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Host specialization by *Cotesia* wasps (Hymenoptera: Braconidae) parasitizing species-rich Melitaeini (Lepidoptera: Nymphalidae) communities in north-eastern Spain

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In order to investigate parasitoids of the genus *Cotesia* (Hymenoptera: Braconidae), larvae of a speciose group of butterflies, the tribe Melitaeini (Lepidoptera: Nymphalidae), were collected from several sites in Catalonia, northern Spain, a region that harbours ten out of the 20 European species of Melitaeini. New information on the natural history of the butterflies is presented, and the structure of their communities and patterns of larval parasitism are described. On the basis of mtDNA sequence data (COI gene), microsatellite data (ten loci) and behavioural experiments, we recognize seven biologically distinct species of *Cotesia* parasitizing the Melitaeini communities within this relatively small geographical area. In particular, the notional species *C. melitaearum* and *C. acuminata* each represents a series of cryptic species with narrow host associations. The possibility of direct competition among the parasitoids and/or indirect interactions between butterflies mediated by *Cotesia* parasitoids is explored. *Biological Journal of the Linnean Society*, 2005, **86**, 45–65.

ADDITIONAL KEYWORDS: checkerspot – community structure – cryptic species – microsatellites – mtDNA – natural history – parasitoids – speciation.

INTRODUCTION

The nymphalid tribe Melitaeini comprises a biologically as well as taxonomically compact group of about 250 butterfly species that are found throughout the Holarctic and Neotropical regions (Higgins, 1981; Kons, 2000; Wahlberg & Zimmermann, 2000). Adults of most species are relatively sedentary and females usually lay eggs in clusters. They diapause during climatic extremes (summer or winter) as larvae and tend to live gregariously in silk nests for at least the first few instars (Kuussaari *et al*., 2004). The larvae feed on just a few plant families belonging to the subclass Asteridae (*sensu* Olmstead *et al*., 1993). Although over its geographical range a single butterfly species may use many plant species in several genera, larvae are generally locally restricted to one or a few species (Singer, 2004).

There are records of egg, larval and pupal parasitism of Melitaeini by both hymenopteran and dipteran parasitoids (van Nouhuys & Hanski, 2004). While some records may be questionable or reflect only an incidental relationship, in general it is evident that the parasitoid complexes associated with Melitaeini

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largely involve specialized primary parasitoids, particularly in the genus *Cotesia* (Hymenoptera: Braconidae: Microgastrinae), that may have a central role in the population dynamics and larval ecology of their hosts. The *Cotesia* species that use Melitaeini butterfly hosts are restricted to that group (van Nouhuys & Hanski, 2004).

The interactions between several species of Melitaeini and their parasitoids have been studied in detail. These include *Euphydryas editha* (Boisduval) (White, 1973; Moore, 1989a, b) and *E. phaeton* (Drury) (Stamp, 1981a, b, 1982) in North America, and *E. aurinia* (Rottemburg) (Ford & Ford, 1930; Porter, 1981, 1983; Eliasson & Shaw, 2003), *E. maturna* (L.) (Eliasson & Shaw, 2003) and *Melitaea cinxia* (L.) (Lei *et al*., 1997; van Nouhuys & Hanski, 2004) in Europe. In most of these cases parasitoids in the genus *Cotesia* are a dominant part of the parasitoid community. Multiple generations of *Cotesia* occur during a single (often univoltine) host generation, forming small broods of typically 1–5 individuals from early instar hosts and of about 12–70 (depending on the *Cotesia* species) when using the later instars. In these systems the high intrinsic rate of population increase of the parasitoid, together with its narrow host range, can lead to great fluctuations of both the parasitoid and host population sizes (Lei & Hanski, 1997; van Nouhuys & Hanski, 2004).

To date, in-depth studies on parasitism of Melitaeini butterflies have focused on areas where only one or a few hosts co-occur (but see Eliasson & Shaw, 2003). However, there are many localities in southern Europe and Asia where more than five Melitaeini species may co-occur, sharing the same or overlapping habitats, or even the same food-plant species (e.g. Komonen, 1998; Wahlberg, Kullberg & Hanski, 2001). In these communities there is the potential for the population dynamics of Melitaeini species to be linked with one another through shared food plants and shared parasitoids (van Nouhuys & Hanski, 2004, 2005). The degree to which this occurs may offer clues to the ecological and evolutionary processes involved in host specialization. While food-plant specialization by butterflies and other herbivores has been extensively studied (Ehrlich & Raven, 1964; Futuyma, 1991; Bernays & Chapman, 1994; Janz & Nylin, 1998), there is very little understanding of the ecological and evolutionary context of host specialization by parasitoids (Godfray, 1994; Shaw, 1994).

The butterfly fauna of Catalonia, in north-eastern Spain, is particularly rich (Martín & Gurrea, 1990; Dennis & Williams, 1995; Stefanescu, Herrando & Páramo, 2004) and contains ten out of the 20 European species of Melitaeini (Tolman & Lewington, 1997), many of them co-occurring at some localities, providing an excellent setting in which to investigate community interactions. One aim of the present study was to characterize these communities, looking also at the phenology and food-plant associations of the butterflies, as little systematic research on them has yet been carried out in southern Europe. Another aim was to elucidate the ecology of the community as a whole, in particular to investigate evidence for direct and indirect competitive interactions.

On the basis of DNA sequence data (mtDNA COI and NADH1 genes, and nuclear ITS2 region) and 12 microsatellite loci, Kankare & Shaw (2004) surveyed the *Cotesia* populations associated with species of Melitaeini on a Eurasian scale (including some samples drawn from the present study). That study showed that two major clades exist in Europe, the first including the nominal taxa *C. acuminata* (Reinhard) and *C. bignellii* (Marshall), and the second including *C. melitaearum* (Wilkinson) and *C. lycophron* (Nixon). These two clades are morphologically dissimilar and almost certainly colonized Melitaeini independently. However, within each clade, Kankare & Shaw (2004) advanced evidence for the existence of cryptic species each having relatively narrow host ranges – that is, several species that have been lumped into each of the *C. acuminata* and *C. melitaearum* morphospecies (here called *C. acuminata* agg. and *C. melitaearum* agg.).

The evidence for the existence of these cryptic species – derived from phylogenetic analyses based on DNA data – is essentially that over wide geographical areas of Eurasia, *Cotesia* specimens reared from particular Melitaeini species, or from the same small groups of host species, consistently group together. Accompanying morphological investigations have provided support for these segregates to varying degrees. In some cases differences were fairly easy to see, while in others a reliable expression of morphological characters to separate them remains elusive (M. R. Shaw, unpubl. data).

In this paper, we present data focusing on these genetically distinct *Cotesia* segregates in a relatively small geographical area that should pose little physical barrier to gene flow; that is, under circumstances in which genuinely isolated biological species should be easiest to detect and characterize ecologically. Experimental data on the behaviour of adult female *Cotesia* towards Melitaeini larvae of species other than those from which the *Cotesia* were reared are also included. Once we distinguish between the cryptic species, the ecological structure of the Melitaeini–*Cotesia* system as a whole is addressed.

MATERIAL AND METHODS

STUDY SITES, SAMPLING AND REARING

Melitaeini larvae were collected from 17 sites in Catalonia, NE Spain (Fig. 1), during the spring and

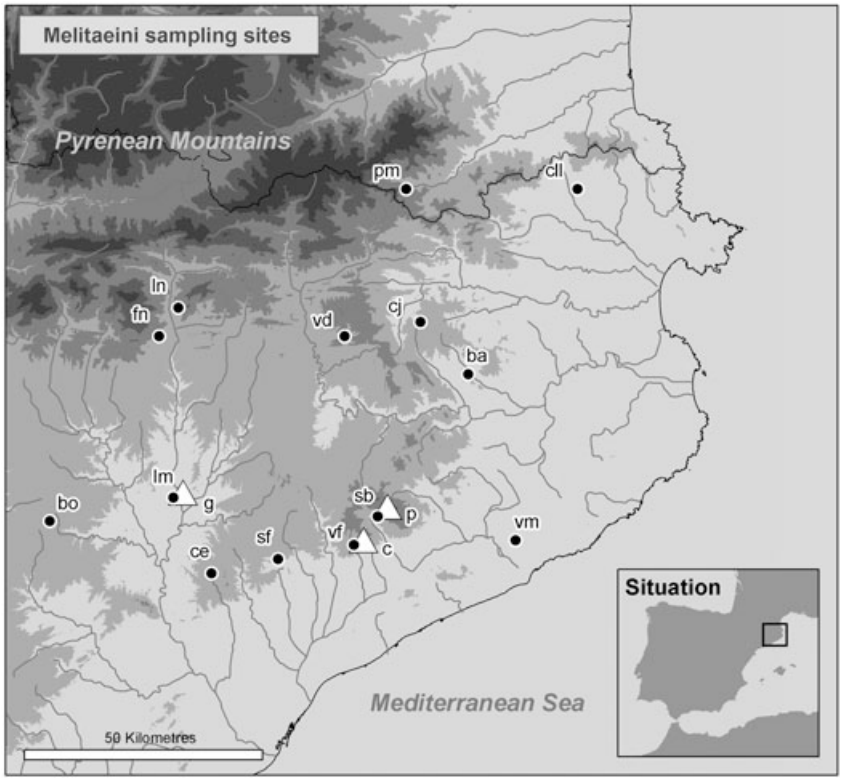

Figure 1. Map showing the sampling locations in Catalonia. Main sites (△): p, El Puig; c, El Cortès; g, El Guix. Secondary sites (●): bo, Boixadors; vm, Bosc de Valldemaria; cj, Can Jordà; cll, Cantallops; ce, Coll d'Estenalles; fn, Font Negra; ba, La Barroca; lm, La Malesa; ln, La Nou de Berguedà; pm, Prats de Molló; sb, Sant Bernat; sf, Sant Feliu de Codines; vf, Vallforners; vd, Vidrà.

