Genetic Divergence Among Host-Specific Cryptic Species in *Cotesia melitaearum* Aggregate (Hymenoptera: Braconidae), Parasitoids of Checkerspot Butterflies

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Ann. Entomol. Soc. Am. 98(3): 382–394 (2005)

ABSTRACT We used mitochondrial DNA sequence data and 12 microsatellite loci to examine the genetic structure of *Cotesia melitaearum* (Wilkinson) (Hymenoptera: Braconidae), a parasitoid wasp reared from two common butterfly species, *Melitaea cinxia* (L.) (Lepidoptera: Nymphalidae) and *Euphydryas aurinia* (Rottemburg) (Lepidoptera: Nymphalidae), across many localities in Europe and Asia, as well as from four more narrowly distributed related European species. The haplotypes of wasps reared from *M. cinxia* and *E. aurinia* show a complex geographic pattern presumably reflecting long-term history, but the microsatellite data yield two host-associated groups, each including populations across Europe and suggesting that currently there is no gene flow between the parasitoid populations attacking these two widely distributed host species. The cryptic species attacking *E. aurinia* also parasitizes the closely related *Euphydryas desfontainii* (Godart), but the three remaining host species have yet another, or possibly several, previously unrecognized parasitoid species. We support the cryptic species status of two *C. melitaearum* aggregate forms parasitizing *M. cinxia* and *Melitaea athalia* (Rottemburg) in the Åland Islands in Finland and provide behavioral and ecological data in addition to the molecular data.

KEY WORDS cryptic species, *Melitaeini*, mtDNA, microsatellites, parasitoids

MOLECULAR STUDIES HAVE REVEALED greater species richness in Hymenoptera than has been identified by previous taxonomic studies based on morphological characters (Jager and Menken 1994, Atanassova et al. 1998, Müller et al. 1999, Babcock and Heraty 2000, de Barro et al. 2000, Hoy et al. 2000, Alvarez and Hoy 2002, Chen et al. 2002, Rokas et al. 2003). For example, by using random amplification of polymorphic DNA-polymerase chain reaction (PCR), Jager and Menken (1994) found that the parasitoid wasp *Ageniaspis fuscicollis* (Dalman) (Hymenoptera: Encyrtidae) reared from different host species from one locality in Europe were different cryptic species. Hoy et al. (2000) used conserved coding genes (Actin 1 and 2) to show that the parasitoid *Ageniaspis* imported into the United States from Australia and Taiwan were actually two cryptic species. Using mitochondrial DNA (mtDNA) and allozyme data, Rokas et al. (2003) demonstrated that the oak gall wasp, *Andricus quercustozae* (Bosc) (Hymenoptera: Cynipidae), that had colonized novel oak (*Quercus* spp.) hosts represented genetically and biologically discrete entities within the species.

Far fewer studies have used microsatellite markers to study possible closely related species, but microsatellite data are useful in conjunction with sequence data to test for current or recent gene flow among populations. For example, Molbo et al. (2003) reported on a complex of previously unrecognized cryptic fig wasp species, some of which seem to be sister taxa with long-term coexistence on a shared host species, whereas others probably colonized novel host species. In another study using microsatellites, McCoy et al. (2001) measured highly significant F_{ST} values among ticks collected from different sympatric seabird hosts but little differentiation among ticks sampled from the same host species. These results suggest that host-related selection restricts gene flow and may eventually lead to the formation of host races. Finally, a molecular phylogeny of *Cotesia* parasitoids parasitizing checkerspot butterflies, by using both used mtDNA sequence data and microsatellite markers, suggests the presence of several previously unrecognized co-occurring host-associated cryptic species (Kankare and Shaw 2004, Kankare et al. 2005a).

Knowledge of possible cryptic species is essential for any ecological and evolutionary study, including host–parasitoid dynamics in multispecies communities (Althoff and Thompson 1999, van Nouhuys and Hanski 2002a, Kankare et al. 2005a) and the effect of local mate competition for the coexistence of species (Zhang et al. 2004). Knowledge of possible cryptic species is also critical for evaluating parasitoids for

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Table 1. Host butterfly species, country of origin, number of sampling sites, sample size (number of females in parentheses), and parameters of microsatellite diversity

Host	Country	No. localities	*n*	Broods	Loci	MNA	Allele range	H_e	H_o	HWE
E. aurinia	France	1	17 (5)	2	12	3.92	1–8	0.37	0.20	
E. aurinia	Italy	?	6 (5)	2	12	2.25	1–3	0.40	0.25	*
E. aurinia	Spain	6	54 (28)	15	11	5.27	1–14	0.44	0.27	*
E. aurinia	Sweden	2	2 (2)	1	11	1.73	1–3	0.35	0.32	
E. desfontainii	Spain	4	38 (25)	12	11	4.45	1–14	0.45	0.25	*
E. aurinia davidi	Siberia	1	6 (5)	1	12	1.92	1–3	0.32	0.41	
M. cinxia	Finland	32	151 (68)	30	12	4.00	1–15	0.31	0.15	*
M. cinxia	China	1	2 (2)	1	12	1.08	1–2	0.04	0.04	
M. cinxia	Estonia	3	26 (8)	8	12	2.58	1–6	0.32	0.17	*
M. cinxia	France	5	121 (70)	13	12	4.33	1–16	0.39	0.29	*
M. cinxia	Russia	6	29 (16)	8	12	4.33	1–11	0.49	0.39	
M. cinxia	Spain	1	2 (0)	1	11	1.00	1–1			
M. cinxia	Sweden	1	5 (0)	1	12	1.27	1–2			
M. cinxia	England	1	2 (2)	1	10	1.50	1–3	0.23	0.20	
M. athalia	Finland	2	5 (5)	2	11	2.00	1–3	0.38	0.30	
M. deione	France	1	5 (5)	1	10	1.80	1–3	0.28	0.24	
M. deione	Spain	5	55 (44)	28	11	2.18	1–3	0.25	0.21	
M. parthenoides	Spain	1	2 (2)	1	9	1.44	1–2	0.30	0.44	

Loci, number of loci; MNA, mean number of alleles over all loci; Allele range, H_e, expected heterozygosity averaged over all loci (Nei 1987); H_o, observed heterozygosity; HWE, test of the Hardy–Weinberg equilibrium (averaged over all loci; significant departure shown by an asterisk [*]).

release as agents of biological control (Rosen 1986, Alvarez and Hoy 2003).

