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Metapopulation genetic structure of two coexisting parasitoids of the Glanville fritillary butterfly

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Abstract We investigated the metapopulation genetic structure of two specialist parasitoids, *Cotesia melitaearum* and *Hyposoter horticola*, attacking the Glanville fritillary butterfly (*Melitaea cinxia*) in the Åland Islands south-western Finland. The host butterfly persists as a classic metapopulation in a network of 4,000 small habitat patches within an area of 50 by 70 km . The two parasitoids are known to differ greatly in their population dynamics and spatial pattern of occupancy in local host populations. Analysis of genetic population structure using F_{ST} and clustering of multilocus genotypes revealed a distinct large-scale spatial structure in *C. melitaearum* but a very weak pattern in *H. horticola*. This result is consistent with the known difference in the dispersal range (much longer in *H. horticola*) and population size (much greater in *H. horticola*) of the two parasitoids.

Keywords *Cotesia* · Dispersal · *Hyposoter* · *Melitaea cinxia* · Spatial structure

Introduction

The genetic structure of metapopulations is influenced by a large number of factors (Harrison and Hastings 1996; Pannell and Charlesworth 2000; Whitlock 2004), including the structure of the landscape (e.g. Manel et al. 2003), the dynamics of local populations, and the rate and range of dispersal of the focal species. The influence of the spatial structure of populations on their genetic structure has been studied in many insect species (e.g. Costa et al. 1996; Roderick 1996; Vaughn and Antolin 1998; Baker et al. 2003; Ingram and Gordon 2003; Sanetra and Crozier 2003). For instance, Massonnet et al. (2002) found high genetic differentiation among and significant deviations from Hardy–Weinberg and linkage equilibria in a third of the local populations of the aphid *Macrosiphoniella tanacetaria* in Germany. They concluded that frequent local extinction and colonization events had created a hierarchical metapopulation structure in the aphid. In contrast, Roslin (2001) showed for the specialist dung beetle *Aphodius fossor* that though the spatial population structure was highly aggregated, due to aggregated distribution of cattle pastures, the genetic population structure was strikingly homogeneous, suggesting extensive gene flow and possibly large and stable populations.

A potentially powerful approach to the study of spatial population structures involves the comparison of two or more species inhabiting the same landscape, because in such a case we may assume that possible differences in population structure are due to the biological properties of the species rather than particular features of the environment. The very few such studies that have been conducted so far have not been conducted in systems that would allow generalizations of the results and have reported only limited genetic differentiation for any of the species examined (e.g. Morehead et al. 2001; Kraushaar et al. 2002; Chapman et al. 2003; Molbo et al. 2004). There are a handful of informative studies comparing the spatial genetic structures of closely interacting species, such as parasitoids and their hosts. Johannesen and Seitz (2003) examined the population genetic structures of the tephritid gall fly *Urophora cardui* and its primary parasitoid *Eurytoma robusta*. They found that the populations of *E. robusta* were genetically more structured than the host populations, probably because the parasitoid

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has a lower rate of dispersal than the host. In an interesting study of *Aganthis* parasitoids and their *Greya* moth hosts, Althoff and Thompson (1999) demonstrated that the two species have incongruent geographical genetic structures, apparently due to dissimilar patterns of selection and gene flow among local populations. Subsequently, the same researchers have demonstrated that patchy distribution of the host food plant and variation in flowering times contribute to the genetic structure of *Aganthis* parasitoids by influencing their movements and gene flow (Althoff and Thompson 2001).

Here, we present an analysis of the spatial genetic structure of the two primary parasitoids of the Glanville fritillary butterfly, *Melitaea cinxia*, in the Åland Islands in south-western Finland. The two parasitoids, *Cotesia melitaearum* (Wilkinson) (Braconidae: Microgastrinae) and *Hyposoter horticola* (Gravenhost) (Ichneumonidae: Campopleginae), are completely specific to the Glanville fritillary in Åland. The natural history and metapopulation biology of the host butterfly (Hanski 1999; Ehrlich and Hanski 2004) and the parasitoids (Lei et al. 1997; Lei and Hanski 1998; van Nouhuys and Hanski 2002a, 2004; van Nouhuys and Ehrnsten 2004) have been studied in great detail, which provides a context in which the genetic population structures of the two parasitoids can be investigated with reference to their contrasting dispersal behaviour and spatial population structures.