summer of 2001, 2002 and 2003. Three areas were sampled intensively during 2002 (henceforward referred to as 'main sites'; Table 1). Each main site consisted of a network of habitat patches occupied by Melitaeini butterflies that are likely to persist as metapopulations (*sensu* Wahlberg *et al*., 2002). Information on butterfly species composition and abundance was available for the communities occurring at these sites because they have been regularly monitored for a number of years (El Puig: 10 years; El Guix and El Cortès: 4 years) as part of the Catalan Butterfly Monitoring Scheme (Stefanescu, 2000).

We visited each of the main sites periodically from February to September 2002 to collect as many larvae of each of the Melitaeini species as we could find, by searching for them on known and likely food plant species (Wahlberg, 2001). The extended sampling period allowed us to collect both prediapause and postdiapause larvae of most species, as well as larvae from different generations in the case of multivoltine butterflies. Additional material was collected from 14 other sites (henceforward referred to as 'secondary sites') distributed throughout the region (Fig. 1) during occasional visits from 2001 to 2003. In most cases, sampling of these secondary sites was restricted to a single Melitaeini species per locality (Table 1), although other species may have been present. In all, Melitaeini larvae were collected from populations distributed over an altitudinal range of 1000 m (*c*. 80–1100 m a.s.l.) and a variety of habitat types, from the typical evergreen oak forest of Mediterranean lowlands to the subalpine meadows of the Pyrenean mountains (Table 1).

The larvae were reared in the laboratories of El Puig and Can Liro in $14 \times 10 \times 6$ cm plastic containers covered with a fine mesh cloth and lined with absorbent tissue paper on the bottom. Each day the larvae were provided with fresh leaves of the food-plant species on which they were found. For larvae collected in a gregarious phase, samples of *c*. ten individuals were kept together in the plastic containers. The fate of each caterpillar was recorded, except for the small fraction of larvae that entered diapause at the end of the season. Larvae that entered diapause, and those that died for unknown reasons, were subtracted from the original sample size.

All of the parasitoids emerging from the host larvae or pupae were reared in the laboratory. One or two

Table 1. Sites from which Melitaeini larvae were collected, with their altitudinal ranges and habitat characteristics. A distinction is made between (A) the main sites that were periodically sampled during February–September 2002, and (B) the secondary sites that were occasionally visited from 2001 to 2003. The Melitaeini species present in each of the main sites is given, ranking the species in decreasing order of adult abundance (C. Stefanescu, unpubl. data). Species in bold type have permanent populations at these sites and were collected as larvae, while those in unboldened type occur in very low numbers and probably represent sink populations dependent on immigration. +: larvae parasitized by *Cotesia*; –: larvae not parasitized by *Cotesia.* Data on voltinism (number of generations completed during a season) and food plants use have been compiled through extensive fieldwork (C. Stefanescu, unpubl. data) and are included to present a comprehensive picture of the natural history of the butterfly species. Only the species collected and the food plants on which they were found are indicated for the secondary sites

Sites	Altitude (m)	Habitat[a]	Melitaeini species	+/–	Voltinism[b]	Food plants[c]
(A)						
El Puig	900–1100	3	***Melitaea cinxia***	+	U	*Plantago lanceolata* (Pl), *Veronica spicata* (Sc)
			Melitaea trivia	+	P	*Verbascum pulverulentum* (Sc), *V. chaixii* (Sc)
			M. athalia celadussa	–	U	*Plantago lanceolata* (Pl), *Veronica chamaedrys* (Sc)
			Melitaea phoebe	+	P	*Centaurea pectinata* (As), *Carduus nigrescens* (As)
			Melitaea deione	+	P	*Antirrhinum majus* (Sc), *Plantago lanceolata* (Pl)
			Melitaea diamina	–	U	*Valeriana officinalis* (Va)
			Euphydryas aurinia		U	*Scabiosa columbaria* (Di)
			Melitaea parthenoides		U	
El Cortès	500–800	1, 2	***Melitaea deione***	+	P	*Plantago lanceolata* (Pl), *Antirrhinum majus* (Sc), *A. orontium* (Sc)
			Melitaea didyma	+	P	*Plantago lanceolata* (Pl)
			Euphydryas aurinia	+	U	*Lonicera implexa* (Ca)
			Melitaea phoebe	+	P	*Centaurea collina* (As), *C. paniculata* (As), *C. pectinata* (As)
			Melitaea cinxia		U	

El Guix	400–450	4	***Euphydryas aurinia***	+	U	*Lonicera implexa* (Ca), *L. etrusca* (Ca)
			Euphydryas desfontainii	+	U	*Cephalaria leucantha* (Di)
			Melitaea didyma	–	P	*Plantago lanceolata* (Pl)
			Melitaea phoebe	–	P	*Centaurea* sp. (As)
			Melitaea cinxia		U	
(B)						
Boixadors	650	4	*Euphydryas desfontainii*	+	U	*Cephalaria leucantha* (Di)
Bosc de Valldemaria	80	1	*Euphydryas aurinia*	+	U	*Lonicera implexa* (Ca)
Can Jordà	540	5	*Euphydryas aurinia*	+	U	*Succisa pratensis* (Di), *Knautia arvensis* (Di)
Cantallops	180	2	*Melitaea didyma*	+	P	*Plantago lanceolata* (Pl)
Coll d'Estenalles	900	4	*Euphydryas aurinia*	+	U	*Lonicera implexa* (Ca), *L. etrusca* (Ca)
Font Negra	1000	5	*Euphydryas desfontainii*	+	U	*Cephalaria leucantha* (Di)
La Barroca	380	4	*Euphydryas aurinia*	+	U	*Lonicera implexa* (Ca), *Succisa pratensis* (Di)
La Malesa	350	4	*Euphydryas aurinia*	+	U	*Lonicera implexa* (Ca), *L. etrusca* (Ca)
			Euphydryas desfontainii	+	U	*Cephalaria leucantha* (Di)
La Nou de Berguedà	1000	5	*Melitaea phoebe*	+	P	*Carlina acaulis* (As)
Prats de Molló	800	5	*Melitaea didyma*	+	P?	*Plantago lanceolata* (Pl)
Sant Bernat	780	1	*Melitaea deione*	+	P	*Antirrhinum majus* (Sc), *Plantago lanceolata* (Pl)
Sant Feliu de Codines	500	1	*Euphydryas aurinia*	+	U	*Lonicera implexa* (Ca)
Vallforners	500–700	1	*Melitaea deione*	+	P	*Antirrhinum majus* (Sc)
Vidrà	1000	5	*Melitaea deione*	+		*Linaria* sp. (Sc)

[a]Habitat types: 1. Evergreen oak forest. 2. Acidic grassland within evergreen oak forest. 3. Acidic grassland and bracken within deciduous forest. 4. Calcareous grassland and scrub within evergreen oak forest. 5. Calcareous grassland and scrub within deciduous forest.
[b]Voltinism: U, univoltine; P, plurivoltine.
[c]Foodplant families: As, Asteraceae; Ca, Caprifoliaceae; Di, Dipsacaceae; Sc, Scrophulariaceae; Pl, Plantaginaceae; Va, Valerianaceae.

days after formation, *Cotesia* cocoons were transferred from the larval rearing boxes into plastic vials where they were kept until adult emergence. Each brood was kept together, but isolated from others. Adults were killed in 100% ethanol for molecular study, or preserved dry for morphological examination, or kept alive to be used in experiments on host acceptance behaviour. Mummified host larvae (i.e. those parasitized by Ichneumonidae: Campopleginae) and puparia of Tachinidae (Diptera) were also kept in individual plastic vials until adults emerged, and were preserved as dry specimens for identification. Most of the adult butterflies reared were released back into their original habitat.