The genus *Cotesia* is a large group of internal gregarious parasitoids of lepidopteran larvae, with 1,500–2,000 species worldwide (Mason 1981). There are several species of *Cotesia* associated with checkerspot butterflies, none of which are known to parasitize any other host species (van Nouhuys and Hanski 2004). Checkerspot butterflies are found in Europe, Asia, North America, and parts of South America, with several species often co-occurring in the same habitat (Murphy et al. 2004). For example, in northern Spain, which has an exceptionally high diversity of butterflies (Stefanescu et al. 2005), up to eight species of checkerspots may co-occur, with each species hosting *Cotesia* parasitoids (Kankare et al. 2005a).

The ecologically best-studied taxon of checkerspot-associated *Cotesia* is the notional species *Cotesia melitaearum* (Wilkinson), which has been recorded from eight different checkerspot species across Europe and Asia (Eliasson and Shaw 2003, van Nouhuys and Hanski 2004, Kankare et al. 2005a). The natural history and population dynamics of *C. melitaearum* agg. have been intensively studied in the Åland Islands in Finland for the past 10 yr (for reviews see van Nouhuys and Hanski 2002a, b, 2004). In Åland, *C. melitaearum* aggregate seems to have two host species, *Melitaea cinxia* (L.) and *Melitaea athelia* (Rottemburg). In large well-connected populations of *M. cinxia*, the parasitoid may attain high densities, parasitize a large fraction of host larvae, and in extreme cases increase the risk of extinction of local host populations (Lei and Hanski 1997).

Kankare and Shaw (2004) used mtDNA sequence data and microsatellite markers to construct a molecular phylogeny of checkerspot-associated *Cotesia* in Europe, Asia, and North America. These species of *Cotesia* seem to have arisen from two independent colonizations of the checkerspot clade and to consist of a greater number of genetically distinct taxa than previously thought based on morphological evidence. Kankare and Shaw (2004) suggested that *C. melitaearum* agg. reared from different host species involve several cryptic species. Our aim here is to present a detailed analysis of the extensive material that is available for *C. melitaearum* agg. from six different host species. We analyze the pattern of genetic variation in, and the evolutionary divergence among, populations sampled over a wide geographic area in Europe and Asia. For *C. melitaearum* agg. parasitizing *M. cinxia* and *M. athalia* in the Åland Islands, we supplement the molecular results with field observations on the rate of parasitism and laboratory experiments on the behavior of parasitoids encountering host larvae.

Materials and Methods

Specimens were obtained by rearing parasitoids from field-collected host larvae. The samples of *C. melitaearum* agg. used in this study, with information on host species and collection sites, are given in Table 1. *Cotesia* reared from a single host species in each country is considered as one sample. Many samples include individuals from different geographic locations within a country. The samples also include multiple individuals from a single host larva, because host larvae may contain the progeny of several parasitoid females or the progeny of a multiply mated female (M.K., personal observations; Table 1). Samples of the *Cotesia* reared from all host species/site combinations have been deposited in the National Museums of Scotland as voucher specimens.

Isolation and Sequencing of DNA. DNA was extracted from the entire body of each wasp individually using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, except that 50 μl of MilliQ water was used in the final elution stage. Universal primers HCO1490,

LCO2198 (Folmer et al. 1994), C1-J-1859, C1-J-2183, and TL2-N-3014 (Simon et al. 1994) were used to amplify both strands of a section of the COI region (1,500 bp). Sequencing was performed as detailed for COI in Kankare and Shaw (2004). Sequences were resolved on an ABI 377 automated DNA sequencer (PerkinElmer Life and Analytical Sciences, Boston, MA), analyzed using Applied Biosystems Prism sequencing analysis software version 3.3 (PerkinElmer Life and Analytical Sciences), manually checked with SEQUENCHER version 3.0 (Gene Codes Corporation, Ann Arbor, MI), and aligned using the program ClustalW (Thompson et al. 1994) with the default alignment parameters. There were no gaps in the alignment. The sequences of the haplotypes not reported previously in Kankare and Shaw (2004) have been deposited in GenBank under accession numbers AY538734–AY538737.

Phylogenetic analyzes were performed with the maximum parsimony (MP) criterion by using PAUP version 4.0b10 (Swofford 2002). Maximum parsimony trees were constructed using the TBR branch swapping method, with simple addition of sequence and equal weight given to transitions and transversions. Bootstrap confidence values (Felsenstein 1985) for each node in the MP trees were calculated with 100 full heuristic searches, and a 50% majority rule consensus tree was computed for these searches. *Microplitis xanthopus* (Ruthe) (Hymenoptera: Braconidae), a parasitoid wasp in another genus of the same subfamily (Microgastrinae), was included as an outgroup in the sequence analyzes.

Microsatellites. Twelve microsatellite loci, *Cme1*, *Cme3*, *Cme4*, *Cme15*, and *Cme17*, isolated from *C. melitaearum* (Kankare et al. 2004), and *Cco1A*, *Cco5A*, *Cco27*, *Cco42*, *Cco65A*, *Cco65B*, and *Cco68*, isolated from *Cotesia congregata* (Say) (Jensen et al. 2002), were used in this study. Microsatellite PCRs were performed as explained by Kankare and Shaw (2004). Diluted and pooled microsatellite PCR products were resolved in an Applied Biosystems 377 automated DNA sequencer (PerkinElmer Life and Analytical Sciences). Gels were analyzed and fragments sized using GENESCAN version 3.1.2 and GENOTYPER version 2.5 programs (PerkinElmer Life and Analytical Sciences), respectively.

The Excel Microsatellite Toolkit (Stephen D.E. Park; http://acer.gen.tcd.ie/≈sdepark/ms-toolkit/) was used to calculate the Nei (1987) expected heterozygosity (H_e), observed number of heterozygotes (H_o), mean number of alleles (MNA), and allele ranges for each *C. melitaearum* agg. sample over all loci (Table 1). Because Hymenoptera are haplodiploid, only data for females were used to calculate Nei's expected gene diversity and observed heterozygosity. Mean number of alleles, allele ranges, and distribution of allele frequencies for each microsatellite locus were calculated using both females and males, taking into account that males are haploid. The program FSTAT 2.9.3.1 (Goudet 2001) was used to estimate deviations from Hardy–Weinberg equilibrium (HWE, assessed by F_{IS}) and from genotypic linkage equilibrium (L-E) by using a log-likelihood test (Goudet et al. 1996). We corrected for multiple tests by using a Bonferroni correction. Only samples made up of at least five females were included in the analyses of deviations from HWE and L-E. FSTAT was used to test for heterozygote deficit among samples (F_{ST}; Weir and Cockerham 1984) and genetic differentiation between samples. Isolation by distance was examined by regressing $F_{ST}/(1 - F_{ST})$ against the logarithm of geographic distance between a pair of samples (Mantel 1967) and tested using the Spearman's rank correlation coefficient (2000 permutations; GENEPOP at http://wbiomed.curtin.edu.au/ genepop; Raymont and Rousset 1995). We analyzed differences in the $F_{ST}/(1 - F_{ST})$ values among host species groups by using analysis of variance (ANOVA). Samples with at least two females were included in the above-mentioned analyses.

