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

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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 Hesperiidae 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 Hesperiidae, but success was lower for parasitoids, in part due to unresolved taxonomy. Potential cases of cryptic diversity, both in Hesperiidae 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.

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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

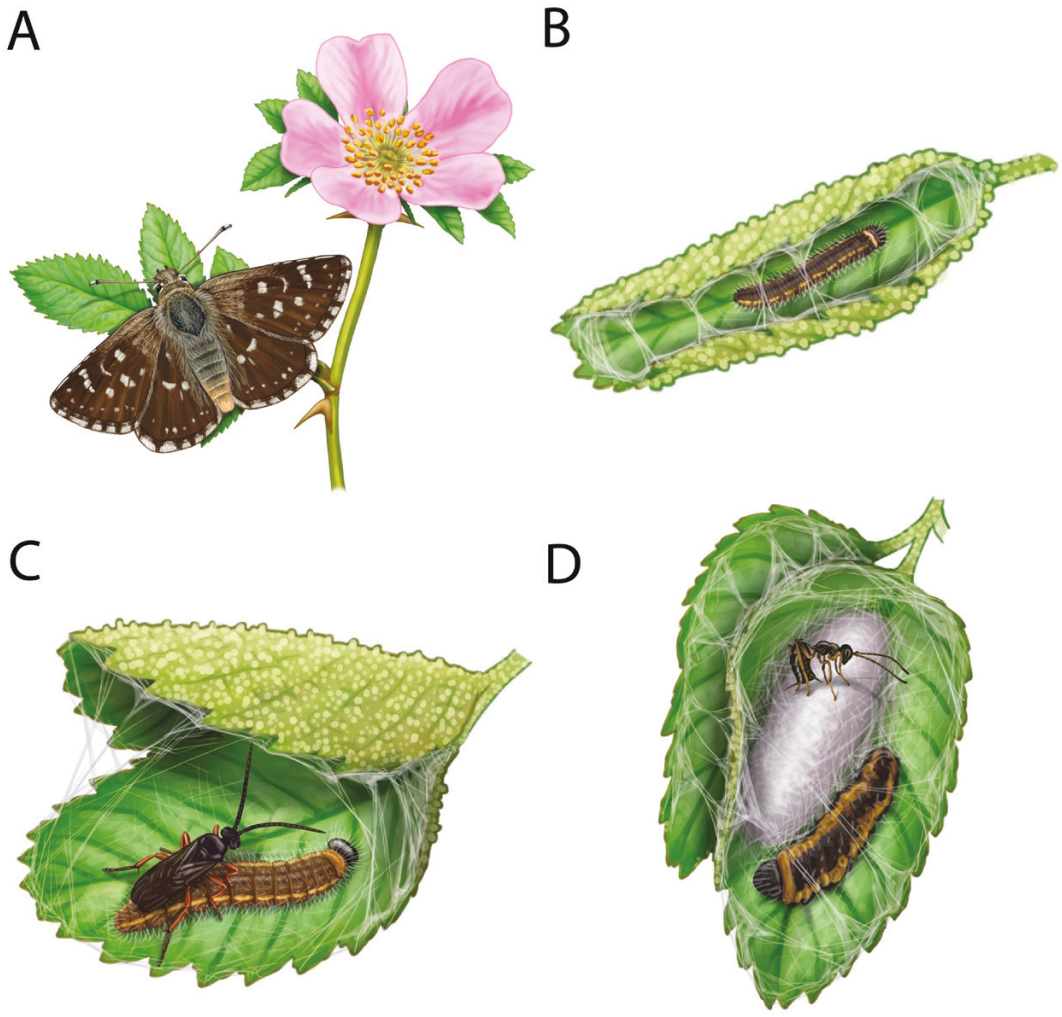

Figure 1. Representation of the study system. Hesperiid larvae feeding on their hostplants can be attacked by a number of parasitoids, which can in turn be attacked by various hyperparasitoids. A, *Spialia rosae* on its hostplant *Rosa sicula*. B, third instar larva of *Sp. rosae* on a silk shelter. 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 hostplant 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, hesperiids 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 hesperiids and their parasitoids were barcoded. The following protocol was carried out at the Institute of Evolutionary Biology (Barcelona, Spain) for both hesperiids 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 $MgCl_2$ 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	5′ ATTCAACCAATCATAAAGATATTGGAAC 3′
	LepR1	5′ TAAACTTCTGGATGTCCAAAAAATCA 3′
Lepidoptera, Hymenoptera	LepF1	5′ ATTCAACCAATCATAAAGATATTGG 3′
	LepR1	5′ TAAACTTCTGGATGTCCAAAAAATCA 3′
Hymenoptera	LCOpar	5′ GGTCAACAAATCATAAAGATATTGGKAT 3′
	Nancy	5′ CCCGGTAAAATTAAAATATAAACTTC 3′
Diptera*, Nematomorpha*	LCO1490	5′ GGTCAACAAATCATAAAGATATTGG 3′
	HCO	5′ TAAACTTCAGGGTGACCAAAAAATCA 3′

