocoon. Solitary parasitoid, usually killing the host as a prepupa. D, *Microgaster nobilis* Reinhard, ex *Carcharodus alceae*, Spain. Adult plus cocoon. Solitary parasitoid, usually killing the host as a prepupa. All specimens are in the collection of the National Museums of Scotland.

Wiemers *et al.* (2018)] and north-western Africa [as defined by Tshikolovets (2011)] were represented, with an average of 54.33 sequences per species. As expected, common, widespread species were represented by more specimens than those that are rare and/or

localized (e.g. *Muschampia mohammed* Oberthür, 1887 and *Mu. leuzeae* Oberthür, 1881 from Maghreb or *Thymelicus hyrax* Lederer, 1861 from the Balkans).

In the neighbour-joining tree (Supporting Information, Fig. S4), 44 species (83%) were

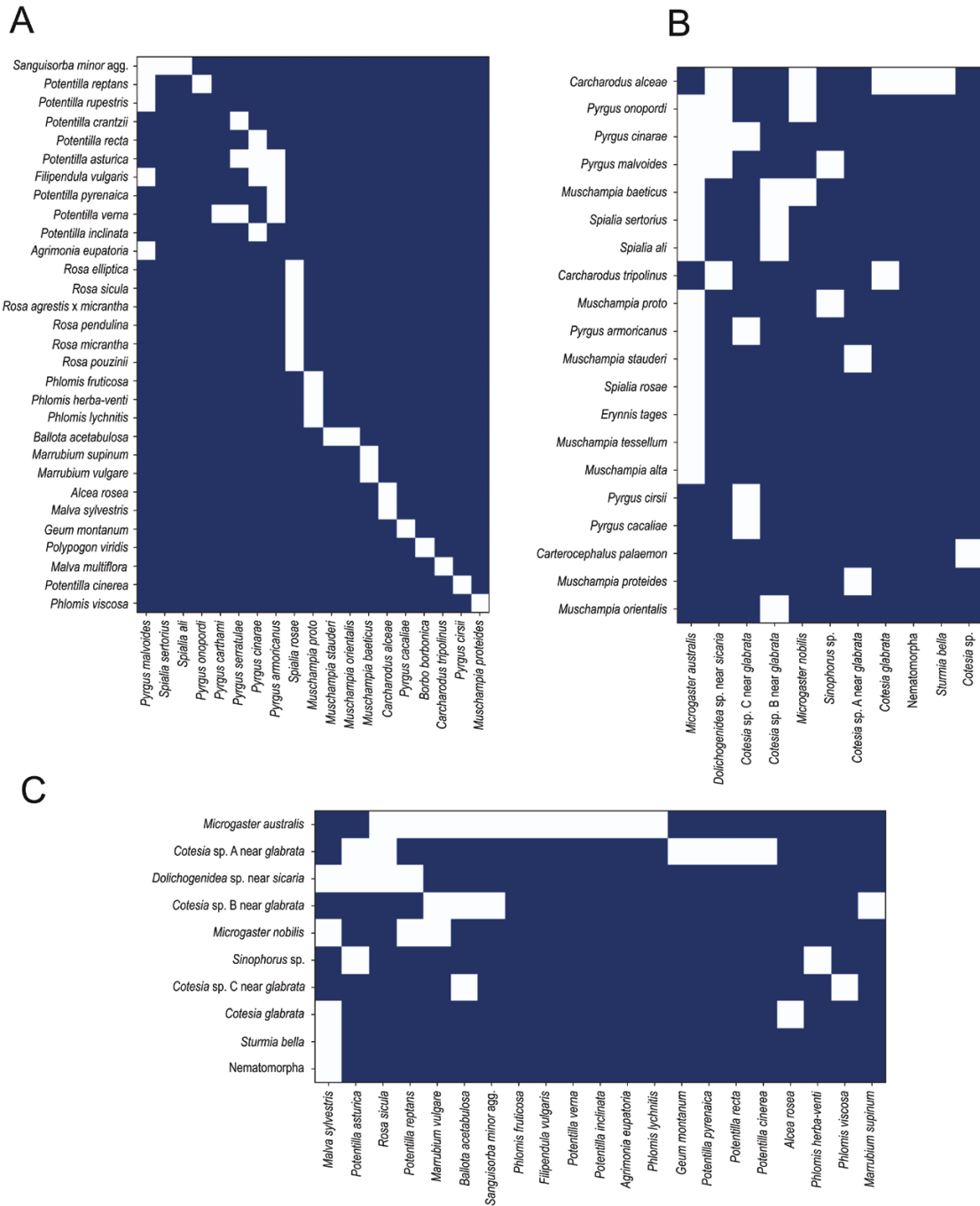


Figure 4. Interaction matrices showing the recorded interactions of HesperIIDae and their hostplants (A), HesperIIDae and their parasitoids (B) and parasitoids and hostplants of HesperIIDae (C). White squares indicate recorded interactions between the taxa in the corresponding row and column, while blue squares indicate lack of interaction.

recovered as monophyletic, six (11%) were recovered as para- or polyphyletic and three were involved in barcode sharing (among the pairs *Pyrgus alveus* Hübner, 1803–*Pyrgus warrenensis* Verity, 1928 and *Py. alveus*–*Pyrgus foulquieri* Oberthür, 1910), giving

an identification success based on species-diagnostic haplotypes (i.e. those not shared among species) of 94.34%. The minimum interspecific distance between *Py. warrenensis* and *Py. foulquieri* was low (0.62%). Six additional species pairs had a minimum *p*-distance

below 1%, and 15 were between 1% and 2% (Supporting Information, Table S5). The BIN analysis recovered seven BINs representing two (in one case three) species, all corresponding to species with less than 1% minimum *p*-distance (Supporting Information, Table S6). In addition, seven species were split into multiple BINs (Supporting Information, Table S7; *Pyrgus armoricanus* Oberthür, 1910, *Py. cinarae* Rambur, 1839, *Py. alveus*, *Py. serratulae* Rambur, 1839, *Spialia ali* Oberthür, 1881, *Thymelicus lineola* Ochsenheimer, 1808 and *T. sylvestris* Poda, 1761).

DISCUSSION

UTILITY OF DNA BARCODING TO STUDY ECOLOGICAL INTERACTIONS

DNA barcoding has shown its utility for clarifying ecological interactions (e.g. Hrček & Godfray, 2015) and our study extends this evidence by focusing on the plant–hesperiid–parasitoid system. Barcodes were obtained for 128 of 233 hesperiid specimens with recorded interactions, and all barcoded specimens were assigned to a species with the taxonomically curated DNA barcode reference library. The remaining 106 records were identified based on their morphology, ecology and distribution. In total, identification success to species level was close to 95%, with the 13 records that remained at genus level being cases in which barcodes could not be obtained.

The capacity of DNA barcodes to deliver species assignments for parasitoids was limited because often the best BLAST matches did not reach species level or they were attributed to potential complexes of cryptic species. These barriers to the use of barcoding indicate the importance of both geographically and taxonomically comprehensive DNA barcode reference libraries, but also the importance of alpha taxonomy and the deposition of voucher specimens in public collections. Among all insect superfamilies, the Ichneumonoidea is one of the most diverse and it is often difficult to identify species morphologically (Quicke, 2015). These difficulties increase the potential utility of DNA barcoding for their identification, but they also complicate the establishment of a solid taxonomic framework.