M. cinxia is an endangered butterfly in Finland inhabiting dry meadows, where its larvae feed gregariously on the host plants *Plantago lanceolata* and *Veronica spicata* (Hanski 1999). In the Åland Islands, *M. cinxia* has mostly very small local populations, and the species persists regionally as a classic metapopulation with a high rate of population turnover (local extinctions and colonizations). Altogether there are about 4,000 habitat patches that are suitable for the butterfly within an area of 50 by 70 km, of which 400–500 are occupied in any 1 year (Hanski 1999; Nieminen et al. 2004). The suitable habitat patches are clustered into semi-independent patch networks (SINs; Hanski et al. 1996; see Discussion and illustrations in Nieminen et al. 2004). Butterflies migrate frequently among local populations within SINs, but their movements among SINs are limited by distance and barriers such as forest and water. The SINs vary in terms of the number, size, and connectivities of individual habitat patches within the network, and only high-quality SINs are occupied (those with a high metapopulation capacity; Hanski and Ovaskainen 2000).

The parasitoids *C. melitaearum* and *H. horticola* differ greatly in their biology. The notional species *C. melitaearum* is in a fact an aggregate of very closely related cryptic species that tend to be specific to just one checkerspot host butterfly species (Kankare and Shaw 2004; Kankare et al. 2004b). The species attacking *M. cinxia* across Europe and Asia, referred to as *C. melitaearum* agg. sp. H by Kankare et al. (2004b), is not known from any other host species. In this paper we refer to it simply as *C. melitaearum*. *C. melitaearum* is a gregarious parasitoid, several individuals developing in each host larva, the number depending on the size of the host larva. In Åland, there are two or three generations per host generation (year) depending on the year (Lei et al. 1997; van Nouhuys and Hanski 2004). *C. melitaearum* has a classic metapopulation structure in Åland, and it has been shown to contribute to local host extinctions when regional host density is high (Lei and Hanski 1997). However, it has a limited range of dispersal, roughly up to 1 km (Lei and Camara 1999; van Nouhuys and Hanski 2002a), and local populations tend to be very small and have a high risk of extinction (van Nouhuys and Tay 2001). It is hence not surprising that *C. melitaearum* only persists in those SINs with a tightly clustered set of sufficiently large host populations (van Nouhuys and Hanski 2002a). The total metapopulation size of *C. melitaearum* in Åland is presently extremely small, with <100 individuals in only 11 local butterfly populations in the spring 2003 (S. van Nouhuys, personal observation). The metapopulation size and distribution of *C. melitaearum* in Åland has declined greatly since the mid 1990s, possibly because the overall host metapopulation size has been relatively small for the past decade (I. Hanski and S. van Nouhuys, personal observation).

H. horticola is a univoltine solitary parasitoid with one generation per host generation (year). In contrast to *C. melitaearum*, this wasp is relatively large and dispersive, with a dispersal range greater (>5 km) than that of the host (3–4 km) and much greater than the dispersal range of *C. melitaearum* (van Nouhuys and Hanski 2002a). Consequently, isolation of host populations at the scale of Åland does not restrict the spatial occurrence of *H. horticola*, in striking contrast to the situation in *C. melitaearum*. Calculations presented in van Nouhuys and Hanski (2002a) suggest that the total metapopulation size of *H. horticola* is about 30% of the total metapopulation size of the host, which is 3,000–4,000 larval families in the autumn, corresponding to the order of 10,000–20,000 adult butterflies.