Samples of the *Cotesia* reared from all host species/site combinations have been deposited in the National Museums of Scotland as mounted specimens.

Molecular analysis of *Cotesia*

DNA was extracted from wasps individually using NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's instructions, except that 50 μL of milliQ water was used in the final elution stage. To verify the taxonomic status of the reared *Cotesia*, a part of the mitochondrial COI gene was sequenced from several individuals from each host species from each sampling site. The sequences were then compared with sequences in the molecular phylogeny of *Cotesia* from Melitaeini hosts from the broader Eurasian study (Kankare & Shaw, 2004). The universal primers used were HCO1490 and LCO2198 (Folmer *et al*., 1994) and C1-J-1859, C1-J-2183 and TL2-N-3014 (Simon *et al*., 1994). Sequencing was performed as detailed for COI in Kankare & Shaw (2004).

Ten microsatellite loci were included in the analysis: *Cme1*, *Cme4* and *Cme17* isolated from *C. melitaearum* agg. (host species *M. inxia* from Åland; Kankare *et al.*, 2004) and *Cco1A*, *Cco5A*, *Cco27*, *Cco42*, *Cco65A*, *Cco65B* and *Cco68*, originally isolated from *Cotesia congregata* (Say) (host species *Ceratomia catalpae* (Boisduval); Jensen *et al.*, 2002). Since two *Cme* loci (*Cme1*, *Cme17*) failed to amplify for *C. acuminata* agg. individuals from both *Melitaea phoebe* (Denis & Schiffermüller) and *M. didyma* (Esper), and two more loci (*Cco1A*, *Cco42*) for *C. acuminata* agg. individuals from *M. didyma*, these loci were removed from the analyses of these populations. Microsatellite PCRs were performed, as detailed in Kankare & Shaw (2004). Diluted and pooled microsatellite PCR products were resolved in three panels in an ABI 377 automated DNA sequencer (PE, Applied Biosystems). Gels were analysed and fragments sized using GENESCAN v. 3.1.2 and GENOTYPER v. 2.5 (PE, Applied Biosystems), respectively.

The Excel Microsatellite Toolkit (http://acer.gen.tcd.i.e./~sdepark/ms-toolkit/) was used to calculate Nei's expected gene diversity (H_e; Nei, 1987), observed heterozygosity (H_o), mean number of alleles (MNA) and allele ranges over all loci for combined *Cotesia* samples from each host species. *Cotesia* reared from a single host species feeding on a single plant species at one collection site are considered a sample. Distribution of allele frequencies was also calculated for combined *Cotesia* samples from each host species for each microsatellite locus.

FSTAT 2.9.3.1 (Goudet, 2001) was used to estimate deviations from Hardy–Weinberg (HW) equilibrium (assessed by F_{IS}, the heterozygote deficit within populations) and from genotypic linkage equilibrium using log-likelihood G-statistics (Goudet *et al*., 1996). Multiple tests were corrected for using Bonferroni correction. Because Hymenoptera are haplodiploid, only the data from females were used to calculate Nei's expected gene diversity and observed heterozygosity. Moreover, only *Cotesia* samples made up of more than four females representing different broods and collected in the same year from one location, were included in the analyses of deviations from HW and genotypic linkage equilibria. FSTAT was used to test for variance in allele frequencies (F_{ST}; Weir & Cockerham, 1984) and for pairwise genetic differentiation (using multilocus genotypes) among combined *Cotesia* samples from each host species, as well as among *Cotesia* reared from the same host species collected from different sites, excluding samples of only two individuals.

Correlations between genetic ($F_{ST}/1–F_{ST}$) and geographical distances (isolation by distance) were tested using Spearman Rank correlation coefficient (r_S). The significance of the correlation was assessed with a Mantel test (2000 permutations) using GENEPOP (http://wbiomed.curtin.edu.au/genepop; Raymont & Rousset, 1995). The chord distance (D_{CE}) of Cavalli-Sforza & Edwards (1967) was used to estimate genetic distances among *Cotesia* based on ten (eight) microsatellite loci using MsatBoot v. 1.2 (Landry, Koskinen & Primmer, 2002). The resulting genetic distance matrices were used to construct Neighbour-joining and consensus trees with NEIGHBOUR and CONSENSE, respectively. Both programmes are implemented in PHYLIP v. 3.75c (Felsenstein, 1995). *Cotesia congregata* was used as an outgroup for the microsatellite distance tree.

Experiments on *Cotesia melitaearum* agg. adult host acceptance behaviour

We observed the behaviour of adult female *C. melitaearum* agg. originating from three host species – *Melitaea trivia* (Denis & Schiffermüller),

M. cinxia and *E. aurinia* – towards their host species of origin as well as towards putative alternate host species. The wasps used were unmated, and fed honey water (1 : 3). To standardize experience and to eliminate inactive wasps, each wasp was observed to (apparently) oviposit into the host species from which it had been reared before the experimental runs. In two treatments, *C. melitaearum* agg. reared from *M. trivia* and tested on *M. cinxia* and *M. athalia* (Rottemburg) from Åland, Finland were tested on *M. cinxia* rather than *M. trivia* because the latter was unavailable. The wasps were placed on a piece of food-plant in the centre of 14 × 10 × 6 cm plastic boxes with three host larvae and fragments of partially eaten food plant and frass. Their behaviour was observed until they appeared to oviposit into a host or for 10 min, whichever came first. An oviposition attempt was scored if the wasp inserted her ovipositor into the host and stayed in a curved position with the wings held back for at least 2 seconds. Other aspects of behaviour recorded include antennation of frass and food plant, duration of oviposition and behaviour during oviposition, as well as avoidance of host larvae by the parasitoids. Each wasp-origin host-species treatment was replicated 5–15 times, each time with a different wasp.

Cotesia melitaearum agg. reared from the overwintering generation of *M. trivia* larvae at El Puig were observed with seven potential host species in the study area, including *M. trivia.* Some of them co-occur with *M. trivia* in El Puig (*M. cinxia, M. athalia celadussa* (Fruhstorfer), *M. phoebe* and, at least in some years, *E. aurinia*), whereas the others occur nearby in El Guix (*E. aurinia*), El Cortès (*M. didyma*), and Sant Bernat (*Melitaea deione* (Geyer)). *Cotesia melitaearum* agg. from *M. trivia* from El Puig were also observed with *M. cinxia* and *M. athalia* from Åland, Finland. *Cotesia melitaearum* agg. reared from *E. aurinia* from El Guix were observed with the co-occurring and closely related *E. desfontainii* (Godart). Additionally, *C. melitaearum* agg. reared from *M. cinxia* from Åland were tested with six potential host species from the Spanish study area, including *M. cinxia* from El Puig. All observations were made between 10:00 and 16:00 h in the laboratory at El Puig, except the tests of *C. melitaearum* agg. from *M. trivia* using *M. cinxia* and *M. athalia* from Åland, which were made under similar laboratory conditions in Finland.

We are for the most part unable to interpret results on the rate of successful parasitism because some of the host larvae that were apparently accepted were late in their last instar, which is still attractive to adult *Cotesia* but unsuitable for their larval development (Eliasson & Shaw, 2003; S. van Nouhuys, pers. observ.), and because of poor laboratory rearing conditions resulting in larvae dying for reasons unrelated to parasitism. Offspring were, however, reared from four out of the seven treatments in which the wasps consistently behaved as if they were ovipositing.

RESULTS

Butterfly and parasitoid communities

We collected a total of 2779 larvae belonging to nine butterfly species, from 20 different food plants (Table 1). At the three main sites, El Puig, El Cortès and El Guix, we recorded eight, five and five co-occurring Melitaeini species, respectively, although some of them have only been seen as adult butterflies and probably do not maintain breeding populations there. All of the species showed a high degree of specialization in food-plant use, with only 1–3 plant species exploited at any site (Table 1).

According to available published data (e.g. Tolman & Lewington, 1997; Wahlberg, 2001, and pers. comm.), some of the host plants we found in this study represent new records for several Melitaeini species. Thus, *Plantago lanceolata* (L.) and *Antirrhinum orontium* (L.) are recorded for the first time as oviposition substrates (i.e. eggs or early instar larvae were observed on these plants, as well as more mobile later instar larvae) for *M. deione*, *Verbascum chaixii* (Villars) for *M. trivia*, and *Centaurea collina* (L.), *Centaurea paniculata* (L.), *Centaurea pectinata* (L.), *Carduus nigrescens* (Villars) and *Carlina acaulis* (L.) for *M. phoebe*. Likewise, *Succisa pratensis* (Moench), *Knautia arvensis* (L.) and *Scabiosa columbaria* (L.) are recorded for the first time as oviposition substrates for the Iberian populations of *E. aurinia* (cf. Mazel, 1986).