The capacity of DNA barcoding to open new sources of information is one of its great strengths. In the present study, we recovered barcode sequences from parasitoid cocoons and larval remains exposed to field conditions for an unknown period of time. The fact that sequences were recovered without using a protocol optimized for degraded DNA suggests that parasitoid cocoons and larval remnants are a good source of information for revealing interactions between hesperiids and their parasitoids. In addition,

the forward primers LepF1b (butterfly-specific, paired with the reverse LepR1) and LCOpar (parasitoid-specific, paired with the reverse Nancy) used in this study successfully discriminated between host and parasitoid, each amplifying 655 bp barcodes from the same DNA extraction. Only on one occasion did LepF1b–LepR1 amplify the parasitoid DNA instead of that of the host. However, it should be noted that all sequences recovered with LCOpar belong to Microgastrinae, which were predominant among our samples, so further study is required to confirm its effectiveness for other groups of parasitoids. While newer approaches, such as next-generation-sequencing technologies, can recover sequences from complex mixes of degraded DNA from different organisms, these methods are technically and bioinformatically more complex. Sanger sequencing of targeted amplicons remains a simpler and widely available alternative and is cost-effective when the number of samples and targeted DNA markers is low.

Other studies have applied similar DNA barcoding approaches to reveal parasitoid links using morphologically unidentifiable material. For example, Rougerie *et al.* (2011) utilized a specific primer pair to amplify host DNA from the gut contents of reared parasitoid wasps preserved in ethanol shortly after their emergence. They achieved a success rate of 24%, demonstrating that host DNA can persist after metamorphosis. More recently, Wirta *et al.* (2014) expanded this approach, recovering both host DNA from parasitoid guts and parasitoid DNA from host tissue using field-collected specimens. They applied this approach to study a community of lepidopteran parasitoids in the High Arctic, achieving a sequencing success rate of *c.* 21% and recovering three times as many interactions as with traditional rearing alone. Additionally, the results had a large impact on web structure, with the variation in some metrics being larger within their webs (i.e. the web with and without the barcoding data) than variation of the same metrics among different food webs from sites around the globe. Given such drastic impacts, and because traditional methods of rearing host larvae are time-consuming (Shaw, 1997; Eveleigh *et al.*, 2007; Smith *et al.*, 2008), DNA barcoding should be routinely incorporated into the study of insect food-webs, as it will significantly increase our ability to understand such systems, both in terms of time efficiency and in the completeness of the recovered webs.

Documenting insect ecological interactions, as well as increasing the available genetic data for the species involved, is likely to provide important knowledge for biodiversity conservation. First, DNA barcode reference libraries can inform species reintroductions, as with *Melanargia russiae* Esper, 1783 in Hungary (Dincă *et al.*, 2018). Second, host–parasite dynamics

are complex, and unpredicted consequences can happen when these are not considered, for example, in species translocations (Northover *et al.*, 2018) or range expansions (Gripenberg *et al.*, 2011). This has resulted in an increasing focus towards parasite conservation, and the same should be done with parasitoids, for which similar dynamics can be expected (Van Nouhuys & Hanski, 2000). Increasing available data on interactions will also help predict and avoid impacts of parasitoids released as biological control agents on non-target species (Hajek *et al.*, 2016), as well as impacts of parasitoids that reduce effectiveness of biological control agents (Paynter *et al.*, 2010). Finally, ecological interactions are themselves a component of biodiversity and their loss may occur at a higher rate than that of the species involved (Valiente-Banuet *et al.*, 2015). In that sense, our study is a step forward in the conservation of Western Palaearctic HesperIIDae and their interactions. European butterflies are in a strong position for conservation, as monitoring programmes are in place in multiple countries. In the case of HesperIIDae, *Hesperia comma catena* Staudinger, 1861 is included in the EU Habitats Directive and Bern Convention, while *Pyrgus cirsii* Rambur, 1839 is on the Red List of the International Union for the Conservation of Nature (IUCN) as Vulnerable A2c (Van Swaay *et al.*, 2010). By contrast, little is known about the conservation status of many parasitoid wasps, despite their ecological and economic importance and their greater vulnerability given their higher trophic position (Shaw & Hochberg, 2001; Thies *et al.*, 2003).

INTERACTIONS AND SPECIES RECOVERED

Most interactions recovered in this study involved members of the subfamily Pyrginae (Table 2), reflecting the higher detectability of their silk shelters compared to those of the other Palaearctic subfamilies (HesperIIDae and Heteropterinae), which feed predominantly on grasses. However, European species of HesperIIDae have been reared often enough to suggest that the general absence of Microgastrinae parasitizing them is a real difference relative to Pyrginae (e.g. Carl, 1968). The current data reinforce this pattern, as most of the parasitoids encountered were microgastrines and none was reared from HesperIIDae. This difference does not seem to be related to hostplant use, because many Microgastrinae attack grass-feeding hosts (Shaw *et al.*, 2009; Shaw, 2012), but it may be geographical, as Microgastrinae have been reported as parasitizing HesperIIDae in other regions (Gupta & Fernández-Triana, 2014).

The *Cotesia* cf. *glabrata* specimens encountered in this study formed four COI sequence clusters with > 2% divergence and each cluster was recovered from a different set of hesperiid species. Host-specific

genetic clades of parasitoids such as these suggest that ecological specialization has been an important force for their diversification. In fact, given the sequence divergence between these clades, they probably represent different, possibly cryptic, species, but a more detailed taxonomic study is needed to confirm this. Indeed, this possibility has already been suggested for the clade from *Pyrgus* spp. (Hernández-Roldán *et al.*, 2012; Obregón *et al.*, 2015).

In addition to these *Cotesia* reared from Pyrginae, another undescribed *Cotesia* species in our dataset was reared from *Carterocephalus palaemon* in Scotland (by P. Eeles), an interaction that is apparently reported for the first time. *Microgaster australis* is also reported from *Erynnis tages* Linnaeus, 1758, *Muschampia baeticus*, *Mu. stauderi*, *Pyrgus malvoides* Elwes & Edwards, 1897, *Py. cinarae*, *Spialia ali* and *Sp. sertorius*. The caterpillar of *Sp. ali* is here illustrated for the first time; images are available in the Supporting Information (Fig. S1C, G, H). Lastly, *Hyposoter ebeninus* Gravenhorst, 1829 is reported from *Pyrgus* sp. This adds to the diversity of its host range (which includes *Pieris* Schrank, 1801 and *Carcharodus*, Shaw *et al.*, 2016), further supporting the possibility of cryptic parasitoid species as suggested by Shaw *et al.* (2009).