Based on the known movement ranges, spatial population dynamics, and (meta)population sizes of the two parasitoid species in the Åland Islands, we expect a pronounced spatial genetic structure in *C. melitaearum* but little spatial genetic subdivision in *H. horticola*. Direct observations of dispersal, especially by small insects that cannot be easily captured in the field and marked, are necessarily based on limited material and have been obtained under a limited set of temporal and environmental conditions. In contrast, the genetic spatial structure of populations integrates processes that have occurred over a prolonged period of time. The combination of the direct observation of movement and dynamics and genetic structure leads to a more comprehensive understanding of the biology of species (Bohonak 1999). If the two sources of information lead to the same conclusion then our conclusions about dispersal and the consequences of dispersal in this system

are strongly supported. On the other hand, new research into the behaviour and life history of the species would be warranted if the genetic population structures differ from what we would expect based on our observations of dispersal and local extinction and colonization dynamics.

Materials and methods

Sampling and rearing

The parasitoids for this study were obtained by sampling post-diapause host larvae in the spring in 1994–2002. Because the host and parasitoid populations were mostly very small, especially in the case of *C. melitaearum*, and we wanted to avoid perturbing them greatly, we were restricted to sampling only a small number of individuals from each population. In the laboratory, immature wasps emerging from the parasitized hosts were kept in ventilated plastic containers at room temperature until the adult wasps emerged. Adults were preserved in 96% ethanol. The host individuals that were not parasitized and which hence developed into butterfly pupae were returned to their natal populations.

The habitat patches suitable for *M. cinxia* make up over 100 SINs (Hanski et al. 1996), which are typically sets of small meadows clustered around small villages and associated cultivated fields and pastures (Nieminen et al. 2004). Because the parasitoid samples per local population are very small, frequently consisting of single individuals, and because both parasitoids can move relatively easily among host populations within a SIN, we use the SIN as a basic spatial unit in our analyses. Altogether, we accumulated a sample of 151 *C. melitaearum* from 30 local host populations in 15 SINs. In ten SINs the sample came from a single local host population, in the remaining SINs wasps were collected from two to five local host populations. Sample size per SIN varied from one to 32 individuals (median 5 individuals). The sample size for *H. horticola* consisted of 217 individuals from 124 local host populations within 37 SINs. The median number of local host populations sampled per SIN was 2 and the median sample size per SIN was 3 individuals (more than ten individuals from five SINs). With one exception, the samples for both species covered the same SINs and the entire ranges of the two parasitoids in Åland (Fig. 1).