Two methods were used to investigate the geographical genetic structure in the samples. First, phylogenetic distances were estimated using Cavalli-Sforza and Edwards (1967) chord distance (D_{CE}), which has been used in phylogenetic studies of other closely related species (Takezaki and Nei 1996). Second, the model-based clustering method of Pritchard et al. (2000) was used to infer the number of genetically distinct clusters in the material. This method estimates the fraction of individual multilocus genotypes belonging to each cluster. The model accounts for the presence of Hardy–Weinberg and linkage disequilibrium by introducing population structure and attempts to find population clustering without such disequilibria. Samples with at least two and five females were used for the microsatellite distance tree and clustering analysis, respectively. *C. melitaearum* males from *M. cinxia* from Sweden and Spain were included in the phylogenetic distance estimates, because there were no females in these samples.

For the phylogenetic analysis, genetic distance estimates based on the microsatellite data and bootstrapping procedures were performed using the program MsatBoot version 1.2 (Landry et al. 2002). The genetic distance matrices thus obtained were used to construct Neighbor-Joining (N-J) and consensus trees with the programs NEIGHBOR and CONSENSE, respectively, implemented in PHYLIP version 3.75c (Felsenstein 1995). Treeview version 1.6.6 (R.D.M. Page 2001, http://taxonomy.zoology.gla.ac.uk/rod/rod.html) was used to draw the trees. *C. congregata* was used as an outgroup for the microsatellite distance tree.

An admixture model with correlated allele frequencies was used for the clustering analysis. Several runs of various lengths (10,000–100,000) were performed for each number of clusters (K), testing K values from 3 to 22. Finally, to choose the best value of K, two independent runs (100,000 steps, after a burn-in period of 100,000 steps) were done using K values from 18 to 22. An individual was assigned to a cluster if the fraction of its genotype assigned to that cluster was >75% and did not exceed 15% for any other cluster.

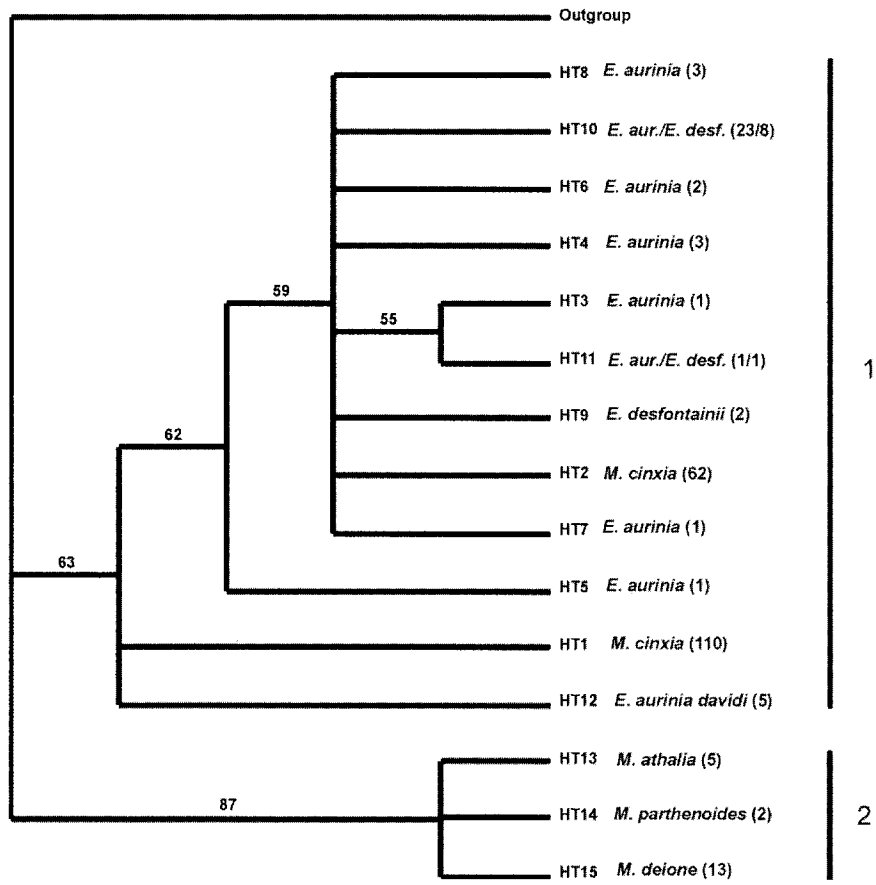

Fig. 1. The 50% majority rule consensus tree for *C. melitaearum* agg. haplotypes derived from the maximum parsimony analysis based on the part of COI gene (188 trees, length 211 steps). Numbers represent bootstrap support values (100 replicates) and are shown for branches with support >50%. Host species from which the different haplotypes were reared are shown to the right of the tree. Sample sizes are given in brackets and the two main clades are indicated by thick vertical lines.

Host Specificity of *C. melitaearum* agg. Reared from Co-occurring *M. cinxia* and *M. athalia*. The hypothesis that *C. melitaearum* agg. parasitizing *M. cinxia* and *M. athalia* in the Åland Islands in Finland in fact consists of two distinct taxa was tested using field and laboratory observations. All *M. athalia* larvae found during yearly spring surveys of *M. cinxia* (Nieminen et al. 2004) were collected and reared (n = 168, averaging 20 larvae in 1997–2004). Nine of the 168 larvae were parasitized by *Cotesia*. These specimens were analyzed genetically and morphologically, along with *C. melitaearum* reared from *M. cinxia*. We observed the oviposition behavior of *C. melitaearum* agg. reared from both *M. cinxia* and *M. athalia* on each host species in the laboratory and recorded rates of successful parasitism.

Results

Haplotype Composition. The alignments of mtDNA COI sequence involved 1,500 nucleotide sites, of which 159 (11%) were variable and 27 (1.8%) were parsimony informative. Maximum parsimony analysis of the data recovered 188 equally parsimonious trees from which we computed the 50% majority rule consensus tree (length 211 steps) presented in the Fig. 1.

Analysis of the sequence data yielded 15 distinct haplotypes from the six host species and the subspecies *Euphydryas aurinia davidi* (Oberthür) (Fig. 1). Two distinct haplotypes, HT1 and HT2, were reared from *M. cinxia*. The more common haplotype (HT1) was found in samples from northern Europe, England, and France as well as from Siberia and China. Haplotype HT2 was present in samples from southwestern Europe but also from Siberia (17 individuals from five distinct localities). The two haplotypes co-occurred in three of six host populations in France, but they were collected from different localities in Siberia, separated by tens of kilometers (Fig. 2a).