While most parasitoid species used one or two host genera, species of *Microgaster* used three to four host genera with *Mi. australis* being found on 13 hosts, far more than any other parasitoid in our dataset. In addition, the only hesperIIDae parasitized by more than one parasitoid species of the same genus were parasitized by both *Mi. australis* and *Mi. nobilis*. This result may suggest that *Microgaster* species are less specialized than *Cotesia*, at least for those taxa parasitizing butterflies. Thus, our data agrees with a tailed distribution of host ranges, such as those reported in Nylin *et al.* (2018), in which a large number of highly specialized species is followed by few, increasingly generalist species. On the other hand, *Mi. australis* was separated into multiple lineages in the neighbour-joining analysis (Supporting Information, Fig. S2), so the possibility of cryptic species needs consideration, although no clear differences in host use relating to this genetic divergence was found.

Hostplant interactions correspond with known relationships, although some of the previous knowledge was already generated using DNA barcoding (e.g. Hernández-Roldán *et al.*, 2016). With the recent rearrangement of the subtribe Carcharodina by Zhang *et al.* (2020), there is higher phylogenetic taxonomic congruence in hostplant use, as now all Lamiaceae-feeders belong to *Muschampia*, restricting *Carcharodus* to Malvaceae. It is also worth noting that a pupal skin of *Spialia sertorius* recovered from *Stachys officinalis* (L.) Franch., 1885 is likely due to the larva abandoning its hostplant to pupate elsewhere; a common behaviour.

Table 2. Species-level interactions reported in this study. New Hesperidae-parasitoid interactions are highlighted in bold

Hesperidae	Individuals	Hostplants	Parasitoids
<i>Borbo borbonica</i>	2	<i>Polygogon viridis</i>	<i>Cotesia glabrata</i> , <i>Dolichogenidea</i> sp. near <i>sicaria</i> , <i>Microgaster nobilis</i> , <i>Sturmia bella</i> , Nematomorpha
<i>Carcharodus alceae</i>	14	<i>Alcea rosea</i> , <i>Malva sylvestris</i>	<i>Cotesia glabrata</i> , <i>Dolichogenidea</i> sp. near <i>sicaria</i>
<i>Carcharodus tripolinus</i>	17	<i>Malva multiflora</i>	<i>Cotesia</i> sp. B near <i>glabrata</i> , <i>Microgaster australis</i> , <i>Microgaster nobilis</i>
<i>Muschampia baeticus</i>	14	<i>Marrubium supinum</i> , <i>M. vulgare</i>	<i>Cotesia</i> sp. B near <i>glabrata</i> , <i>Microgaster australis</i> , <i>Microgaster nobilis</i>
<i>Muschampia floccifera</i>	1	<i>Stachys alpina</i>	<i>Cotesia</i> sp. B near <i>glabrata</i>
<i>Muschampia orientalis</i>	3	<i>Ballota acetabulosa</i>	<i>Cotesia</i> sp. C near <i>glabrata</i>
<i>Muschampia proteides</i>	3	<i>Phlomis viscosa</i>	<i>Cotesia</i> sp. C near <i>glabrata</i> , <i>Microgaster australis</i>
<i>Muschampia proto</i>	16	<i>Phlomis fruticosa</i> , <i>Phlomis herba-venti</i> , <i>Phlomis lychnitis</i>	<i>Microgaster australis</i> , <i>Sinophorus</i> sp.
<i>Muschampia tessellum</i>	1		<i>Microgaster australis</i>
<i>Muschampia alta</i>	3		<i>Cotesia</i> sp. C near <i>glabrata</i> , <i>Microgaster australis</i>
<i>Muschampia stauderi</i>	7	<i>Ballota acetabulosa</i>	<i>Cotesia</i> sp. C near <i>glabrata</i> , <i>Microgaster australis</i>
<i>Pyrgus armoricanus</i>	7	<i>Filipendula vulgaris</i> , <i>Potentilla asturica</i> , <i>Potentilla pyrenaica</i> , <i>Potentilla verna</i>	<i>Cotesia</i> sp. A near <i>glabrata</i> , <i>Microgaster australis</i>
<i>Pyrgus cacaliae</i>	23	<i>Geum montanum</i>	<i>Cotesia</i> sp. A near <i>glabrata</i>
<i>Pyrgus carthami</i>	1	<i>Potentilla verna</i>	<i>Cotesia</i> sp. A near <i>glabrata</i> , <i>Dolichogenidea</i> sp. near <i>sicaria</i> , <i>Microgaster australis</i>
<i>Pyrgus cinaræ</i>	16	<i>Filipendula vulgaris</i> , <i>Potentilla asturica</i> , <i>Potentilla recta</i>	<i>Cotesia</i> sp. A near <i>glabrata</i>
<i>Pyrgus cirsii</i>	1	<i>Potentilla inclinata</i> , <i>Potentilla recta</i>	<i>Cotesia</i> sp. A near <i>glabrata</i> , <i>Dolichogenidea</i> sp. near <i>sicaria</i> , <i>Microgaster australis</i>
<i>Pyrgus malvoides</i>	13	<i>Potentilla cinerea</i>	<i>Cotesia</i> sp. A near <i>glabrata</i>
		<i>Agrimonia eupatoria</i> , <i>Filipendula vulgaris</i> , <i>Potentilla rupestris</i> , <i>Sanguisorba minor</i> agg.	<i>Dolichogenidea</i> sp. near <i>sicaria</i> , <i>Microgaster australis</i> , <i>Sinophorus</i> sp., <i>Gelis</i> sp.
<i>Pyrgus onopordi</i>	4	<i>Potentilla reptans</i>	<i>Dolichogenidea</i> sp. near <i>sicaria</i> , <i>Microgaster nobilis</i> , <i>Microgaster australis</i>
<i>Pyrgus serratulae</i>	3	<i>Potentilla asturica</i> , <i>Potentilla crantzii</i> , <i>Potentilla verna</i>	<i>Dolichogenidea</i> sp. near <i>sicaria</i> , <i>Microgaster nobilis</i> , <i>Microgaster australis</i>
<i>Pyrgus</i> sp.	9	<i>Sanguisorba minor</i> agg.	<i>Catolaccus ater</i> , <i>Hyposoter ebeninus</i> , <i>Sinophorus</i> sp.
<i>Spialia ali</i>	9	<i>Rosa agrestis</i> × <i>micrantha</i> , <i>Rosa elliptica</i> , <i>Rosa micrantha</i> , <i>Rosa pendulina</i> , <i>Rosa pouzinii</i> , <i>Rosa sicula</i>	<i>Cotesia</i> sp. B near <i>glabrata</i> , <i>Microgaster australis</i>
<i>Spialia rosae</i>	32	<i>Sanguisorba minor</i> agg.	<i>Microgaster australis</i>
<i>Spialia sertorius</i>	29		<i>Cotesia</i> sp. B near <i>glabrata</i> , <i>Microgaster australis</i>
<i>Erynnis tages</i>	1		<i>Microgaster australis</i>
<i>Carterocephalus palaemon</i>	1		<i>Cotesia</i> sp. (undescribed species)
<i>Ochlodes sylvanus</i>	1	Poaceae	Chalcidoidea