Of the 2779 larvae collected, 862 died for unknown reasons during rearing and 287 entered diapause, their fate not being further assessed. Therefore, the rates of parasitism were estimated from an effective sample of 1630 larvae (1301 from primary sites and 329 from secondary sites) (Table 2). The number of larvae collected from three secondary sites (Bosc de Valldemaria, Coll d'Estenalles and La Barroca) was not recorded, so the rates of parasitism are unknown for these samples.

We reared parasitoids from each of the Melitaeini species that was collected, with the exception of *M. diamina* (Lang) (but only four larvae of this species were found) (Table 2). Larval *Cotesia* were the most frequently recorded parasitoids, followed by six species of Tachinidae. In all, seven out of nine Melitaeini species sampled from the main sites were attacked by *Cotesia*. The complete absence of *Cotesia* from the large *M. athalia celadussa* sample, and the very low level of parasitism achieved by *Cotesia* in the co-occurring *M. cinxia* population, are important

Table 2. Sample sizes and rate of parasitism. Rate of parasitism was calculated considering only the effective larval sample (discounting those larvae that died for unknown reasons during rearing, or entered diapause). *Cotesia melitaearum* agg. was also reared from an unknown number of *Euphydryas aurinia* from Bosc de Valldemaria, Coll d'Estenalles and La Barroca; *C. bignellii* was also reared from an unknown number of *E. aurinia* from La Barroca. (A) main sites; (B) secondary sites

		Number of					Per cent parasitized by		
Melitaeni species	Site	larvae	pupae	*Cotesia*	Tachinidae	others	*Cotesia*	Tachinidae	others
(A)									
Melitaea cinxia	El Puig	168	162	1[1]	0	5*	0.6	0.0	3.0
Melitaea phoebe	El Puig	38	25	13[2]	0	0	34.2	0.0	0.0
	El Cortès	18	15	3[2]	0	0	16.7	0.0	0.0
Melitaea trivia	El Puig	176	160	16[1]	0	0	9.1	0.0	0.0
Melitaea didyma	El Cortès	19	12	7[1,2]	0	0	36.8	0.0	0.0
Melitaea diamina	El Puig	4	4	0	0	0	0.0	0.0	0.0
Melitaea deione	El Puig	57	47	9[1]	1[e]	0	15.8	1.8	0.0
	El Cortès	154	147	3[1]	4[b]	0	1.9	2.6	0.0
M. athalia celadussa	El Puig	273	265	0	8[b,f]	0	0.0	2.9	0.0
Euphydryas aurinia	El Cortès	6	3	3[1]	0	0	50.0	0.0	0.0
	El Guix	170	160	6[1]	4[a,c]	0	3.5	2.4	0.0
E. desfontainii	El Guix	218	208	10[1]	0	0	4.6	0.0	0.0
(B)									
Melitaea phoebe	La Nou de Berguedà	1	0	1[2]	0	0	100.0	0.0	0.0
Melitaea didyma	Sant Bernat	5	5	0	0	0	0.0	0.0	0.0
	Cantallops	43	0	42[1,2]	1[d]	0	97.7	2.3	0.0
	Prats de Molló	1	0	1[2]	0	0	100.0	0.0	0.0
Melitaea deione									
	Sant Bernat	40	18	8[1]	14[b]	0	20.0	35.0	0.0
	Vallforners	13	10	3[1]	0	0	23.0	0.0	0.0
	Vidrà	1	0	1[1]	0	0	100.0	0.0	0.0
Euphydryas aurinia	La Malesa	40	39	1[1]	0	0	2.5	0.0	0.0
	S. Feliu de Codines	4	2	2[1]	0	0	50.0	0.0	0.0
	Can Jordà	11	0	11[3]	0	0	100.0	0.0	0.0
Euphydryas desfontainii	La Malesa	63	63	0	0	0	0.0	0.0	0.0
	Boixadors	94	93	1[1]	0	0	1.1	0.0	0.0
	Font Negra	13	8	5[1]	0	0	38.5	0.0	0.0

[1]*Cotesia melitaearum* agg., [2]*Cotesia acuminata* agg., [3]*Cotesia bignellii*; [a]*Compsilura concinnata*, [b]*Erycia fatua*, [c]*Erycia furibunda*, [d]*Exorista larvarum*, [e]*Exorista segregata*, [f]*Pales pavida*, **Hyposoter horticola*.

exceptions to the generally ubiquitous presence of *Cotesia* in the samples. Five of the seven species parasitized by *Cotesia* at the main sites were also sampled at secondary sites; all five were parasitized by *Cotesia* at most localities. However, the low rate of parasitism of *Euphydryas* species at La Malesa and Boixadors is of interest. At one of the main sites, El Puig, the sampling revealed much less consistent use of the host assemblage by *Cotesia* species, though differences in the host spectrum present at the various sites makes this result difficult to interpret.

Tachinidae achieved lower levels of parasitism than *Cotesia* in general, and were recorded from only three species at the main sites, and two from secondary sites (including one species from which Tachinidae were not reared at the main sites). Because of the generally low level of parasitism achieved by Tachinidae, the qualitative differences recorded between the main and secondary sites may be merely stochastic. Similarly, we do not read much into the apparent absence of Tachinidae from some populations. Two of the tachinids reared, *Compsilura concinnata* (Meigen) and *Pales pavida* (Meigen), are among the most abundant, widespread and polyphagous of all the European species of Tachinidae, but neither appears to have been recorded previously from the present host species (*E. aurinia* and *M. athalia celadussa*, respectively; H.P. Tschorsnig, pers. comm.). Apart from single rearings of *Exorista segregata* (Rondani) and *Exorista larvarum* (L.), both common, widespread and polyphagous species, the remaining Tachinidae reared belonged to two species of *Erycia*, a genus which is entirely restricted to Melitaeini (Herting, 1960). The presence of these species, *E. furibunda* (Zetterstedt) and *E. fatua* (Meigen), in the Melitaeini populations is accordingly of greater significance. *Erycia furibunda* is widespread as a parasitoid of *E. aurinia* (one record from *E. desfontainii*: Ford, Shaw & Robertson, 2000) and is a univoltine species, while *E. fatua* has been recorded from a wider range of Melitaeini and is potentially multivoltine. Both gain access to the host larva (probably while it is very small; Ford *et al*., 2000) but kill the host in its pupal stage.

The only other primary parasitoids reared from hosts collected in the larval stage were several individuals of a solitary species of Ichneumonidae (Campopleginae) – *Hyposoter horticola* (Gravenhorst), from *M. cinxia* at El Puig. This is a regular parasitoid of *M. cinxia* in many parts of Europe (e.g. Lei *et al*., 1997) and at least locally apparently restricted to this host species. It is a remarkable species among Campopleginae for ovipositing into the host larva before the latter has hatched from its egg (van Nouhuys & Ehrnsten, 2004). The parasitoid larva makes a 'mummy' out of the half-grown host larva, inside which the parasitoid pupates. In these respects its behaviour is similar to that of the related genus *Benjaminia*, which is entirely associated with Melitaeini (Wahl, 1989), but which we did not find in our study.

Secondary parasitism (hyperparasitism) was insufficiently sampled to be included in our analyses.

Genetic diversity among *Cotesia* reared from different host species and localities

The taxonomic status of the *Cotesia* investigated in this study was elucidated using mtDNA COI sequences. The *Cotesia* populations fell into the host-associated clades expected on the basis of the more comprehensive phylogeny of *Cotesia* using Melitaeini (Kankare & Shaw, 2004).