available (dx.doi.org/10.5883/DS-HESPPAR and dx.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; Bendiksby *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 where 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 Hesperiidae 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 Hesperiidae 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 Hesperiidae 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 Hesperiinae 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 Hesperiidae–hostplant network showed a clearly modular structure (Fig. 4A), with a cluster

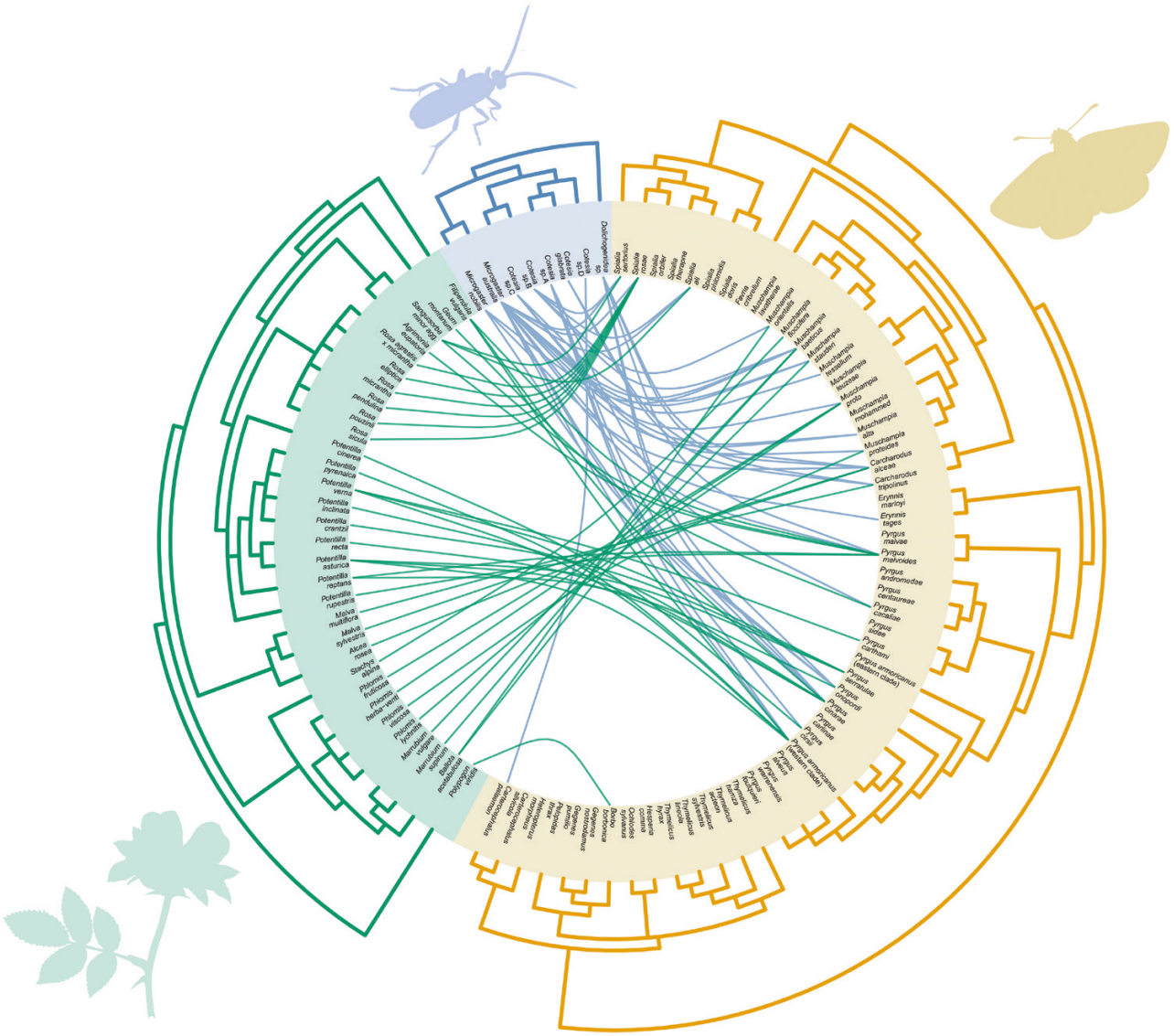

Figure 2. Circular cladogram showing ecological interactions among European and North African Hesperiidae, their hostplants, and their microgastrine parasitoids, recovered through DNA barcoding for Hesperiidae and/or parasitoids. Hesperiid, parasitoid and plant cladograms are coloured in orange, blue 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 hesperiids. 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