While the HesperIIDae–hostplant network was clearly modular, the two networks involving parasitoids had a more nested structure, although this was not statistically significant. Modularity has been associated with high-intimacy interactions (Pires & Guimarães, 2013), as well as with competition and local adaptation (Valverde *et al.*, 2020). The genetic structure underlying the interactions also affects the network structure, with matching-alleles models generally expected to cause modularity and gene-for-gene models causing nestedness (Fortuna *et al.*, 2019). In addition, the observed structure is also dependent on the spatial (Valverde *et al.*, 2020) and phylogenetic (Beckett & Williams, 2013) scales considered, and nestedness can also arise from neutral mechanisms of community assembly. Modularity in the HesperIIDae–plant network could be explained by the strong butterfly–plant coevolutionary interactions (Edger *et al.*, 2015; Van der Linden *et al.*, 2021), although evidence for this is scarcer in HesperIIDae compared to other families. This modular pattern is in general agreement with the framework of Braga *et al.* (2018), which also suggests that high modularity may arise from adaptive radiations. On the other hand, they also suggest that, despite the prevalence of phylogenetic conservatism, variation in host ranges over time generates global network nestedness through both within-module nestedness and between-module connectivity (Braga *et al.*, 2018; Nylin *et al.*, 2018). Although the butterfly–plant network presented here does not support this (there is no connectivity between modules and nestedness was lower than expected by chance), additional sampling may provide the links necessary to connect the modules. Indeed, the same study by Braga *et al.* also suggests that nestedness may be harder to detect than modularity.

The nested structure in the parasitoid networks is more surprising, as one would expect similarly strong coevolutionary dynamics, but it is likely that multiple factors are at play. Parasitism on *Drosophila* Fallén, 1823 species seems to follow gene-for-gene dynamics with costs of virulence and variations in host diversity affecting the interactions (Dupas *et al.*, 2003), which would facilitate nestedness, but a different situation may occur in different taxonomic groups and phylogenetic scales. Nestedness could be the product of substantially different levels of host specialization of the parasitoid genera included in our dataset (as seems to be the case in *Cotesia* vs. *Microgaster*). The parasitoid–plant network may be even more complex, as it may be both an indirect result of the other two and a direct result of mutualistic interactions (Van Loon *et al.*, 2000). In addition, it must be kept in mind that these networks are not complete. More species could be added (Askew & Shaw, 2022) and interactions

may be missing. In particular, interactions involving *Carcharodus alceae* or *Mi. australis* would increase the nestedness of the butterfly–parasitoid network. Finally, reducing the taxonomic uncertainty in the parasitoid taxa would also improve the accuracy of future analysis.

UTILITY OF THE REFERENCE LIBRARY FOR EUROPEAN AND MAGHREB HESPERIIDAE

The DNA barcode reference library for HesperIIDae assembled in this study includes all currently recognized species for the study area, representing a powerful tool for studies aiming to extend understanding of the ecology of this family. This is particularly true for larval ecology, but it can also aid the identification of other life stages, as many hesperiid taxa are difficult to identify by non-specialists.

The geographic coverage is greater for Europe, especially the Mediterranean peninsulas where species diversity is highest. By contrast, sampling coverage for the Maghreb is comparatively lower, despite its possession of a diverse fauna with multiple endemic taxa. Future efforts to improve DNA barcode reference libraries in the Western Palaearctic should focus on this region.

The present reference library can correctly identify 94% (50 out of 53) of the hesperiid species from the study area. Three closely related species (*Pyrgus alveus*–*Py. warrenensis*, *Py. alveus*–*Py. foulquieri*) share barcodes and cannot be reliably distinguished with *COI*. This may be due to operational factors such as unresolved taxonomy, since the taxonomy of the *Pyrgus alveus* species complex is much debated. On the one hand, the specific status of *Py. warrenensis* and *Py. foulquieri* is not universally accepted, although likely in our opinion based on current evidence. On the other hand, some authors consider that *Py. alveus* may represent several cryptic species. Thus, it is possible that these simply represent cases of incomplete lineage sorting due to recent speciation. It must be kept in mind that 22 species pairs involving 23 species had a minimum interspecific barcode divergence of < 2%, and seven pairs involving 14 species had < 1%. These usually involve recent speciation events that have been studied in detail (e.g. *Sp. rosae*–*Sp. orbifer*, *Carcharodus alceae*–*Carcharodus tripolinus*). Recently, Dincă *et al.* (2021) estimated that the available barcodes for European butterflies represent 62% of their total *COI* haplotype diversity, but most haplotypes (typically representing single-base mutations) are present at low frequencies and, for each species, only a few haplotypes are abundant. Therefore, future studies may reveal new haplotypes as well as new cases of barcode sharing, but such discoveries are unlikely to alter the efficacy of DNA barcoding in practice, because of the rarity of the yet-to-be discovered haplotypes.

The BIN analysis split seven hesperiid species (*Pyrgus alveus*, *Py. armoricanus*, *Py. cinarae*, *Py. serratulae*, *Spialia ali*, *Thymelicus lineola* and *T. sylvestris*) into two or more BINs (Supporting Information, Table S6). The case of *T. sylvestris* has already been studied in detail by Hinojosa *et al.* (2019) who concluded, based on nuclear data, that it is indeed a single species. The same study also suggests that *T. lineola* is a single species, although sampling was low for this taxon. The two BINs that form *Py. armoricanus* match with the two lineages of this species recovered in the neighbour-joining analysis (Supporting Information, Fig. S4). These lineages seem to be parapatric, occurring in sympatry at some localities in south-eastern Romania (Dincă *et al.*, 2011, 2021). A similar situation occurs with *P. serratulae* with one BIN comprising sequences from Western Europe and the other from Eastern Europe. In the case of *Py. alveus*, most sequences fall in the same BIN as *Py. foulquieri* and *Py. warrenensis*, with a smaller BIN consisting of specimens of *Py. alveus numidus* Oberthür, 1910 from Morocco. The separation of the latter taxon from the rest of *Py. alveus* has also been supported by Pitteloud *et al.* (2017). Finally, *Sp. ali* is also divided into two BINs; this was previously shown by Hernández-Roldán *et al.* (2016), whose species delimitation analyses suggested potential cryptic taxa, but they concluded that further analyses were required to confirm this assertion.

CONCLUSION

This study represents the first compilation of hostplant and parasitoid interactions for European and North African Hesperidae relying on molecular techniques. It sets a reference for future studies seeking either to improve DNA barcode libraries or to elucidate food webs. As current DNA barcode reference libraries allow for the identification of most European butterflies, future efforts should be directed towards expanding coverage to neighbouring regions, such as North Africa. Nevertheless, some potential cases of cryptic butterfly species remain to be studied in the Western Palaearctic. In addition, future DNA barcoding projects should focus on natural enemies of butterflies, particularly parasitoid wasps, whose taxonomic framework and barcoding reference libraries are poorly developed. The increased application of DNA barcoding and other molecular techniques will undoubtedly extend our ability to understand ecological interactions in general, and host–parasitoid systems in particular, both by improving knowledge of parasitoid diversity and by increasing our capacity to construct interaction networks. Regardless, conducting field surveys and ecological observations will remain essential. Such progress will provide important knowledge for conservation, not only of the species themselves but also of their interactions.