Microsatellite diversity among combined *Cotesia* samples from different host species is given in Table 3. The mean number of alleles (across 6–10 microsatellite loci) ranged from 1.1 to 5.5. The allele range was highest (1–14) in *C. melitaearum* agg. individuals

Table 3. *Cotesia* species, host butterfly species, number of sampling locations, sample sizes (females in parentheses), number of broods used and microsatellite diversity estimates of *Cotesia* reared from each of the host species. *C. a*., *Cotesia acuminata*; *C. m*., *Cotesia melitaearum*. MNA, mean number of alleles; H_E, Nei's (1987) expected gene diversity; H_O, observed heterozygosity. *Only one location was used in the molecular analyses

Cotesia species	Host	Location	*N*(*N*f)	Broods	Loci	MNA	Allele range	H_E	H_O
C. a. agg.	*M. didyma*	2	15 (14)	15	6	1.17	1–2	0.027	0.028
C. a. agg.	*M. phoebe*	3	36 (29)	19	8	3.00	1–8	0.250	0.222
C. m. agg.	*E. aurinia*	6	52 (24)	30	10	5.50	1–14	0.449	0.282
C. m. agg.	*E. desfontainii*	4	38 (24)	19	10	4.50	1–14	0.444	0.286
C. m. agg.	*M. deione*	5	49 (38)	29	10	2.10	1–3	0.240	0.220
C. m. agg.	*M. didyma*	2	15 (15)	5	10	3.00	1–6	0.458	0.423
C. m. agg.	*M. trivia*	1	1 (9)	13	10	2.10	1–5	0.300	0.344
C. m. agg.	*M. cinxia*	1	2 (0)	1	10	1.1	1–2	–	–
C. bignellii	*E. aurinia*	2*	7 (3)	7	10	1.30	1–2	0.067	0.111

from *E. aurinia* and *E. desfontainii*. Expected gene diversity among all *Cotesia* samples ranged from 0.027 to 0.458 and the average observed heterozygosity from 0.028 to 0.423 (Table 3). All the *Cotesia* samples tested were in Hardy–Weinberg equilibrium; in other words, none of the populations had a deficiency of heterozygotes. Moreover, no departure from linkage disequilibrium was observed between pairs of loci in any of the samples (after correcting for multiple tests), suggesting that genotype distributions were independent.

Distribution of microsatellite allele frequencies among *Cotesia* reared from different host species showed a large amount of host-specific variation (Table 4). Variation was most pronounced in *C. acuminata* agg. individuals from the host species *M. didyma* and *M. phoebe*, which had host-specific alleles, with no overlap in size, in all the six microsatellite loci that amplified in both species. Moreover, two loci (*Cco1A* and *Cco42*) amplified only in *C. acuminata* agg. individuals from *M. phoebe*. In *C. melitaearum* agg. from *M. didyma* and *M. trivia*, two microsatellite loci (*Cco5A* and *Cme4*) were diagnostic with host-specific alleles and several other loci had unique alleles at lower frequencies. *Cotesia melitaearum* agg. from *M. deione* had host-specific alleles in *Cco65B*, *Cco65A*, and *Cme1*. In addition, *C. melitaearum* agg. from *E. aurinia* and *E. desfontainii*, which shared alleles in all ten microsatellite loci, had host-specific alleles in four loci (*Cco68*, *Cco65A*, *Cme1*, *Cme4*) that were not shared with any other *C. melitaearum* agg. individuals. Host-specific alleles with very low frequency were also present in other microsatellite loci. Interestingly, *C. bignellii* from *E. aurinia* had only one diagnostic locus (*Cco5A*) and host-specific alleles in two more loci.

Significant ($P = 0.05$) genetic differentiation was found between the *Cotesia* samples from each host species; the F_{ST} values were generally very high, from 0.10 to 0.96 with the mean of 0.64 (Table 5A). When comparisons were made between *Cotesia* populations from a single host species, significant genetic differentiation ($P = 0.05$) was found between *C. acuminata* agg. populations from *M. phoebe* as well as between *C. melitaearum* agg. populations from *M. didyma* (Table 5B). In addition, two out of three comparisons between *C. melitaearum* agg. populations from *M. deione* showed a significant genetic differentiation. F_{ST} estimates in these particular cases ranged

Table 4. Allele frequencies (%) for *Cotesia* populations from different host species in ten (6/8 scored for *Cotesia acuminata* agg) microsatellite loci. *N* (b) = number of individuals (broods). *Abbreviations:* Ca, *C. acuminata* agg.; Cb, *C. bignellii*; Cm, *C. melitaearum* agg.; Ea, *Euphydryas aurinia*; Ed, *E. desfontainii*; Mc, *Melitaea cinxia*; Mdei, *M. deione*; Mdid, *M. didyma*; Mph, *M. phoebe*; Mt, *M. trivia*

Locus	Allele length (repeat nos.) *N* (b)	Populations								
		Ca/Mdid 15 (15)	Ca/Mph 36 (19)	Cb/Ea 7 (7)	Cm/Ea 52 (30)	Cm/Ed 38 (19)	Cm/Mc 2 (1)	Cm/Mdei 49 (29)	Cm/Mdid 15 (5)	Cm/Mt 13 (13)
Cco1A	39									69
	42			58	93	93	100	92	34	31
	43				2	6			31	
	44								35	
	45		12		5	1		8		
	49			42						
	51		62							
	53		5							
	54		9							
	56		12							
Cco5A	28				1					
	29	100								
	30		100							
	31			100						
	33				99	100	100	100		
	34								32	
	35								32	
	36								25	
	37								11	
	38									96
	41									4

Table 4. *Continued*

Locus	Allele length (repeat nos.) *N* (b)	Populations								
		Ca/Mdid 15 (15)	Ca/Mph 36 (19)	Cb/Ea 7 (7)	Cm/Ea 52 (30)	Cm/Ed 38 (19)	Cm/Mc 2 (1)	Cm/Mdei 49 (29)	Cm/Mdid 15 (5)	Cm/Mt 13 (13)
Cco65B	38				2					
	39									58
	41			100					29	
	42	100								42
	43				39	14		67	64	
	44				59	86	100	3	7	
	45		100							
	48							30		
Cco68	51	8							100	100
	52	92		100	15	18	100	100		
	53		100		74	70				
	54				1	7				
	57				8	5				
	61				2					
Cco27	35				1					
	36		100		1					
	37			100				12		100
	38	100								
	41				89	100	100		46	
	42								16	
	43							16	15	
	44									
	45				9			72		
	49								23	
Cco42	29				1					
	30								19	
	31								4	
	33								4	
	34				2	12				
	35				22	34			57	
	36				25	1		27	8	46
	37				13	11		73	8	
	38				37	38				54
	39			80		4	100			
				20						
	46		18							
	48		5							
	49		70							
	52		7							
Cco65A	42							69		
	43							14		
	44		4							
	45							17	11	
	46				2					
	48		90		15	19	100			
	49		6	25	57	33				
	50				21	39				
	51				3	2			35	
	53			75	2	7				
	56	100								38
	57								36	62
	60								18	

Table 4. *Continued*

Locus	Allele length (repeat nos.) *N* (b)	Populations								
		Ca/Mdid 15 (15)	Ca/Mph 36 (19)	Cb/Ea 7 (7)	Cm/Ea 52 (30)	Cm/Ed 38 (19)	Cm/Mc 2 (1)	Cm/Mdei 49 (29)	Cm/Mdid 15 (5)	Cm/Mt 13 (13)
Cme1	75								75	
	83						100		25	
	88				66	6				
	89			100	2	49				
	90				3	12				
	91				20	8				
	92				3	17				
	93				1			8		
	96							77		
	98							15		
	101				5	8				
	115									30
	116									5
	118									15
	123									15
	124									35
Cme17	62				1					
	63				2					
	65				87	84	100	64		85
	66			100	4	11		18	100	15
	67				1			18		
	70					5				
	72				5					
Cme4	114									8
	116									92
	124								88	
	125								12	
	127		1							
	128	100								
	129							100		
	132						50			
	133		10	100	18	6	50			
	134		42		16	24				
	135				4	6				
	136		6		1	8				
	137		26			6				
	138		7							
	139				9	4				
	140				24	2				
	141		1			8				
	142					8				
	143					12				
	144				2					
	145		7		3	4				
	146				7	4				
	153				4					
	155				7					
	156				1					
	161				3					
	162					4				
	163				1	4				

Table 5. Pairwise F_{ST} estimates (above the diagonal) and the significance of genetic differentiation based on multilocus genotypes (below diagonal) among the combined *Cotesia* samples from each host species (A) and among *Cotesia* populations from different host species at different sites (B). B1, *C. melitaearum*/*Euphydryas aurinia* (Ea)/*E. desfontainii* (Ed); B2, *C. melitaearum*/*Melitaea deione*; B3, *C. acuminata*/*M. phoebe*; B4, *C. melitaearum*/*M. didyma*. *P = 0.05, NS, not significant; *N,* number of broods. The numbers after El Guix and El Cortès refer to individual habitat patches within the main sites

	1	2	3	4	5	6	7	8
(A)								
1 *C. a.* agg./*M. didyma*		0.89	0.68	0.73	0.80	0.75	0.88	0.96
2 *C. a.* agg./*M .phoebe*	*		0.57	0.62	0.74	0.62	0.71	0.76
3 *C. m.* agg./*E. aurinia*	*	*		0.10	0.49	0.49	0.54	0.59
4 *C. m.* agg./*E. desfontainii*	*	*	*		0.52	0.49	0.56	0.57
5 *C. m.* agg./*M. deione*	*	*	*	*		0.62	0.66	0.68
6 *C. m.* agg./*M. didyma*	*	*	*	*	*		0.53	0.59
7 *C. m.* agg./*M. trivia*	*	*	*	*	*	*		0.69
8 *C. bignellii*/*E. aurinia*	*	*	*	*	*	*	*	