C. melitaearum agg. samples reared from *E. aurinia* yielded a total of eight haplotypes, two of which (HT10 and HT11) also were recorded from the closely related *E. desfontainii* (Fig. 1). Additionally, *E. desfontainii* samples included yet another haplotype

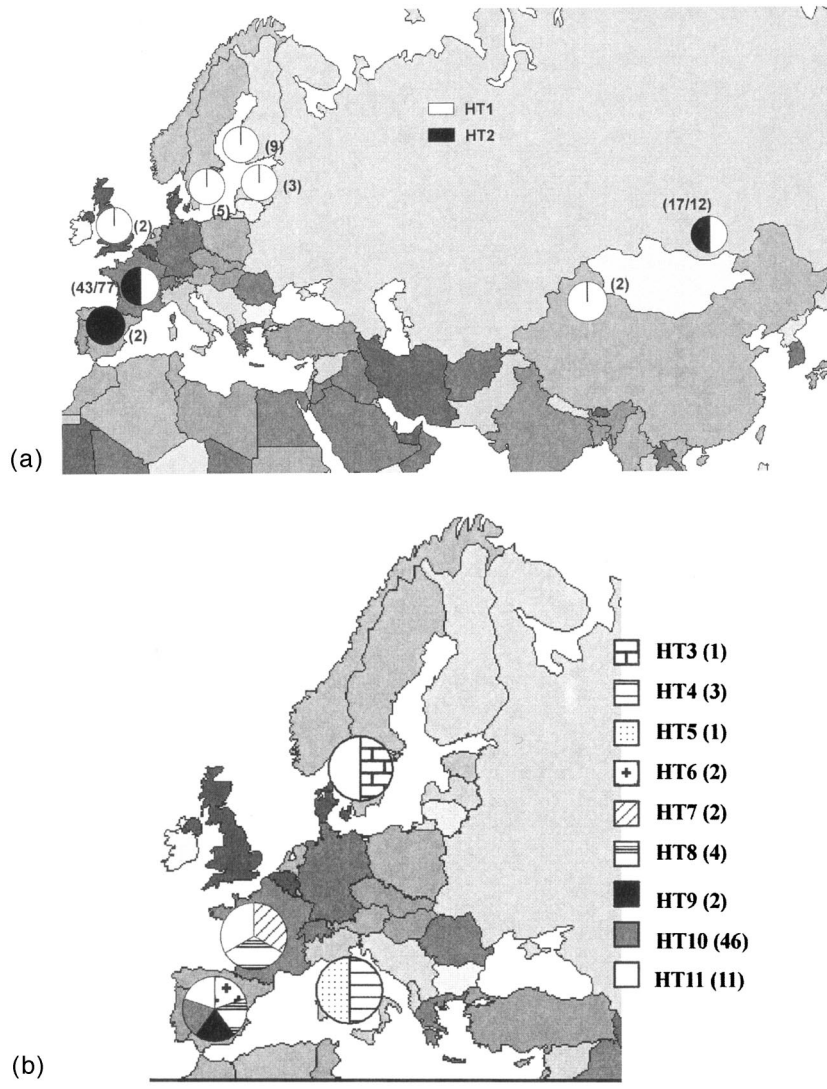

Fig. 2. (a) Geographical occurrence of the two haplotypes of *C. melitaearum* agg. (HT1 and HT2) reared from *M. cinxia* in Europe and Asia. (b) Geographical occurrence of the nine haplotypes reared from *E. aurinia* and *E. desfontainii* in Europe. Sample sizes are given in brackets.

(HT9), which we have not recorded from *E. aurinia.* Seven of these nine haplotypes from *E. aurinia* and *E. desfontainii* were each restricted to samples from a single country (Fig. 2b), whereas the remaining two haplotypes were collected from multiple localities in France and Spain (HT8) and from *E. aurinia* in Sweden, France, and Spain (HT11).

In the haplotype tree constructed using the mtDNA data, HT2 from *M. cinxia* and all the haplotypes from *E. aurinia* and *E. desfontainii* clustered together, whereas the second haplotype from *M. cinxia* (HT1) and HT12 from the Asian subspecies *E. aurinia davidi* remained outside this cluster (Fig. 1).

All the haplotypes mentioned so far cluster together to form the first of the two main clades in Fig. 1. The second main clade includes three more haplotypes, each reared from three other host species, *Melitaea deione* (Geyer) (HT15) and *Melitaea parthenoides* (Keferstein) in Spain (HT14) and *M. athalia* in Finland (HT13). The pairwise nucleotide divergences between these three haplotypes and all the others are fairly large, between 1 and 2%. Among the remaining haplotypes, HT1 and HT12 stand out with a pairwise divergence of ≈1% from the others, which had pairwise divergences of <0.5% among one another (Table 2).

Microsatellite Diversity and Population Differentiation. Of the 18 species-locality samples in Table 1, seven included >25 individuals collected from at least three different local populations. Among these seven relatively large samples, there is no systematic relationship between sample size and the average number

Table 2. Pairwise nucleotide divergence (%) between different *C. melitaearum* agg. haplotypes

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	xx														
2	**1.07**	xx													
3	**1.07**	0.27	xx												
4	**1.00**	0.20	0.20	xx											
5	**1.00**	0.28	0.28	0.21	xx										
6	**1.34**	0.20	0.41	0.30	0.44	xx									
7	**1.10**	0.06	0.26	0.20	0.28	0.20	xx								
8	**1.02**	0.07	0.21	0.14	0.21	0.21	0.07	xx							
9	**1.00**	0.13	0.20	0.13	0.21	0.20	0.13	0.13	xx						
10	0.93	0.13	0.13	0.07	0.14	0.20	0.13	0.07	0.07	xx					
11	**1.00**	0.20	0.07	0.13	0.21	0.30	0.20	0.14	0.13	0.07	xx				
12	**1.27**	**1.00**	**1.00**	0.94	0.86	**1.17**	**1.00**	0.95	0.94	0.87	0.94	xx			
13	**1.67**	**1.54**	**1.40**	**1.47**	**1.31**	**1.84**	**1.50**	**1.50**	**1.47**	**1.40**	**1.33**	**1.74**	xx		
14	**1.82**	**1.55**	**1.41**	**1.48**	**1.37**	**2.08**	**1.50**	**1.50**	**1.48**	**1.41**	**1.35**	**1.82**	0.81	xx	
15	**1.87**	**1.74**	**1.61**	**1.67**	**1.53**	**1.67**	**1.80**	**1.71**	**1.64**	**1.61**	**1.54**	**1.94**	**1.00**	0.95	xx

Values ≥ 1.00 are shown in bold.

of alleles or observed heterozygosity. Three samples stand out as having particularly low genetic diversity. Among the four large samples reared from *M. cinxia*, the two samples from the large islands in the Baltic, Åland in Finland and Saaremaa in Estonia, have lower diversity than the two mainland samples from France and Siberia (Table 1). The third low-diversity sample is from *M. deione* in Spain, which is a surprising result, because the *M. deione* sample was collected from 18 different broods (presumed families of wasp siblings) from five localities, separated by up to 45 km.