ACKNOWLEDGEMENTS

We thank botanists Llorenç Sáez (Autonomous University of Barcelona) and Modesto Luceño (Pablo de Olavide University, Seville), who helped us in the identification of hostplants, as well as David M. Robertson for the identification of Tachinidae, and Ángel Blázquez, Peter Eeles, Tristan Lafranchis, Peter J.C. Russell, Miloš Popović and Constantí Stefanescu, for the samples contributed to the dataset. Support for this research was provided by the Spanish National Research Council (CSIC) with a JAE-Intro fellowship for the introduction to research to ETD (reference numbers JAEINT_20_00248 and JAEINT20_EX_0638) and by projects PID2019-107078GB-I00/MCIN/AEI/10.13039/501100011033 and 2017-SGR-991 (Generalitat de Catalunya) to RV, and PID2020-117739GA-I00/MCIN/AEI/10.13039/501100011033 to GT. We thank the Rachadaphiseksomphot Fund, Graduate School, Chulalongkorn University, for the award of a Senior Postdoctoral Fellowship to DLJQ. Further support for this research was provided by the Academy of Finland (Academy Research Fellow, decision no. 328895) to VD. VD also acknowledges the Visiting Professor Fellowship awarded by the Research Institute of the University of Bucharest. PDNH acknowledges support from Genome Canada through Ontario Genomics. BV has been funded by the CERCA Programme of the Generalitat de Catalunya and by the Grant RYC-22243-2017, whose PI is Josep Sardanyés. SV was supported by the Spanish Ministry of Economy and Competitiveness, grant PID2020-117822GB-I00 MINEICO/AEI/ FEDER and the European Union. We also thank Keith Willmott and an anonymous reviewer for their suggestions on the manuscript.

DATA AVAILABILITY

All sequences used in this study are available in the BOLD datasets DS-HESPPAR (Hesperidae sequences) and DS-HESPPARB (parasitoid sequences), which are publicly available (dx.doi.org/10.5883/DS-HESPPAR and dx.doi.org/10.5883/DS-HESPPARB).

REFERENCES

- Abdelfattah A, Malacrino A, Wisniewski M, Cacciola SO, Schena L. 2018. Metabarcoding: a powerful tool to investigate microbial communities and shape future plant protection strategies. *Biological Control* **120**: 1–10.
- Akrim F, Mahmood T, Max T, Nadeem MS, Qasim S, Andleeb S. 2018. Assessment of bias in morphological identification of carnivore scats confirmed with molecular scatology in north-eastern Himalayan region of Pakistan. *PeerJ* **2018**: e5262.

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403–410.
- Askew RR, Shaw MR. 2022. *Sympiesis notata* (Hymenoptera: Chalcidoidea: Eulophidae), a gregarious parasitoid of HesperIIDae (Lepidoptera), and some records of other Chalcidoidea associated with skipper butterflies in the Western Palearctic. *Entomologist's Monthly Magazine* **158**: 28–32.
- Beckett SJ, Williams HTP. 2013. Coevolutionary diversification creates nested-modular structure in phage–Bacteria interaction networks. *Interface Focus* **3**: 20130033.
- Bell KL, Fowler J, Burgess KS, Dobbs EK, Gruenewald D, Lawley B, Morozumi C, Brosi BJ. 2017. Applying pollen DNA metabarcoding to the study of plant–pollinator interactions. *Applications in Plant Sciences* **5**: 1600124.
- Bendiksby M, Thorbek L, Scheen AC, Lindqvist C, Ryding O. 2011. An updated phylogeny and classification of Lamiaceae subfamily Lamioideae. *Taxon* **60**: 471–484.
- Bergsten J, Bilton DT, Fujisawa T, Elliott M, Monaghan MT, Balke M, Hendrich L, Geijer J, Herrmann J, Foster GN, Ribera I, Nilsson AN, Barraclough TG, Vogler AP. 2012. The effect of geographical scale of sampling on DNA barcoding. *Systematic Biology* **61**: 851–869.
- Braga MP, Guimarães PR, Wheat CW, Nylín S, Janz N. 2018. Unifying host-associated diversification processes using butterfly–plant networks. *Nature Communications* **9**: 5155.
- Bunholi IV, da Ferrette BL S, De Biasi JB, de Magalhães C O, Rotundo MM, Oliveira C, Foresti F, Mendonça FF. 2018. The fishing and illegal trade of the angel shark: DNA barcoding against misleading identifications. *Fisheries Research* **206**: 193–197.
- Burtseva O, Kublanovskaya A, Fedorenko T, Lobakova E, Chekanov K. 2021. Gut microbiome of the White Sea fish revealed by 16S rRNA metabarcoding. *Aquaculture* **533**: 736175.
- Byng JW, Smets EF, Van Vugt R, Bidault E, Davidson C, Kenicer G, Chase MW, Christenhusz MJM. 2018. The phylogeny of angiosperms poster: a visual summary of APG IV family relationships and floral diversity. *The Global Flora* **4**: 4–7.
- Carl KP. 1968. *Thymelicus lineola* (Lepidoptera: HesperIIDae) and its parasites in Europe. *The Canadian Entomologist* **100**: 785–801.
- Dapporto L, Cini A, Vodá R, Dincă V, Wiemers M, Menchetti M, Magini G, Talavera G, Shreeve T, Bonelli S, CL Pietro, Balletto E, Scalercio S, Vila R. 2019. Integrating three comprehensive data sets shows that mitochondrial DNA variation is linked to species traits and paleogeographic events in European butterflies. *Molecular Ecology Resources* **19**: 1623–1636.
- DeWaard JR, Ivanova N V, Hajibabaei M, Hebert PD. 2008. Assembling DNA barcodes: analytical protocols. In: Cristofre M, ed. *Methods in molecular biology: environmental genetics*. Totowa: Humana Press, 275–293.
- Dincă V, Zakharov EV, Hebert PDN, Vila R. 2011. Complete DNA barcode reference library for a country's butterfly fauna reveals high performance for temperate Europe. *Proceedings of the Royal Society B: Biological Sciences* **278**: 347–355.
- Dincă V, Montagud S, Talavera G, Hernández-Roldán J, Munguira ML, García-Barros E, Hebert PDN, Vila R. 2015. DNA barcode reference library for Iberian butterflies enables a continental-scale preview of potential cryptic diversity. *Scientific Reports* **5**: 1–12.
- Dincă V, Bálint Z, Vodá R, Dapporto L, Hebert PDN, Vila R. 2018. Use of genetic, climatic, and microbiological data to inform reintroduction of a regionally extinct butterfly. *Conservation Biology* **32**: 828–837.
- Dincă V, Dapporto L, Somervuo P, Vodá R, Cuvelier S, Gascoigne-Pees M, Huemer P, Mutanen M, Hebert PDN, Vila R. 2021. High resolution DNA barcode library for European butterflies reveals continental patterns of mitochondrial genetic diversity. *Communications Biology* **4**: 315.
- Dobeš C, Paule J. 2010. A comprehensive chloroplast DNA-based phylogeny of the genus *Potentilla* (Rosaceae): implications for its geographic origin, phylogeography and generic circumscription. *Molecular Phylogenetics and Evolution* **56**: 156–175.
- Dupas S, Carton Y, Poiriè M. 2003. Genetic dimension of the coevolution of virulence–resistance in *Drosophila*–parasitoid wasp relationships. *Heredity* **90**: 84–89.
- Edger PP, Heidel-Fischer HM, Bekaert M, Rota J, Glöckner G, Platts AE, Heckel DG, Der JP, Wafula EK, Tang M, Hofberger JA, Smithson A, Hall JC, Blanchette M, Bureau TE, Wright SI, DePamphilis CW, Eric Schranz M, Barker MS, Conant GC, Wahlberg N, Vogel H, Pires JC, Wheat CW. 2015. The butterfly plant arms-race escalated by gene and genome duplications. *Proceedings of the National Academy of Sciences of the USA* **112**: 8362–8366.
- Eveleigh ES, McCann KS, McCarthy PC, Pollock SJ, Lucarotti CJ, Morin B, McDougall GA, Strongman DB, Huber JT, Umbanhowar J, Faria LDB. 2007. Fluctuations in density of an outbreak species drive diversity cascades in food webs. *Proceedings of the National Academy of Sciences of the USA* **104**: 16976–16981.
- Fernandez-Triana J, Shaw MR, Boudreault C, Beaudin M, Broad GR. 2020. Annotated and illustrated world checklist of Microgastrinae parasitoid wasps (Hymenoptera, Braconidae). *ZooKeys* **920**: 1–1090.
- Flores CO, Meyer JR, Valverde S, Farr L, Weitz JS. 2011. Statistical structure of host-phage interactions. *Proceedings of the National Academy of Sciences of the USA* **108**: E288–E297.
- Fortuna MA, Barbour MA, Zaman L, Hall AR, Buckling A, Bascompte J. 2019. Coevolutionary dynamics shape the structure of bacteria-phage infection networks. *Evolution* **73**: 1001–1011.
- Goldstein PZ, DeSalle R. 2011. Integrating DNA barcode data and taxonomic practice: determination, discovery, and description. *BioEssays* **33**: 135–147.
- González-Varo JP, Arroyo JM, Jordano P. 2014. Who dispersed the seeds? The use of DNA barcoding in frugivory and seed dispersal studies (M Gilbert, Ed.). *Methods in Ecology and Evolution* **5**: 806–814.