(B1)	*N*	1	2	3	4	5	6	7	8	9
1 Ea/El Guix-1	18 (9)		0.00	0.11	0.03	0.16	0.17	0.12	0.20	0.18
2 Ea/La Barroca	4 (4)	NS		0.04	0.08	0.08	0.21	0.12	0.21	0.19
3 Ea/Bosc de Valdemaria	4 (4)	NS	NS		0.10	0.24	0.16	0.06	0.08	0.15
4 Ea/El Guix-2	11 (4)	NS	NS	NS		0.21	0.17	0.17	0.19	0.21
5 Ea/El Cortès-3	9 (3)	NS	NS	NS	NS		0.29	0.21	0.32	0.29
6 Ea/Sant Feliu de Codines	6 (2)	NS	NS	NS	NS	NS		0.19	0.24	0.23
7 Ed/El Guix-1	21 (7)	NS	NS	NS	*	NS	NS		0.16	0.10
8 Ed/La Malesa	5 (2)	NS	NS	NS	NS	NS	NS	NS		0.18
9 Ed/Font Negra	9 (4)	NS	NS	NS	NS	NS	NS	*	NS	

(B2)	*N*	1	2	3
1 Vallforners	14 (4)		0.29	0.13
2 Sant Bernat	17 (8)	*		0.06
3 El Puig-2	9 (3)	*	NS	

(B3)	*N*	1	2
1 El Puig-1	26 (10)		0.20
2 El Cortès-2	7 (3)	*	

(B4)	*N*	1	2
1 Cantallops	9 (3)		0.26
2 El Cortès-1	6 (2)	*	

from 0.20 (*C. acuminata* agg./*M. phoebe*) to 0.26 (*C. melitaearum* agg./*M. didyma*) and from 0.06 to 0.29 (*C. melitaearum* agg./*M. deione*). On the other hand, in *C. melitaearum* agg. from *E. aurinia* and *E. desfontainii*, a significant genetic differentiation was found in only 2 of 36 comparisons involving nine populations (F_{ST} = 0.00–0.32; Table 5B).

The Neighbour-joining consensus tree based on the microsatellite data shows the phylogenetic relationships between *Cotesia* reared from different host spe-

cies and from different collection localities (Fig. 2). Most of the *Cotesia* fall into five clades, labelled 1–5 in Figure 2. Clade 1 contained all *C. melitaearum* agg. populations reared from *E. aurinia* and *E. desfontainii*. Clade 2 was formed by *C. melitaearum* agg. from *M. deione*, clade 3 by *C. melitaearum* agg. from *M. didyma* and clades 4 and 5 by *C. acuminata* agg. from *M. phoebe* and from *M. didyma*, respectively. Clades 2, 3, 4 and 5 are supported with very high (≥ 98%) bootstrap support values, while clade 1 had only 61% support. *Cotesia melitaearum* agg. individuals from *M. cinxia*, *Melitaea parthenoides* (Keferstein) (a species that occurs in our study area but was too scarce to be adequately sampled) and *M. trivia*, which remained outside the five clades, also appear to represent distinct entities.

None of the *Cotesia* samples from the same host species in different locations showed isolation by distance as measured by pairwise $F_{ST}/(1–F_{ST})$ values in Mantel's test: *C. melitaearum* agg. from *E. aurinia* (five populations, $r_S = –0.19$, $P = 0.64$), *C. melitaearum* agg. from *E. desfontainii* (four populations, $r_S = –0.41$, $P = 0.76$), *C. melitaearum* agg. from *M. deione* (six populations, $r_S = 0.06$, $P = 0.56$), and *C. acuminata* agg. from *M. phoebe* (three populations, $r_S = 23.9$, $P = 0.18$).

Experiments on adult oviposition behaviour

All of the *C. melitaearum* agg. individuals attempted to parasitize the host species from which they came (Table 6). They all actively antennated frass and partially eaten food plant when presented with any of the

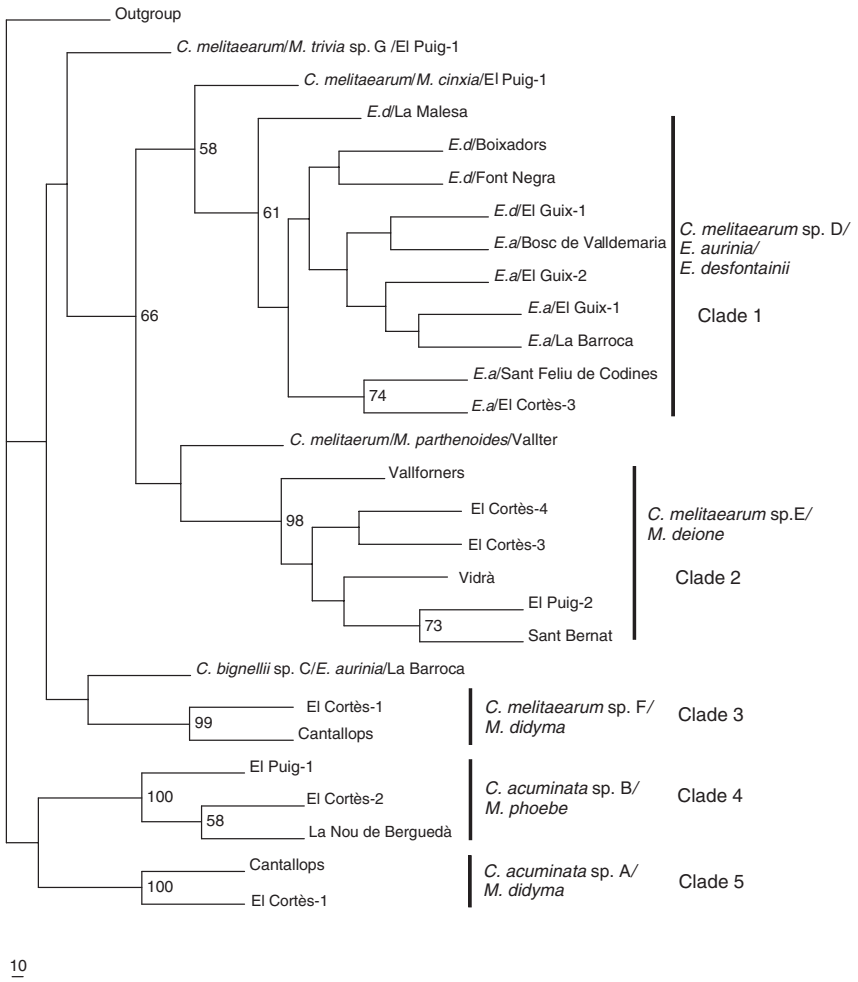

Figure 2. Neighbour-joining consensus tree of the relationships among *Cotesia* based on the microsatellite data. Distances are calculated with the chord distance (D_{CE}) of Cavalli-Sforza & Edwards (1967) based on ten (eight) microsatellite loci. Bootstrap support estimates (100 replicates) are indicated for statistically supported groups (= 50%). The numbers attached to El Puig, El Cortès and El Guix refer to individual habitat patches within the main sites. Vertical bars indicate clades 1–5 (see Results) and letters A–G the seven recognized *Cotesia* species (see Discussion). The linear scale relates the branch lengths to D_{CE} units.