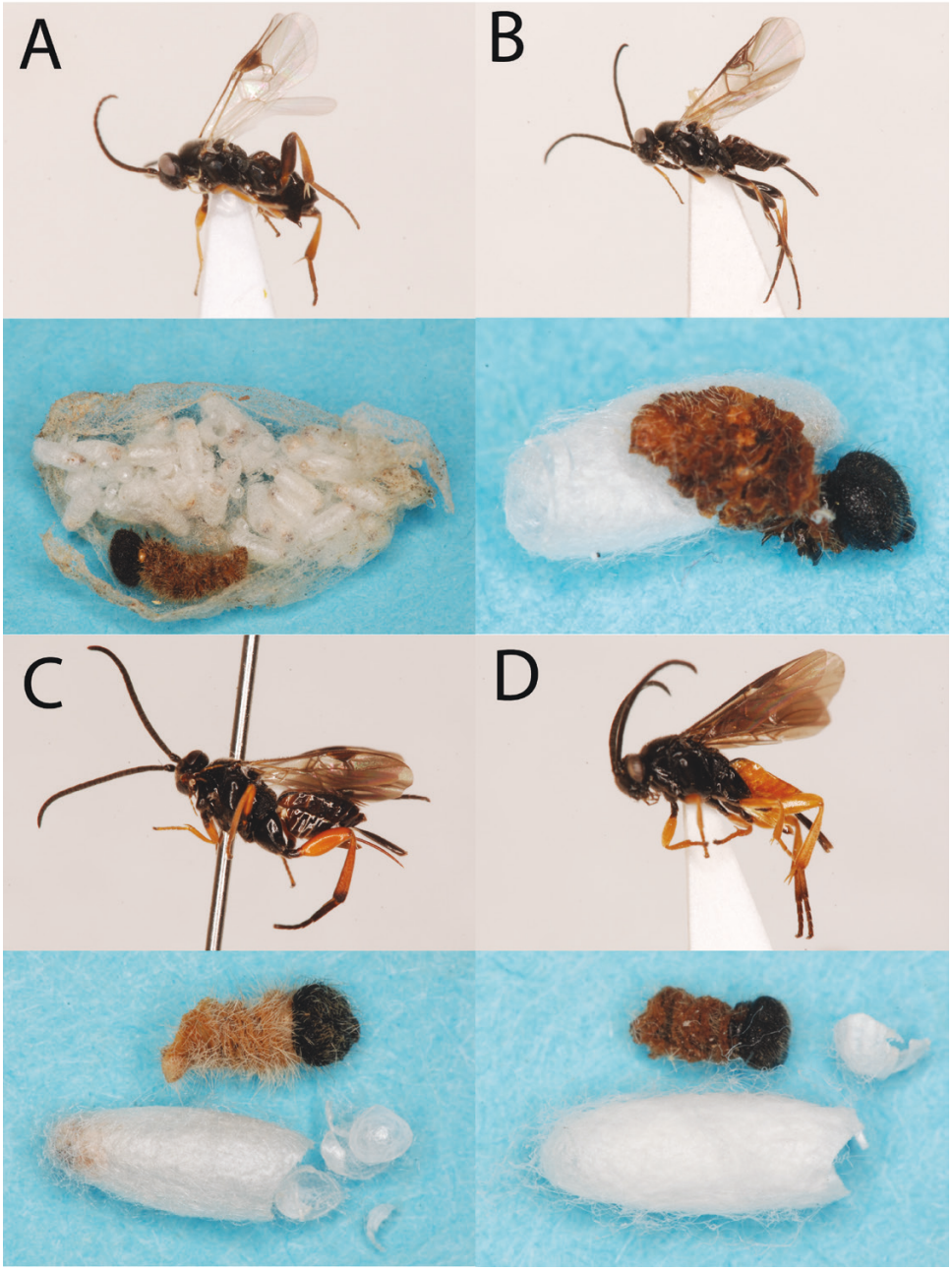

Figure 3. Mounted specimens illustrating the species of Microgastrinae recovered in this study. A, *Cotesia glabrata* Telenga ex *Carcharodus alceae*, Italy. Adult plus cocoons. Gregarious parasitoid; brood sizes vary considerably, host usually well grown or prepupal when killed. The other *Cotesia* 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