- Greeney HF. 2009.** A revised classification scheme for larval hesperiid shelters, with comments on shelter diversity in the Pyrginae. *Journal of Research on the Lepidoptera* **41**: 53–59.
- Greeney HF, Jones MT. 2003.** Shelter building in the HesperIIDae: a classification scheme for larval shelters. *Journal of Research on the Lepidoptera* **37**: 27–36.
- Gripenberg S, Hamer N, Brereton T, Roy DB, Lewis OT. 2011.** A novel parasitoid and a declining butterfly: cause or coincidence? *Ecological Entomology* **36**: 271–281.
- Gupta A, Fernández-Triana JL. 2014.** Diversity, host association, and cocoon variability of reared Indian Microgastrinae (Hymenoptera: Braconidae). *Zootaxa* **3800**: 1–101.
- Hajek AE, Hurley BP, Kenis M, Garnas JR, Bush SJ, Wingfield MJ, Van Lenteren JC, Cock MJW. 2016.** Exotic biological control agents: a solution or contribution to arthropod invasions? *Biological Invasions* **18**: 953–969.
- Hausmann A, Haszprunar G, Segerer AH, Speidel W, Behounek G, Hebert PDN. 2011.** Now DNA-barcoded: the butterflies and larger moths of Germany. *Spixiana* **34**: 47–58.
- Hawkins BA. 1994.** *Pattern and process in host-parasitoid interactions*. Cambridge: Cambridge University Press.
- Hebert PDN, Cywinska A, Ball SL, DeWaard JR. 2003.** Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* **270**: 313–321.
- Hebert PDN, deWaard JR, Zakharov E V, Prosser SWJ, Sones JE, McKeown JTA, Mantle B, La Salle J. 2013.** A DNA ‘Barcode Blitz’: rapid digitization and sequencing of a natural history collection. *PLoS One* **8**: e68535.
- Hernández-Roldán JL, Munguira ML, Wagner W, Vila R. 2012.** Comparative analysis and taxonomic use of the morphology of immature stages and natural history traits in European species of *Pyrgus* Hübner (Lepidoptera: HesperIIDae, Pyrginae). *Zootaxa* **3470**: 1–71.
- Hernández-Roldán JL, Dapporto L, Dincă V, Vicente JC, Hornett EA, Šíchová J, Lukhtanov VA, Talavera G, Vila R. 2016.** Integrative analyses unveil speciation linked to hostplant shift in *Spialia* butterflies. *Molecular Ecology* **25**: 4267–4284.
- Hernández-Roldán JL, Vicente JC, Vila R, Munguira ML. 2018.** Natural history and immature stage morphology of *Spialia* Swinhoe, 1912 in the Iberian Peninsula (Lepidoptera, HesperIIDae). *Nota Lepidopterologica* **41**: 1–22.
- Hinojosa JC, Koubínová D, Szenteczki MA, Pitteloud C, Dincă V, Alvarez N, Vila R. 2019.** A mirage of cryptic species: genomics uncover striking mitonuclear discordance in the butterfly *Thymelicus sylvestris*. *Molecular Ecology* **28**: 3857–3868.
- Hinojosa JC, Dapporto L, Brockmann E, Dincă V, Tikhonov V, Grishin N, Lukhtanov VA, Vila R. 2021.** Overlooked cryptic diversity in *Muschampia* (Lepidoptera: HesperIIDae) adds two species to the European butterfly fauna. *Zoological Journal of the Linnean Society* **193**: 847–859.
- Hrček J, Godfray HCJ. 2015.** What do molecular methods bring to host–parasitoid food webs? *Trends in Parasitology* **31**: 30–35.
- Ivanova NV, DeWaard JR, Hebert PDN. 2006.** An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes* **6**: 998–1002.
- Jurado-Rivera JA, Vogler AP, Reid CAM, Petitpierre E, Gómez-Zurita J. 2009.** DNA barcoding insect–hostplant associations. *Proceedings of the Royal Society B: Biological Sciences* **276**: 639–648.
- Kaartinen R, Stone GN, Hearn J, Lohse K, Roslin T. 2010.** Revealing secret liaisons: DNA barcoding changes our understanding of food webs. *Ecological Entomology* **35**: 623–638.
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018.** MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**: 1547–1549.
- Lafferty KD, Kuris AM. 2002.** Trophic strategies, animal diversity and body size. *Trends in Ecology & Evolution* **17**: 507–513.
- Li B, Cantino PD, Olmstead RG, Bramley GLC, Xiang CL, Ma ZH, Tan YH, Zhang DX. 2016.** A large-scale chloroplast phylogeny of the Lamiaceae sheds new light on its subfamilial classification. *Scientific Reports* **6**: 1–18.
- Litman J, Chittaro Y, Birrer S, Praz C, Wermeille E, Fluri M, Stalling T, Schmid S, Wyler S, Gonseth Y. 2018.** A DNA barcode reference library for Swiss butterflies and forester moths as a tool for species identification, systematics and conservation. *PLoS One* **13**: e0208639.
- Lukhtanov VA, Sourakov A, Zakharov EV, Hebert PDN. 2009.** DNA barcoding Central Asian butterflies: increasing geographical dimension does not significantly reduce the success of species identification. *Molecular Ecology Resources* **9**: 1302–1310.
- Mathiesen C, Scheen AC, Lindqvist C. 2011.** Phylogeny and biogeography of the lamioid genus *Phlomis* (Lamiaceae). *Kew Bulletin* **66**: 83–99.
- Menchetti M, Talavera G, Cini A, Salvati V, Dincă V, Platania L, Bonelli S, Balletto E, Vila R, Dapporto L. 2021.** Two ways to be endemic. Alps and Apennines are different functional refugia during climatic cycles. *Molecular Ecology* **30**: 1297–1310.
- Moran Z, Orth DJ, Schmitt JD, Hallerman EM, Aguilar R. 2015.** Effectiveness of DNA barcoding for identifying piscine prey items in stomach contents of piscivorous catfishes. *Environmental Biology of Fishes* **99**: 161–167.
- Nobile AB, Freitas-Souza D, Ruiz-Ruano FJ, Nobile MLM, Costa GO, De Lima FP, Camacho JPM, Foresti F, Oliveira C. 2019.** DNA metabarcoding of Neotropical ichthyoplankton: enabling high accuracy with lower cost. *Metabarcoding and Metagenomics* **3**: 69–76.
- Northover AS, Lymbery AJ, Wayne AF, Godfrey SS, Thompson RCA. 2018.** The hidden consequences of altering host–parasite relationships during fauna translocations. *Biological Conservation* **220**: 140–148.
- Nylin S, Agosta S, Bensch S, Boeger W, Braga MP, Brooks DR, Forister ML, Hambäck PA, Hoberg EP, Nyman T, Schäpers A, Stigall AL, Wheat CW, Österling M, Janz N. 2018.** Embracing colonizations: a new paradigm for species association dynamics. *Trends in Ecology and Evolution* **33**: 4–14.
- Obregón R, Shaw MR, Fernández-Haeger J, Jordano D. 2015.** Parasitoid and ant interactions of some Iberian