Six *C. melitaearum* agg. samples of the 12 that included at least five females showed a significant ($P < 0.05$) departure from the Hardy–Weinberg equilibrium caused by heterozygote deficit over all microsatellite loci (Table 1; correcting for multiple tests). All except one of these samples (we do not know the spatial structure of the *E. aurinia* sample from Italy) consisted of individuals originating from several local populations within a region rather than from one panmictic population, which probably explains the departure from the HWE. Among the remaining six samples with no significant deviation from the HWE, three samples came from a single population.

Permutation tests for each pair of loci across samples revealed 21 significant ($P < 0.05$) departures from linkage equilibrium after correcting for multiple tests. However, only four of the 12 samples contributed to the departure (*C. melitaearum* agg. from *E. desfontainii*, *M. deione* from Spain, and *M. cinxia* from Finland and France), and in 17 locus pair combinations the departure was due to only one sample (*M. cinxia* from France). These results suggest that the departure from the linkage equilibrium is not due to physical linkage of the loci but rather to an admixture in these particular samples.

Substantial to very high genetic differentiation was detected between samples collected from different host species, with F_{ST} estimates ranging from 0.16 to 0.92. The neighbor-joining tree based on chord distance (D_{CE}) indicates a clear genetic structure with three main groups (Fig. 3). Group 1 includes all samples reared from *E. aurinia* and *E. desfontainii*, group 2 samples from *M. cinxia*, and group 3 includes samples reared from *M. parthenoides*, *M. deione*, and *M. athalia*. *C. melitaearum* agg. from the Asian subspecies *E. aurinia davidi* remained outside these main groups. It is noteworthy that while in the haplotype tree (Fig. 1) HT2 from *M. cinxia* is located in the major clade with all the *E. aurinia* and *E. desfontainii* haplotypes, in the tree based on microsatellites the samples reared from *M. cinxia* comprise one group regardless of the haplotype (Fig. 3). One microsatellite locus, *Cme1*, shows a particularly revealing pattern, because there is hardly any overlap in the alleles among samples reared from *M. cinxia* and *E. aurinia*/*E. desfontainii* (Table 3). The similarity of *Cotesia* reared from *E. aurinia* and *E. desfontainii* is underscored by the very similar allele frequencies of samples from these two species in Spain, compared with *E. aurinia* collected from a wider geographic range (Table 3).

We detected no isolation by distance within the *Cotesia* groups identified in Fig. 3 (Fig. 4). Genetic differentiation as measured with pairwise $F_{ST}/(1 - F_{ST})$ values was significantly greater in between-group comparisons than in comparisons within group 1 (associated with *E. aurinia* and *E. desfontainii*, $P < 0.001$) and group 2 (associated with *M. cinxia*, $P < 0.001$; Bonferroni-corrected multiple comparisons).

Clustering of Multilocus Genotypes. Multilocus genotypes in the pooled data set were clustered into groups that largely agree with host species and locality; 17 of the 21 clusters consisted of a single *Cotesia* sample. The remaining four clusters each contained two samples, two of which included the species pair *E. aurinia* and *E. desfontainii* from Spain (Table 3). One might expect that these two clusters (5 and 7 in Table 4) correspond to populations of the two host species sampled at the same localities, but in fact the individuals originated from several populations. Regardless, this result suggests the close similarity of *C. melitaearum* agg. from these two host species, which is also apparent in Fig. 3. The two other mixed clusters were *C. melitaearum* agg. reared from *M. deione* in France and Spain, and *C. melitaearum* reared from *M. cinxia* in Finland and France. This latter cluster is the only really unexpected one, but

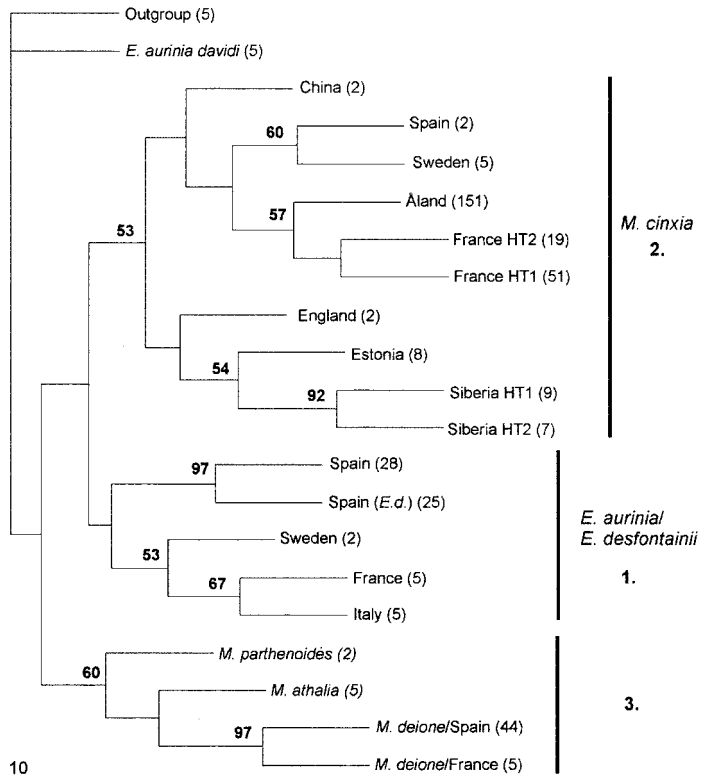

Fig. 3. Neighbor-joining tree based on 12 microsatellite loci and the chord distance (D_{CE}) of Cavalli-Sforza and Edwards. Numbers represent bootstrap support values (100 replicates) and are shown for branches for which the value was >50%. Sample sizes are given in brackets, and thick vertical lines indicate the three main groups. Branch lengths are linearly related to the D_{CE} values.

it should be noted that of the 10 clusters into which the parasitoids reared from *M. cinxia* in Finland and France fell, only one was mixed and only a few individuals were assigned to this cluster.