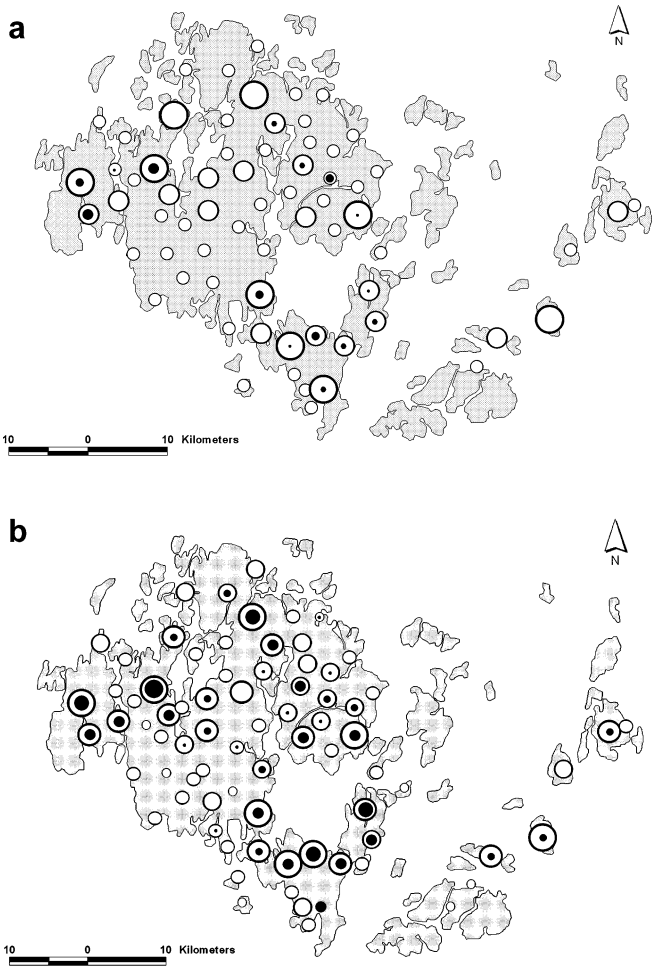

Fig. 1 A map of the Åland Islands showing the locations of the semi-independent patch networks (SINs) that have been occupied by the host butterfly and the samples sizes for *Cotesia melitaearum* (**a**) and *Hyposoter horticola* (**b**). The size of the *white circle* is proportional to the logarithm of the mean number of hosts (*Melitaea cinxia*) larvae in the SIN in the years 1996–2002 (**a**) and in the years 1994–2002 (**b**). The size of the *black circle* represents the sample size of the parasitoid on a logarithmic scale

Molecular analyses

DNA was extracted from a single *C. melitaearum* individual or one *H. horticola* hind leg using the NucleoSpin tissue kit (Macherey-Nagel) according to the manufacture's instructions except that 50 µl of milliQ water was used in the final elution stage for both species.

DNA variation in *C. melitaearum* was assayed at three microsatellite loci (*Cme1, Cme4*, and *Cme15*) isolated from *C. melitaearum* (Kankare et al. 2004a) and at six loci (*Cco1A, Cco27*, *Cco42, Cco65A*, *Cco65B*, and *Cco68*) originally isolated from *C. congregata* (Say) (Jensen et al. 2002). Four microsatellite loci were available for *H. horticola*: *Hho1*, *Hho3*, and *Hho5* isolated from *H. horticola* (Kankare et al. 2004a) and one, *VGT1*, isolated from *Venturia canescens* (Gravenhorst) (R. Butcher et al., unpublished data). Microsatellite polymerase chain reactions (PCRs) for *Cotesia* were performed as described by Kankare and Shaw (2004) and for *Hyposoter* according to the same protocols with locus specific primer concentrations and annealing temperatures as given in Kankare et al. (2004a). Diluted and pooled microsatellite PCR products were resolved in an ABI 377 automated DNA sequencer (PE; Applied Biosystems). Gels were analysed and fragments sized using GENESCAN version 3.1.2 and GENOTYPER version 2.5 programmes (PE; Applied Biosystems), respectively.

Statistical analyses

To characterize genetic diversity in the two parasitoid species, we calculated Nei's average gene diversity (H_e; Nei 1987) over all loci, observed heterozygosity (H_o), mean number of alleles (MNA), and the range of the number of alleles over all loci using the Excel Microsatellite toolkit (Stephen D. E. Park, http://acer.gen.tcd.ie/~sdepark/ms-toolkit/). Because of haplodiploidy in Hymenoptera, only data for females were used to calculate average gene diversity and the observed number of heterozygotes. The mean number of alleles and allele range were calculated using all individuals, taking into account that males are haploid.

We estimated deviations from Hardy–Weinberg equilibrium assessed by F_{IS} and from genotypic linkage equilibrium within SINs using the log-likelihood test (Goudet et al. 1996) in the program FSTAT 2.9.3.1 (Goudet 2001). Only SIN samples with more than two *C. melitaearum* or two *H. horticola* females were included in these analyses ($n = 7$ SINs for *C. melitaearum* and $n = 21$ SINs for *H. horticola*). We used Bonferroni correction for multiple tests. We calculated partial correlations between H_e and sample size, number of patches within SINs, and average connectivity (Hanski 1999) among the habitat patches within SINs. Only those SINs from which two or more females were available were included in this analysis. We calculated pairwise F_{ST} values (Weir and Cockerham 1984) and genetic differentiation between SINs using FSTAT. The effect of geographic distance on the degree of genetic divergence between pairs of SINs was examined by regressing $F_{ST}/(1-F_{ST})$ against the natural logarithm of the geographic distance. The significance of the Spearman correlation between the two distances was tested using the Mantel test (2,000 permutations; Mantel 1967) as implemented in GENEPOP (http://wbiomed.curtin.edu.au/ genepop; Raymont and Rousset 1995).