- butterflies (Insecta: Lepidoptera). *Shilap. Revista de Lepidopterologia* **43**: 439–454.
- Paynter Q, Fowler S V, Hugh Gourlay A, Groenteman R, Peterson PG, Smith L, Winks CJ. 2010.** Predicting parasitoid accumulation on biological control agents of weeds. *Journal of Applied Ecology* **47**: 575–582.
- Peoples BK, Cooper P, Frimpong EA, Hallerman EM. 2017.** DNA barcoding elucidates cyprinid reproductive interactions in a southwest Virginia stream. *Transactions of the American Fisheries Society* **146**: 84–91.
- Persson NL, Toresen I, Andersen HL, Smedmark JEE, Eriksson T. 2020.** Detecting destabilizing species in the phylogenetic backbone of *Potentilla* (Rosaceae) using low-copy nuclear markers. *AoB Plants* **12**: plaa017.
- Pires MM, Guimarães PR. 2013.** Interaction intimacy organizes networks of antagonistic interactions in different ways. *Journal of the Royal Society Interface* **10**: 20120649.
- Pitteloud C, Arrigo N, Suchan T, Mastretta-yanes A, Herna J, Brockmann E, Vila R, Dincă V, Chittaro Y, Kleckova I, Fumagalli L, Buerki S. 2017.** Climatic niche evolution is faster in sympatric than allopatric lineages of the butterfly genus *Pyrgus*. *Proceedings of the Royal Society B: Biological Sciences* **284**: 20170208.
- Potter D, Eriksson T, Evans RC, Oh S, Smedmark JEE, Morgan DR, Kerr M, Robertson KR, Arsenault M, Dickinson TA, Campbell CS. 2007.** Phylogeny and classification of Rosaceae. *Plant Systematics and Evolution* **266**: 5–43.
- POWO. 2022.** Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; <http://www.plantsoftheworldonline.org/> Retrieved 08 June 2022.
- Quicke DLJ. 2015.** *The braconid and ichneumonid parasitoid wasps: biology, systematics, evolution and ecology*. Chichester: John Wiley & Sons.
- Quicke DLJ, Smith MA, Janzen DH, Hallwachs W, Fernandez-Triana J, Laurence NM, Zaldívar-Riverón A, Shaw MR, Broad GR, Klopstein S, Shaw SR, Hrcek J, Hebert PDN, Miller SE, Rodriguez JJ, Whitfield JB, Sharkey MJ, Sharanowski BJ, Jussila R, Gauld ID, Chesters D, Vogler AP. 2012.** Utility of the DNA barcoding gene fragment for parasitic wasp phylogeny (Hymenoptera: Ichneumonoidea): data release and new measure of taxonomic congruence. *Molecular Ecology Resources* **12**: 676–685.
- Ratnasingham S, Hebert PDN. 2007.** BOLD: the barcode of life data system (<http://www.barcodinglife.org>). *Molecular Ecology Notes* **7**: 355–364.
- Ratnasingham S, Hebert PDN. 2013.** A DNA-based registry for all animal species: the Barcode Index Number (BIN) system. *PLoS One* **8**: e66213.
- Rougerie R, Smith MA, Fernandez-Triana J, Lopez-Vaamonde C, Ratnasingham S, Hebert PDN. 2011.** Molecular analysis of parasitoid linkages (MAPL): gut contents of adult parasitoid wasps reveal larval host. *Molecular Ecology* **20**: 179–186.
- Sharkey MJ, Janzen DH, Hallwachs W, Chapman EG, Smith MA, Dapkey T, Brown A, Ratnasingham S, Naik S, Manjunath R, Perez K, Milton M, Hebert P, Shaw SR, Kittel RN, Solis MA, Metz MA, Goldstein PZ, Brown JW, Quicke DLJ, Van Achterberg C, Brown BV, Burns JM. 2021.** Minimalist revision and description of 403 new species in 11 subfamilies of Costa Rican braconid parasitoid wasps, including host records for 219 species. *ZooKeys* **1013**: 1–665.
- Shaw MR. 1997.** Rearing parasitic Hymenoptera. *Amateur Entomologists' Society* **25**: 1–46.
- Shaw MR. 2012.** Notes on some European Microgastrinae (Hymenoptera: Braconidae) in the National Museums of Scotland, with twenty species new to Britain, new host data, taxonomic changes and remarks, and descriptions of two new species of *Microgaster* Latreille. *Entomologists' Gazette* **63**: 173–201.
- Shaw MR, Hochberg ME. 2001.** The neglect of parasitic Hymenoptera in insect conservation strategies: the British fauna as a prime example. *Journal of Insect Conservation* **5**: 253–263.
- Shaw MR, Stefanescu C, Van Nouhuys S. 2009.** Parasitoids of European butterflies. In: Settele J, Van Dyck H, Shreeve T, Konvička M, eds. *Ecology of butterflies in Europe*. Cambridge: Cambridge University Press, 130–156.
- Shaw MR, Horstmann K, Whiffin AL. 2016.** Two hundred and twenty-five species of reared western Palearctic Campopleginae (Hymenoptera: Ichneumonidae) in the National Museums of Scotland, with descriptions of new species of *Campoplex* and *Diadegma*, and records of fifty-five species new to Britain. *Entomologist's Gazette* **67**: 177–222.
- Smith MA, Rodriguez JJ, Whitfield JB, Deans AR, Janzen DH, Hallwachs W, Hebert PDN. 2008.** Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. *Proceedings of the National Academy of Sciences of the USA* **105**: 12359–12364.
- Smith MA, Fernández-Triana JL, Eveleigh E, Gómez J, Guclu C, Hallwachs W, Hebert PDN, Hrcek J, Huber JT, Janzen D, Mason PG, Miller S, Quicke DLJ, Rodriguez JJ, Rougerie R, Shaw MR, Várkonyi G, Ward DF, Whitfield JB, Zaldívar-Riverón A. 2013.** DNA barcoding and the taxonomy of Microgastrinae wasps (Hymenoptera, Braconidae): impacts after 8 years and nearly 20 000 sequences. *Molecular Ecology Resources* **13**: 168–176.
- Suchan T, Talavera G, Sáez L, Ronikier M, Vila R. 2019.** Pollen metabarcoding as a tool for tracking long-distance insect migrations. *Molecular Ecology Resources* **19**: 149–162.
- R Core Team. 2021.** *R: a language and environment for statistical computing*. Vienna: R Core Team.
- Talavera G, Lukhtanov V, Pierce NE, Vila R. 2022.** DNA barcodes combined with multi-locus data of representative taxa can generate reliable higher-level phylogenies. *Systematic Biology* **71**: 382–395.
- Thies C, Steffan-Dewenter I, Tscharrntke T. 2003.** Effects of landscape context on herbivory and parasitism at different spatial scales. *Oikos* **101**: 18–25.
- Tshikolovets VV. 2011.** *Butterflies of Europe and the Mediterranean area*. Kyiv: Tshikolovets Publications.