The assignment success was high (>80%) in seven of the 12 *Cotesia* samples (Table 4). For example, all parasitoid individuals from the host species *M. deione* (Spain) were assigned to a single cluster. However,

Table 3. Allele frequencies (%) in the *Cme1* locus for the pooled material for *M. cinxia*, and for *E. aurinia* and *E. desfontainii* from the localities indicated in table

Alleles	*M. cinxia*	*E. aurinia*	*E. aurinia*	*E. aurinia*	*E. aurinia*	*E. desfontainii*
	Pooled	France	Italy	Sweden	Spain	Spain
n	335	17	6	2	54	38
82	0.20					
83	96.04	3.70				
84	3.37					
85	0.20					
88					67.69	5.97
89			18.18		1.54	46.27
90					4.62	13.43
91					18.46	5.97
92					1.54	19.40
93		11.11	27.27		1.54	
94		7.41				
95		3.70				
98		14.81				
99		22.22	54.55	25.00		
100		33.33				
101		3.70			4.62	8.96
103				75.00		

Alleles are given in repeat numbers. *n*, sample size.

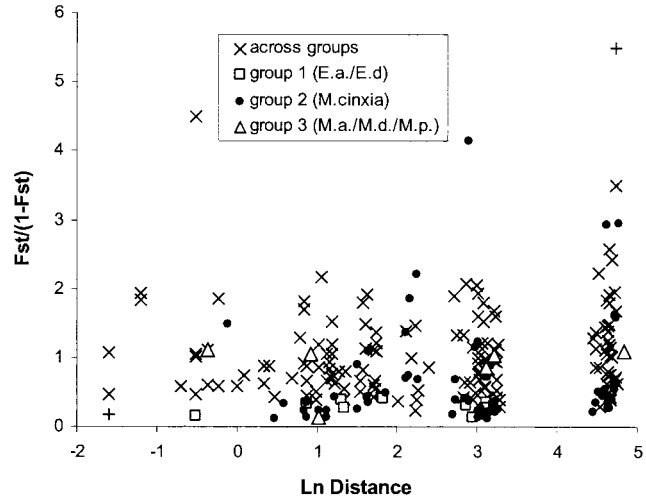

Fig. 4. Value of $F_{ST}/(1 - F_{ST})$ plotted against the logarithm of the geographical distance for pairs of samples. Four kinds of comparisons are indicated by different symbols explained in the legend (two points from across-groups comparisons shown by + had smaller or larger values than indicated and were displaced along the axes to improve clarity). None of the relationships within groups was significant (Mantel tests, group 1: $r_S = 0.005$, $P = 0.52$; group 2: $r_S = 0.310$, $P = 0.24$; and group 3: $r_S = 0.132$, $P = 0.71$). Geographical and genetic distances between *C. melitaearum* agg. reared from *M. cinxia* from France were calculated separately for several collecting localities.

>90% of *Cotesia* from *E. desfontainii* from Spain, and 89% of *M. cinxia* from France, were assigned to several clusters. In particular, the pattern among parasitoids reared from *M. cinxia* was more complex than the pattern for parasitoids reared from the other host species. The former were placed in a total of 12 clusters, of which seven occurred in France only. It is noteworthy that all the samples with individuals assigned to several clusters showed a significant departure from HWE (Table 4, samples marked with [a]; also see Table 1), supporting the interpretation that these samples did not come from single panmictic populations.

***Cotesia melitaearum* Aggregate Host Specificity in Åland Islands.** *C. melitaearum* agg. reared from *M. cinxia* and *M. athalia* in the Åland Islands have clearly distinct haplotypes (1.7% difference, Table 2; also see Fig. 1). In the microsatellite data, there are five loci with different fixed alleles in the two species as well as some species-specific alleles at two other loci (Table 5). The molecular data therefore conclusively demonstrate that two distinct genetic forms are involved.

C. melitaearum agg. emerged from nine of the 168 *M. athalia* larvae collected from *M. cinxia* populations. Two broods of cocoons yielded only hyperparasitoids, the remaining broods produced adult *Cotesia.* In six of seven trials, these wasps successfully parasitized postdiapause *M. athalia* larvae in the laboratory. However, for the most part they showed little or no interest in parasitizing *M. cinxia* larvae of any instar. Of the six *M. cinxia* larvae that seemed to have been parasitized in

Table 4. Percentage of *C. melitaearum* agg. reared from different host species and belonging to the 21 clusters inferred by the multilocus clustering method

Host	Country	No. localities	*n*	Broods	Clusters																				Total %
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
E. aurinia	France	1	5	2																					80	80
E. aurinia[a]	Italy	1	5	2				80																		80
E. aurinia[a]	Spain	6	28	15					4		11										50					65
E. desfontainii[a]	Spain	4	25	12					24		56	12														92
E. adavidi	Siberia	1	5	1	100																					100
M. cinxia[a]	Finland	20	68	30		24				6				13	13											59
M. cinxia[a]	Estonia	1	8	8												43										43
M. cinxia[a]	France	4	70	13						9							16	17	9	19		16	3			89
M. cinxia	Russia	4	16	8			50																			50
M. athalia	Finland	2	5	2																				100		100
M. deione	France	1	5	1									40													40
M. deione	Spain	5	44	28									100													100

No. localities, number of sampling localities; *n*, number of individuals in the analyzes; Broods, number of host larvae from which the *Cotesia* individuals were collected; Total %, percentage of *Cotesia* individuals assigned to one or more clusters.

[a] Significant departure from Hardy–Weinberg equilibrium; see Table 1.

Table 5. Allele frequence data for *C. melitaearum* agg. samples from *M. cinxia* and *M. athalia* (sample size in parentheses) in the Åland Islands

Locus	Alleles[a]	*M. cinxia* (151)	*M. athalia* (5)	Locus	Alleles[a]	*M. cinxia* (151)	*M. athalia* (5)
Cco1A	35	1.12		***Cme1***	83	91.63	
	42	88.27	100.00		84	57.44	
	43	3.35			85	0.47	
	45	7.26			93		33.33
Cco5A	33	100.00	100.00		95		66.67
Cco65B	44	97.31		*Cme15*	46	1.44	
	45	2.69			47	7.66	
	46		20.00		48	5.26	
	47		40.00		49	21.05	
	48		40.00		50	16.75	37.50
Cco68	51	0.88	40.00		51	9.57	
	52	99.12	60.00		52	4.31	25.00
Cco27	40		10.00		53	4.31	
	41	78.16	50.00		54	4.78	
	42	6.31			55	7.18	
	43	15.53			56	1.44	
	45		40.00		57	0.48	37.50
Cco42	35		60.00		59	7.66	
	36	0.92	10.00		60	0.96	
	37	8.29	30.00		61	7.18	
	38	46.08		***Cme17***	65	100.00	
	39	16.59			66		100.00
	40	27.65		***Cme4***	128		80.00
	41	0.46			129		20.00
Cco65A	44	0.47			132	28.71	
	46		100.00		133	37.80	
	48	26.64			134	9.09	
	50	26.54			136	22.49	
	51	48.34			137	1.91	