Table 6. Summary of the behavioural observations of *Cotesia melitaearum* agg. females offered different species of Melitaeini larvae to parasitize. *Very short ovipositor insertion, no characteristic wing position

Host species	Collection site	Test species	Collection site	No. *Cotesia* tested	No. attacking (No. broods emerged)
Melitaea trivia	El Puig	*Melitaea cinxia*	El Puig	5	5
Melitaea trivia	El Puig	*Melitaea trivia*	El Puig	5	5 (2)
Melitaea trivia	El Puig	*Euphydryas aurinia*	El Guix	15	2*
Melitaea trivia	El Puig	*Melitaea athalia celadussa*	El Puig	11	3
Melitaea trivia	El Puig	*Melitaea phoebe*	El Puig	8	0
Melitaea trivia	El Puig	*Melitaea didyma*	El Cortès	8	2
Melitaea trivia	El Puig	*Melitaea deione*	Sant Bernat	9	0
Melitaea trivia	El Puig	*Melitaea cinxia*	Åland, Finland	6	6 (3)
Melitaea trivia	El Puig	*Melitaea athalia*	Åland, Finland	6	5
Euphydryas aurinia	El Guix	*Euphydryas aurinia*	El Guix	4	4 (3)
Euphydryas aurinia	El Guix	*Euphydryas desfontainii*	El Guix	4	4 (2)
Melitaea cinxia	Åland, Finland	*Melitaea phoebe*	El Puig	11	0
Melitaea cinxia	Åland, Finland	*Melitaea cinxia*	El Puig	7	6
Melitaea cinxia	Åland, Finland	*Melitaea trivia*	El Puig	10	1
Melitaea cinxia	Åland, Finland	*Euphydryas aurinia*	El Guix	8	1*
Melitaea cinxia	Åland, Finland	*Melitaea athalia*	El Puig	12	0
Melitaea cinxia	Åland, Finland	*Melitaea didyma*	El Cortès	8	0

(putative) host species, and each wasp appeared to notice the host larvae. In general, although not all host combinations were tested, our behavioural observations support the genetic differences detected among *C. melitaearum* agg. individuals collected from different hosts (see also Kankare & Shaw, 2004). *Cotesia melitaearum* agg. individuals originating from *M. trivia* at El Puig did not attempt to parasitize most other Melitaeini (*M. phoebe*, *M. didyma*, *M. deione* and *E. aurinia*), all but *M. didyma* co-occurring in El Puig. They did however, readily attempt to parasitize coexisting *M. cinxia* as well as *M. cinxia* from Finland. Interestingly, offspring successfully developed from at least some of the *M. cinxia* from Finland. Unfortunately, the Spanish *M. cinxia* were probably parasitized too late in their final instar for *Cotesia* to develop successfully. A few individuals (3/11) also appeared to oviposit into co-occurring *M. athalia celadussa* and 5/6 were willing to parasitize *M. athalia* from Finland, but no progeny resulted in either case. Two of these wasps very briefly inserted their ovipositors into *E. aurinia*, but they were for the most part reluctant even to get near this species. The *C. melitaearum* agg. individuals reared from *E. aurinia* from El Guix readily attempted to parasitize *E. aurinia* as well as the co-occurring *E. desfontainii*. Offspring were reared from some of the larvae in each of these two treatments (Table 6), suggesting that both of the co-occurring *Euphydryas* are viable hosts for *C. melitaearum* agg. reared from *E. aurinia*.

DISCUSSION

MELITAEINI COMMUNITIES IN CATALONIA

The nymphalid tribe Melitaeini constitutes a guild of ecologically and morphologically similar butterfly species, on which no detailed studies have been previously conducted in the Mediterranean Basin. Our intensive sampling at a few sites provides new information on the natural history of most of these butterflies, including reliable data on food plants and phenology, as well as on the species composition of local communities and on parasitism of the larvae (Tables 1, 2). These data in themselves have value for the conservation biology of the butterfly taxa concerned, as well as their specialized parasitoids, in this part of Europe. More generally, we use this information along with molecular data to begin to expose the structure of this community of butterflies, food plants, and parasitoids. More importantly, we distinguish seven biologically distinct species of *Cotesia* attacking the Melitaeini in our study area, rather than the three notional 'morphospecies' previously recognized. In particular, the notional *C. melitaearum* and *C. acuminata* each represent a series of cryptic species with narrow host associations.

Among the most notable findings of this study is the co-occurrence of as many as eight species of Melitaeini butterflies at a single site, a pattern that is probably not uncommon in relatively humid and cool Mediterranean climates where butterfly species richness is

high (cf. Stefanescu *et al*., 2004). Where so many ecologically similar species co-occur there is the potential for direct and indirect interactions among them, and among their natural enemies, including direct and apparent competition that can contribute to explaining community structure (Holt, 1977; Holt & Lawton, 1994; Bonsall & Hassell, 1997). We have sampled the parasitoid complexes only at the level of primary parasitoids, and it is possible that secondary parasitoids may have strong structuring effects on the primary parasitoids (van Nouhuys & Hanski, 2000; van Nouhuys & Tay, 2001). Also, we have sampled only the larval stages of the hosts. While we have no knowledge of the importance of egg parasitism in these communities, we do have limited data (J. Planas, C. Stefanescu & M. R. Shaw, unpubl. data) to suggest that levels of pupal parasitism (i.e. by idiobiont parasitoids) are not only high but also include some specialized parasitoids of Melitaeini. Despite these limitations, and other general problems of obtaining representative samples in the study of parasitism (Shaw, 1997), clear qualitative patterns have emerged on larval parasitism affecting the host butterflies. It is evident that species in the genus *Cotesia* interact strongly with most though not all of the Melitaeini taxa and populations we studied.

Genetic differentiation among *Cotesia* reared from Melitaeini hosts

Our main approach to studying the *Cotesia* parasitoids of Melitaeini was genetic, through the examination of microsatellite loci present in the samples that we reared. This follows from the wider, Eurasian scale study by Kankare & Shaw (2004), but involves more detailed sampling of the Catalonian Melitaeini community, where parasitoid populations lie in a relatively small geographical area in which the physical barriers to gene flow are limited, providing a means to take Kankare & Shaw's (2004) study into the fields of community and evolutionary ecology. The prerequisite for detailed study of community interactions, however, is to establish how many biological species of *Cotesia* we are dealing with, their host-specificity, and the extent to which we must still recognize uncertainty. We have four potential sources of data on which to draw, though some are only patchy: (1) DNA (genetic); (2) oviposition behaviour towards a range of hosts under laboratory conditions; (3) records of which co-occurring host species we have reared *Cotesia* from, and (4) morphology. A fifth source of data, the host ranges given for the segregates recognized by Kankare & Shaw (2004), is used to help to define the plausible host ranges of the taxa seen in this part of Spain.

Several studies have used mtDNA and/or allozyme data to distinguish among host-specific insect races or species (e.g. Atanassova *et al*., 1998; Babcock & Heraty, 2000; de Barro *et al*., 2000; Stone *et al*., 2001; Alvarez & Hoy, 2002; Chen, Giles & Greenstone, 2002; Abrahamson *et al*., 2003; Rokas *et al*., 2003). Far fewer studies have used microsatellite markers to investigate host-specific races or the presence of cryptic species. Molbo *et al*. (2003) reported the coexistence of previously unknown cryptic fig wasp species in more than half of the species they surveyed. In another work, Bucheli, Gautschi & Shykoff (2000) used microsatellites to study host-specific differentiation in the anther fungus *Microbotryum violaceum* (Pers.) Deml & Oberwinkler on many species of Caryophyllaceae. Their analyses revealed almost perfect isolation among samples from different host-plant species with a highly significant F_{ST} value of 0.56.

In our study, we found substantial to very high genetic differentiation (F_{ST} values ranging from 0.1 to 0.96) between *Cotesia* reared from most host species, while F_{ST} values among *Cotesia* reared from the same host species ranged from 0 to 0.32 (Table 5). Furthermore, different *Cotesia* populations clustered according to their host species in the microsatellite distance tree (Fig. 2). Indicative host-associated microsatellite allele frequencies were observed in all *Cotesia* samples. In addition, all samples from each host included at least one, and in some cases several, unique alleles that were not observed in samples reared from other hosts (Table 4). It should be noted that these comparisons are between *Cotesia* reared from each host species. If we compare the microsatellite allele frequencies only among *C. acuminata* agg. or among *C. melitaearum* agg., the genetic differentiation is even more pronounced.

The one exception to the pattern of host-specificity is the *C. melitaearum* agg. reared from *E. aurinia* and *E. desfontainii*, which group together in a distinct clade in the microsatellite distance tree. Correspondingly, very similar allele frequency distributions and low frequency of private alleles between these hosts in the microsatellite data suggest ongoing gene flow, implying that *C. melitaearum* from *E. aurinia* and *E. desfontainii* is a single species.

On the basis of the data presented here and in Kankare & Shaw (2004), we recognize seven *Cotesia* species parasitizing the Melitaeini communities in this part of Spain; that is, seven biological entities that do not interbreed even though they are all found within a relatively small geographical area, some even co-occurring in the same meadow. These species (henceforth referred to by letters), and a summary of the evidence by which we distinguish among them, are presented in Table 7.