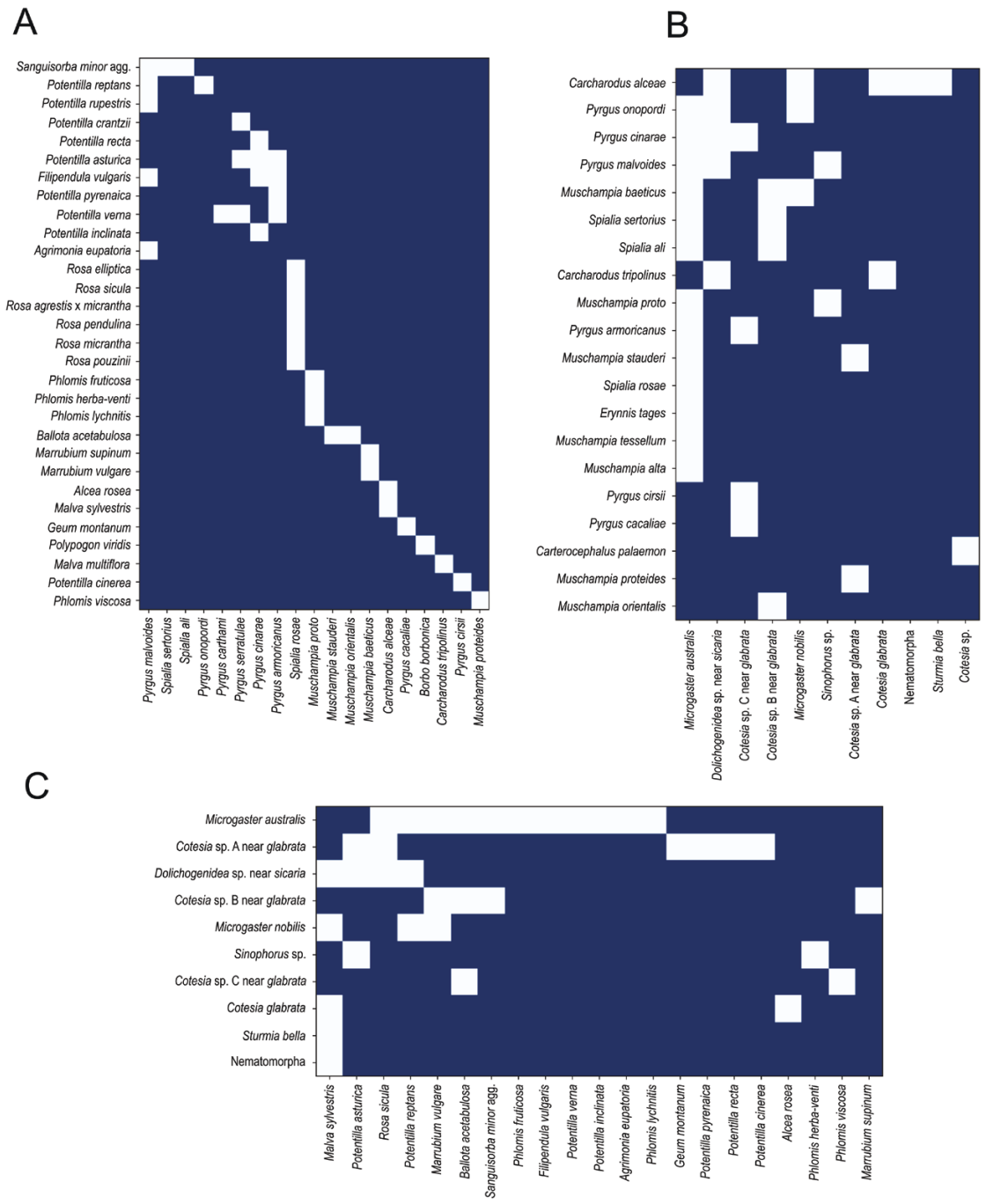

Figure 4. Interaction matrices showing the recorded interactions of Hesperiidae and their hostplants (A), Hesperiidae and their parasitoids (B) and parasitoids and hostplants of Hesperiidae (C). White squares indicate recorded interactions between the taxa in the corresponding row and column, while blue 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 (Hesperiinae and Heteropterinae), which feed predominantly on grasses. However, European species of Hesperiinae 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 Hesperiinae. 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 Hesperiinae 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 hesperiids 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 Hesperiidae-parasitoid interactions are highlighted in bold

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

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DATA AVAILABILITY

All sequences used in this study are available in the BOLD datasets DS-HESPPAR (Hesperiidae 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).

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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 Hesperiidae 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 Hesperiidae.
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 Hesperiidae–hostplants, Hesperiidae–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.