[a] Alleles are given in repeat numbers. Loci with diagnostic host-specific alleles are shown in bold.

the laboratory, five died and one pupated successfully to an adult butterfly. Similarly, *C. melitaearum* agg. reared from *M. cinxia* seems to be equally restricted to its own host and unsuited to using the alternative host, *M. athalia.* Forty-five of the 53 *C. melitaearum* agg. reared from *M. cinxia* and offered *M. athalia* larvae did not even attempt to oviposit. A few touched *M. athalia* larvae with their antennae, without proceeding to oviposit, but all showed interest in frass and partially eaten host plant leaves (typical behavior when exposed to *M. cinxia* larvae). Two wasps briefly inserted their ovipositors into *M. athalia* larvae, but no progeny emerged (the host larvae pupated). The remaining six wasps were persuaded to attempt to parasitize *M. athalia* by first being stimulated to oviposit with an *M. cinxia* larva that was quickly removed and replaced by an *M. athalia* larva. Although oviposition seemed to take place, once again the hosts pupated successfully and no parasitoids emerged. In summary, not a single trial using the alternate host produced viable wasp progeny.

Discussion

In this study, we use combined sequence data, microsatellite data, and field and laboratory observations on parasitism at multiple spatial scales to show that what was once thought to be a single parasitoid species is actually a complex of cryptic species. Furthermore, genetic differentiation within this group of parasitoids is associated with host species rather than with spatial isolation, although geography naturally affects genetic structure within each cryptic species. Below, we discuss the host-associated cryptic species that we think exist and then contrast the genetic population structures among some of them through Europe and Asia.

Cryptic Species in the Åland Islands. In the Åland Islands in Finland, *C. melitaearum* agg. has two host species, *M. cinxia* and *M. athalia.* There are ≈4,000 habitat patches suitable for *M. cinxia*, of which 400–500 are occupied in any one year (Hanski 1999, Nieminen et al. 2004). *C. melitaearum* agg. in turn occupies 10–20% of the extant *M. cinxia* populations (van Nouhuys and Hanski 1999). *M. athalia* is a more abundant butterfly than *M. cinxia* in Åland, but it has a different primary habitat, forest edges and sparsely wooded meadows. Much less is known about the distribution and dynamics of *M. athalia* than of *M. cinxia*, and the relationship between *M. athalia* and *C. melitaearum* agg. is poorly known. The parasitoid has been reared from only three populations of *M. athalia*, in connection with the research on *M. cinxia.* We have not attempted to sample *M. athalia* larvae in its main habitats in the Åland islands because the larvae are cryptic and hence difficult to find.

The molecular results demonstrate conclusively that the two host butterflies in Åland are attacked by two different parasitoid species, though morphological characters to separate them have not been found (M. R. Shaw, personal communication). First, the respective parasitoids have distinct haplotypes of the mtDNA sequence, with a large nucleotide divergence

of 1.7% (Fig. 1). Second, the two species show no overlap at all in the allele frequencies of five microsatellite loci (Table 4). Third, our laboratory and field studies indicate that parasitoids reared from one host species do not recognize the other species as suitable hosts. A previous experiment by Wahlberg et al. (2001) found that *C. melitaearum* agg. reared from *M. cinxia* from Siberia did not parasitize *M. athalia* from Åland. And fourth, one more indication that *C. melitaearum* agg. parasitizing *M. cinxia* and *M. athalia* includes two distinct species is the spatial distribution and dynamics of the well-studied *Cotesia* attacking *M. cinxia.* This wasp has a limited spatial distribution in Åland, being present only in areas where the density of local *M. cinxia* populations is high (van Nouhuys and Hanski 2002b). If *M. athalia* were a regular host of the wasp using *M. cinxia,* it would be difficult to understand the spatially restricted occurrence of the *M. cinxia*-associated wasp, given that *M. athalia* occurs widely in the Åland Islands.

Although there is little doubt that the two host species are attacked by distinct parasitoid species, the biology of the *M. athelia*-associated taxon remains a challenge for further research. All existing records of *Cotesia* from *M. athalia* in Europe indicate a very low level of parasitism, suggestive of the respective *Cotesia* using some other host(s) and only occasionally attacking *M. athalia* (Eliasson and Shaw 2003, Kankare et al. 2005a). But such a biology would be surprising in view of this group of *Cotesia* being otherwise entirely restricted to checkerspots (Kankare and Shaw 2004).

Cryptic *C. melitaearum* Species Parasitizing Other Host Species. The microsatellite data provide ample evidence of significant gene flow between *C. melitaearum* agg. using *E. aurinia* and *E. desfontainii* in Spain (group 1 in Fig. 3). The conclusion that this parasitoid is a single species is further reinforced by the observation (Kankare et al. 2005a) that *C. melitaearum* agg. reared from *E. aurinia* successfully parasitizes *E. desfontainii* under laboratory conditions, whereas *C. melitaearum* agg. reared from other checkerspot species are unwilling to attack either *Euphydryas* species. Because all our samples from *E. desfontainii* were from northern Spain we do not know whether *E. aurinia* and *E. desfontainii* are shared hosts only locally or throughout their ranges.

The relationship between wasps reared from *E. aurinia*/*E. desfontainii* and those reared from *M. cinxia* is more ambiguous. Parasitoid samples across Europe group according to host species, not according to locality (Table 4, clustering analysis; Fig. 3, microsatellite distance tree). These findings suggest that though there is not much difference in the sequence data between *C. melitaearum* agg. from *E. aurinia*/*E. desfontainii* and from *M. cinxia,* there is nonetheless no or only very limited current gene flow between them.