We collected too few samples to classify *C. melitaearum* agg. reared from *M. cinxia* and from *M. parthenoides*. Based on genetic data from *C. melitaearum* agg. reared from *M. cinxia* and

Table 7. Summary of characteristics of the seven *Cotesia* species

Cotesia species	Host species	No. host-specific loci/ (allele frequency)/ total no. loci*	No. diagnostic loci*	Morphological differentiation from nearest taxa	Host range
C. acuminata agg. sp. A	*M. didyma*	6/(100)/6	6	yes	locally monophagous
C. acuminata agg. sp. B[1]	*M. phoebe*	8/(100)/8	8	yes	locally monophagous
C. bignellii (sp. C[2])	*E. aurinia*	3/(> 13)/10	1	yes	locally monophagous
C. melitaearum agg. sp. D[3]	*E. aurinia* and *E. desfontainii*	4/(> 79)/10	1	yes[3]	*E. aurinia* and *E. desfontainii*
C. melitaearum agg. sp. E	*M. deione*	4/(> 30)/10	0	weak	locally monophagous[4]
C. melitaearum agg. sp. F[5]	*M. didyma*	7/(> 18)/10	2	yes	locally monophagous
C. melitaearum agg. sp. G	*M. trivia*	5/(> 58)/10	3	weak	?locally monophagous[6]

*Comparisons were made within the *C. acuminata* agg. for species A and B, within all the groups for species C and within the *C. melitaearum* agg. for species D–G.

[1]*C. acuminata* (Reinhard) was described from material reared from both *Euphydryas maturna* and *Melitaea phoebe*, and the correct application of the name needs further investigation.

[2]Corresponds to *C. bignellii* (Marshall), which was described from material reared from *E. aurinia* in England.

[3]*C. melitaearum* (Wilkinson) was described as a parasitoid of *E. aurinia* in England. Molecular evidence to distinguish morphologically similar *C. melitaearum* agg. parasitizing *E. aurinia* and *M. cinxia* across Europe is inconsistent (Kankare & Shaw, 2004; M. Kankare *et al*., 2005a). However, sp. D differs morphologically from *C. melitaearum s.s.* and may have diverged as an endemic Iberian taxon using only *Euphydryas*.

[4]Although it appears to be locally monophagous in our samples, Kankare & Shaw (2004) link it with *Melitaea parthenoides*.

[5]Kankare & Shaw (2004) tentatively identified this taxon as the poorly understood species *Cotesia lycophron* (Nixon, 1974), the types of which were reared from *M. didyma* but appear to be aberrant specimens with a shortened hypopygium.

[6]Under laboratory conditions species G did not attack most possible Melitaeini hosts, supporting the idea that it is host-specific. However, it did attack *M. cinxia*, from which progeny were reared. Our failure to rear it from a large sample of *M. cinxia* collected at the site where it was abundant as a parasitoid of *M. trivia* is therefore surprising and deserves further investigation.

E. aurinia throughout Europe and Asia, it appears that there is limited gene flow among wasps reared from each of the two host species. However, there is also not a consistent pattern of DNA sequence data separating them into host-associated taxa (M. Kankare *et al.*, 2005a).

Host and parasitoid community structure

It is notable that with the exception of species D (from *E. aurinia* and *E. desfontainii*), we found no hosts sharing the same *Cotesia* species, and with two exceptions (discussed below) butterflies did not host more than one *Cotesia* species in any single location. Of course this may not be the entire story, because we may have missed sampling some parasitoid taxa, but it does indicate that currently there is little direct interspecific competition locally among *Cotesia*, nor could there be current indirect interaction (apparent competition) among butterfly species due to *Cotesia*. This leads to two interesting questions: (1) what drives specialization in *Cotesia* that parasitize Melitaeini? (2) To what extent can the lack of coexistence that we observed be attributed to past competition or competitive exclusion (Connell, 1980; Hawkins, 2000), other local biotic interactions (enemies, including hyperparasitoids) (Holt & Lawton, 1994; Bonsall & Hassell, 1997), or stochasticity and spatial dynamics (Hanski & Ranta, 1983; Hochberg & Ives, 1999; Holt, 2002)?

Host-specificity

We do not suggest that all koinobiont parasitoids have diversified into morphologically and ecologically similar sister species groups as the *Cotesia* of Melitaeini appear to have, because it is clear that at least some of them have more or less extensive host ranges (Shaw, 1994). Perhaps the explanation for the evolutionary radiations seen here is the presence of an array of abundant, ecologically and physiologically similar, potential hosts. That is, the Melitaeini may present a combination of similarity and diversity that promotes parasitoid specialization. It is interesting, however, that throughout its range one host species, *M. athalia* (and *M. athalia celadussa*), is at most only very seldom successfully parasitized by *Cotesia* species (Eliasson & Shaw, 2003; M. Kankare *et al.*, 2005b). Unfortunately, we have been unable to obtain sufficient and fresh samples even to investigate whether or not these rare events always involve the same cryptic *Cotesia* taxa.

The mechanisms by which gene flow between these *Cotesia* species was restricted in their incipient phase of speciation are unclear, and it cannot be determined from our data whether the same isolating mechanisms remain important at present. We found significant genetic differentiation even among populations within some species (Table 5), and no evidence of isolation by distance. This corresponds to the relatively weak dispersal behaviour that has been observed for *C. melitaearum* agg. from *M. cinxia* in Finland (van Nouhuys & Hanski, 2002), leading to its genetically differentiated population structure over a relatively small area (M. Kankare *et al.*, 2005b). In laboratory observations of adult *Cotesia* behaviour here and elsewhere (Eliasson & Shaw, 2003; M. Kankare *et al.*, 2005a), females for the most part only attacked the host species from which they were reared. This is not, however, absolute. For example, we did not rear species G (host species *M. trivia*) from any of our large sample of *M. cinxia*, but under laboratory conditions it attacked co-occurring *M. cinxia* as well as *M. cinxia* from Finland, and progeny were reared in the latter case. Adult female host fidelity in itself could not explain genetic isolation anyway, because hosts of different species commonly occupied the same habitat, and often the same plant, and hence adult parasitoids would certainly have the opportunity to interbreed. As is often the case when evidence of probable sympatric speciation is presented, the mechanisms of isolation are still unknown.

Direct and indirect interactions

Direct competition among Melitaeini that share the same food-plant species (e.g. *M. cinxia*, *M. didyma*, *M. athalia celadussa*, *M. deione* and *M. parthenoides*, all feeding on *P. lanceolata*) could be detrimental to, or eliminate, some or all of the species. Although competition for food would seem likely, especially given the often gregarious behaviour of larvae, food plants are generally abundant where they are used and we observed no evidence of direct competition for food at any of our three main sites, which suggests that direct interspecific competition is not currently structuring the butterfly community. Interspecific competition during exceptional seasons may still provide structure over a longer timescale, but we have observed that these ecologically similar butterfly species coexist sharing the same food plant species at multiple locations.

There is some evidence of direct interspecific competition among the *Cotesia* because with two exceptions, the butterfly species known to support more than one *Cotesia* species hosted only a single *Cotesia* at particular sites. The absence of species F (*C. ?lycophron*) as a parasitoid of *M. trivia* at El Puig, where species G was an abundant parasitoid of *M. trivia*, and the absence of *C. bignellii* (species C) from El Cortès and El Guix, where species D was reared from *E. aurinia*, are each suggestive of competitive exclusion. Interestingly, where we did find *C. bignellii* and species D attacking *E. aurinia* in the

same location (La Barroca), in all except one case, species D was reared from larvae feeding on *Lonicera implexa* (Aiton) and *C. bignellii* was reared from larvae feeding on *Succisa pratensis*. In El Guix and other locations in Spain, species D has always been reared from *E. aurinia* larvae feeding on *Lonicera etrusca* (Santi) or *L. implexa* or both, while *C. bignellii* has only been reared from *E. aurinia* feeding on *S. pratensis*. Therefore resource partitioning is a second possible explanation for the presence or absence of these two parasitoids. Further study of the system would be necessary to ascertain whether competitive exclusion or resource partitioning occur, or whether there is a competitive relationship at all among the parasitoids that could contribute to explaining their distributions (cf. Hawkins, 2000).

For the most part our data provide little evidence of apparent competition, or indirect interaction among butterflies mediated by shared parasitoids (Holt & Lawton, 1993, 1994). The exceptions are *E. aurinia* and *E. desfontainii*, which co-occur and are parasitized by species D. The abundances of the two butterfly species are correlated, a pattern that contrasts with what is observed among other co-occurring Melitaeini species and suggests that perhaps the population dynamics of *Euphydryas* species are linked by their shared parasitoid (C. Stefanescu & S. van Nouhuys, unpubl. data). Given that the *Cotesia* are locally host-specific, at first sight it might be argued that there is little opportunity for apparent competition between the butterflies to be mediated by *Cotesia* species. However, it is precisely this pattern of local host-specificity of the *Cotesia* species that could have resulted from the complete local elimination of other Melitaeini species as a result of apparent competition via shared *Cotesia* parasitoids in the past. To summarize, there are suggestive scenarios for both direct competition among the parasitoids and indirect interaction among the butterflies, but it is hard to determine the extent to which these processes are structuring the communities at present.

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