- Valiente-Banuet A, Aizen MA, Alcántara JM, Arroyo J, Cocucci A, Galetti M, García MB, García D, Gómez JM, Jordano P, Medel R, Navarro L, Obeso JR, Oviedo R, Ramírez N, Rey PJ, Traveset A, Verdú M, Zamora R. 2015.** Beyond species loss: the extinction of ecological interactions in a changing world. *Functional Ecology* **29**: 299–307.
- Valverde S, Vidiella B, Montañez R, Fraile A, Sacristán S, García-Arenal F. 2020.** Coexistence of nestedness and modularity in host–pathogen infection networks. *Nature Ecology and Evolution* **4**: 568–577.
- Van der Linden CFH, Wallis de Vries MF, Simon S. 2021.** Great chemistry between us: the link between plant chemical defenses and butterfly evolution. *Ecology and Evolution* **11**: 8595–8613.
- Van Loon JJA, De Boer JG, Dicke M. 2000.** Parasitoid–plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomologia Experimentalis et Applicata* **97**: 219–227.
- Van Nouhuys S, Hanski I. 2000.** Apparent competition between parasitoids mediated by a shared hyperparasitoid. *Ecology Letters* **3**: 82–84.
- Van Swaay C, Wynhoff I, Verovnik R, Wiemers M, López Munguira M, Maes D, Sasic M, Verstrael T, Warren M, Settle J. 2010.** *Pyrgus cirsii*. *The IUCN Red List of Threatened Species 2010*: e.T39481A10240539. Available from: <https://doi.org/10.2305/IUCN.UK.2010-1.RLTS.T39481A10240539.en> (accessed 25 May 2022).
- Wang JF, Qiao GX. 2009.** DNA barcoding of genus *Toxoptera* Koch (Hemiptera: Aphididae): Identification and molecular phylogeny inferred from mitochondrial *COI* sequences. *Insect Science* **16**: 475–484.
- Wiemers M, Fiedler K. 2007.** Does the DNA barcoding gap exist? A case study in blue butterflies (Lepidoptera: Lycaenidae). *Frontiers in Zoology* **4**: 81–16.
- Wiemers M, Balletto E, Dincă V, Fric ZF, Lamas G, Lukhtanov V, Munguira ML, Van Swaay CAM, Vila R, Vliegenthart A, Wahlberg N, Verovnik R. 2018.** An updated checklist of the European butterflies (Lepidoptera, Papilionoidea). *ZooKeys* **811**: 9–45.
- Wirta HK, Hebert PDN, Kaartinen R, Prosser SW, Várkonyi G, Roslin T. 2014.** Complementary molecular information changes our perception of food web structure. *Proceedings of the National Academy of Sciences of the USA* **111**: 1885–1890.
- Yu G. 2020.** Using ggtree to visualize data on tree-like structures. *Current Protocols in Bioinformatics* **69**: e96.
- Yu G, Smith DK, Zhu H, Guan Y, Lam TTY. 2017.** Ggtree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods in Ecology and Evolution* **8**: 28–36.
- Yu G, Lam TTY, Zhu H, Guan Y. 2018.** Two methods for mapping and visualizing associated data on phylogeny using ggtree. *Molecular Biology and Evolution* **35**: 3041–3043.
- Zeng L, Zhang N, Zhang Q, Endress PK, Huang J, Ma H. 2017.** Resolution of deep eudicot phylogeny and their temporal diversification using nuclear genes from transcriptomic and genomic datasets. *New Phytologist* **214**: 1338–1354.
- Zhang J, Brockmann E, Cong Q, Shen J, Grishin NV. 2020.** A genomic perspective on the taxonomy of the subtribe Carcharodina (Lepidoptera: Hesperidae: Carcharodini). *Zootaxa* **4748**: 182–194.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. List of ecological interactions involving Hesperidae presented in this study.

Table S2. Minimum interspecific distances (p -distance) among parasitoid species, expressed as percentage.

Table S3. Number of host species and host genera per parasitoid species. For each parasitoid, host species were only counted if they had a species-level identification or if there was no other host species in that genus.

Table S4. Number of parasitoid species and genera per hesperiid species. For each hesperiid, parasitoid species were only counted when they had a species-level identification or, if they had an identification above the species level, when there were no other parasitoids in that taxonomic group.

Table S5. Minimum congeneric interspecific distances (p -distance) among Hesperidae.

Table S6. Discordant BINs obtained from the BIN discordance analysis.

Table S7. Concordant BINs obtained from the BIN discordance analysis.

Figure S1. A–I, photographs of the samples used for this study.

Figure S2. Neighbour-joining tree based on uncorrected p -distances of the parasitoid barcodes. Branch labels correspond to bootstrap support values (expressed over 1).

Figure S3. Modularity and nestedness values for the Hesperidae–hostplants, Hesperidae–parasitoids and hostplants–parasitoids interaction networks. The grey bars represent the distribution of values obtained from randomized networks; the red bar indicates the value of the actual network.

Figure S4. Neighbour-joining tree based on uncorrected p -distances of the hesperiid barcodes. Branch labels correspond to bootstrap support values (expressed over 1). Sequences corresponding to the specimens in the table of interactions are highlighted in bold.