Group 3 (Fig. 3), which includes not only *M. athalia* from Åland in Finland but also *M. parthenoides* from Spain and *M. deione* from Spain and France, shows that there are yet more subtle observations to consider. Although wasps from the three host species group together in both mtDNA (Fig. 1) and microsatellite data (Fig. 3) and are morphologically similar (M. R. Shaw, personal communication), there is in fact evidence supporting the view that they are each independent taxa. First, Kankare et al. (2005a) reared many ($n = 273$) *M. athalia celadussa* along with four other co-occurring checkerspot butterflies from one area in Spain. All the latter species were parasitized by *Cotesia,* yet no *Cotesia* were reared from *M. athalia celadussa,* which suggests that *M. athalia celadussa* is not a host for any of them, including *C. melitaearum* agg. reared from *M. deione.* Second, the microsatellite data for the wasps reared from *M. deione* were exceptionally homogeneous (Table 1), with every individual reared from *M. deione* in Spain being classified into the same group (9 in Table 3). This pattern was present even though wasps were collected from 18 different broods from five different local populations separated by up to 45 km. Given these results, it seems likely that *C. melitaearum* agg. reared from *M. deione* in Spain is a distinct species, which also is supported by the results of Kankare et al. (2005a) referred to below. Further research is needed to clarify the relationships among *C. melitaearum* agg. parasitizing *M. athalia* and *M. parthenoides.*

Geographical Population Structures. Considering *C. melitaearum* agg. reared from different host species as different taxa, we can compare their genetic population structures to infer more about their history. Wasps reared from the two widely distributed and common host species, *M. cinxia* and *E. aurinia,* show a contrasting pattern of haplotype diversity, with only two distinct haplotypes from *M. cinxia* but a cluster of eight rather similar haplotypes from *E. aurinia* (plus one more from the closely related *E. desfontainii;* Fig. 1). The additional complication here is that one of the *M. cinxia*-associated haplotypes is similar to the *E. aurinia*-associated haplotypes.

Postglacial history may explain the geographical distribution of the two haplotypes reared from *M. cinxia* (Fig. 2a). Both haplotypes occur in Asia, but only one of them (HT1) has been found in northern Europe. The host butterfly *M. cinxia* in Finland and Estonia represents an eastern clade and most likely migrated from a glacial refugium in the east (probably in Asia) after the last glacial maximum (Saccheri et al. 2004). It is possible that the specialist wasp spread to northern Europe from the same refugium. However, *M. cinxia* butterfly populations in Sweden, which have *C. melitaearum* agg. haplotype HT1 like the Finnish populations, are in fact very different from *M. cinxia* in Finland and belong to a central European clade (Saccheri et al. 2004). However, some *C. melitaearum* agg. individuals from Åland and from France were grouped into the same microsatellite cluster (Table 3), suggesting recent shared ancestry. The apparently dissimilar range expansions between the host and its parasitoids is interesting (Hochberg and Ives 1999) and worthy of further study. How the *M. cinxia*-associated *C. melitaearum* agg. acquired the haplotype that is similar to the haplotypes from *E. aurinia*/*E. desfontainii* remains an interesting open question. One pos-

sibility is a host switch from *E. aurinia* to *M. cinxia* in the past, followed by gene flow among the two *M. cinxia*-associated haplotypes.

At smaller geographic scales, among samples from a single host species, there are various patterns of spatial genetic structure. Some of the *Cotesia* samples consisted of individuals that fell into two or more clusters in the clustering analysis (Table 3). This would be an unexpected result if the samples came from single panmictic populations, but in fact several samples were collected from more than one local host population. *C. melitaearum* agg. is a poor disperser, typically moving distances <1 km per generation (van Nouhuys and Hanski 2002b); hence, significant genetic structure may occur within very small areas. The largest sample from the Åland Islands in Finland includes wasps collected from many local host populations within an area of 30 by 40 km and falling into several multilocus clusters (these data has been analyzed separately; Kankare et al. 2005b). A contrasting pattern is exhibited by *C. melitaearum* agg. reared from a large sample of *M. deione* collected from five distinct localities separated by up to 45 km in Spain and containing surprisingly little genetic diversity. The average number of alleles in the microsatellite loci was roughly twice as large in comparable samples from *E. aurinia*, *E. desfontainii*, and *M. cinxia* than from *M. deione* (Table 1). Perhaps this wasp is actually more dispersive than *C. melitaearum* agg. studied in Finland, or the host population structure possibly is more continuous, facilitating dispersal by the parasitoid.

In conclusion, genetic differentiation among *C. melitaearum* agg. samples reared from different host species across Europe and Asia presents a complex pattern that is definitely not consistent with the hypothesis of a single parasitoid species. The overall pattern, supported by the results of Kankare et al. (2005a) for a checkerspot community in Spain and by the broader phylogeny of checkerspot-associated *Cotesia* (Kankare and Shaw 2004), implies a far higher level of host specificity than previously thought. Kankare et al. (2005a) distinguished five cryptic species of *C. melitaearum* agg. in northern Spain, associated with *E. aurinia*/*E. desfontainii*, *M. cinxia*, *M. deione*, *M. didyma*, and *M. trivia.* Our results generalize these findings beyond the Spanish study area for *E. aurinia*, *M. cinxia*, and *M. deione*, and we can now add to the above list yet another form associated with *M. athalia.* It is apparent that there is no or only limited gene flow among *C. melitaearum* agg. using *E. aurinia* and *M. cinxia* throughout Europe and Asia.

The present results indicate that the radiation of checkerspot-associated *Cotesia* may have been driven by host phylogeny. *E. aurinia* and *E. desfontainii*, the two host species that most clearly share a single *Cotesia* species, are very closely related butterflies (Wahlberg and Zimmermann 2000). *M. athalia*, *M. deione*, and *M. parthenoides*, the hosts of *C. melitaearum* agg. in group 3 (Fig. 3), are all rather closely related, although not as closely related as *E. aurinia* and *E. desfontainii.* Whether host evolution has in turn been affected by the parasitoids remains an open question. Wahlberg et al. (2004) concluded that the radiation of checkerspot butterflies themselves has been affected by host plant taxonomy but that there is no evidence for the reciprocal influence (the host plant taxa are very much older than the butterflies).

Coevolutionary dynamics among checkerspots and their *Cotesia* parasitoids would not be an entirely surprising finding given the strong reciprocal ecological dynamics that can occur in this host–parasitoid system (van Nouhuys and Hanski 2004).

Acknowledgments

C. melitaearum agg. samples used in this study were collected by numerous researchers: H. Decimon, G.-C. Lei, A. Komonen, G. Néve, U. Norberg, J. Planas, D.L.J. Quicke, M. R. Shaw, M. Singer, L. Slaughter, C. Stefanescu, and N. Wahlberg. Parasitoid samples were morphologically identified by Mark R. Shaw (National Museums of Scotland). We thank Toshka Nyman for help in the laboratory and Tim Carr, Charles Godfray, Gavin Hinten, and Mark R. Shaw for comments on the manuscript. This study has been supported by the Academy of Finland (grants 38604 and 44887 to I. H., Finnish Centre of Excellence Programme 2000–2005).

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Received 11 June 2004; accepted 4 January 2005.