We investigated the genetic spatial structures of the two parasitoid species using the program STRUCTURE (Pritchard et al. 2000), which identifies clusters of genetically similar diploid individuals based on their multilocus genotypes without prior knowledge of their population affinities. Only females were used in this analysis. We used an admixture model with correlated allele frequencies to calculate the number of genetic clusters (K value) in the samples. We first performed several runs for each K value from 1 to 16 for *C. melitaerum* and from 1 to 8 for *H. horticola* (using 50,000 iterations, after a burn-in period of 50,000 steps). Based on these results, and to choose the best value of K, independent runs (200,000 iterations, 200,000 steps) were conducted using six to eight clusters for *Cotesia* and two, three, and four clusters for *Hyposoter* (100,000 iterations, 100,000 steps). An individual *C. melitaearum* was assigned to a cluster if the fraction of its genotype assigned to that cluster was >90%. For *H. horticola* the respective criterion was 80%, as there were too few individuals to be assigned with the 90% criterion.

Results

(Meta)population structures in Åland Islands

Sample sizes, H_e, H_o, MNA, and allele ranges in the pooled samples of the two species are presented in Table 1. Within patch networks (SINs), all *H. horticola* samples were in Hardy–Weinberg equilibrium, including samples consisting of individuals from more than one local population (21 out of 37 SINs). Moreover, no departures from linkage equilibrium were observed between pairs of loci after correcting for multiple tests. In contrast, *C. melitaearum* samples from three out of seven SINs showed a significant ($P < 0.05$) departure from the Hardy–Weinberg equilibrium due to heterozygote deficit over all microsatellite loci (after correcting for multiple tests). All these samples consisted of individuals originating from several local host populations within the respective patch networks, suggesting some degree of spatial structure within *Cotesia* metapopulations inhabiting particular SINs. Permutation tests for each locus pair did not indicate significant linkage disequilibrium.

The overall F_{ST} value for *C. melitaearum* was much greater ($F_{ST} = 0.378$) than that for *H. horticola* ($F_{ST} = 0.063$). Furthermore, pairwise F_{ST} values were generally small between SINs for the latter species ($F_{ST} = 0$–0.500, mean 0.100) but substantially greater for *C. melitaearum* ($F_{ST} = 0.113$–0.653, mean 0.297). None of the comparisons of pairwise genetic differentiation was significant for *H. horticola*, whereas six comparisons out of 21 (29%) yielded a significant result for *C. melitaearum*. There was significant isolation by distance in *C. melitaearum* (Mantel test, $r_S = 0.193$, $P = 0.022$; Fig. 2a) as well as in *H. horticola* ($r_S = 0.043$, $P = 0.028$; Fig. 2b), but the regression slope was significantly steeper for the former ($F_{1,\ 227} = 5.29$; $p = 0.022$). It should also be noted that the sample size was greater for *H. horticola*, providing more statistical power.

The sample sizes varied greatly among the SINs, as did the number of local host populations sampled and the connectivities among them. However, partial correlation analysis revealed no significant relationships between genetic diversity and any of these variables.

Table 1 Sample sizes of *Cotesia melitaearum* and *Hyposoter horticola*, and parameters of microsatellite diversity based on nine and four loci for *C. melitaearum* and *H. horticola*, respectively. *n* no. of individuals; n_{fem} no. of females; H_e average gene diversity (Nei 1987); H_o observed heterozygosity; *MNA* mean number of alleles, and the range allele over all microsatellite loci

Parasitoid species	n	n_{fem}	H_e	H_o	MNA	Allele range
Cotesia	151	68	0.31	0.15	4.00	1–15
Hyposoter	217	93	0.46	0.36	6.00	2–12

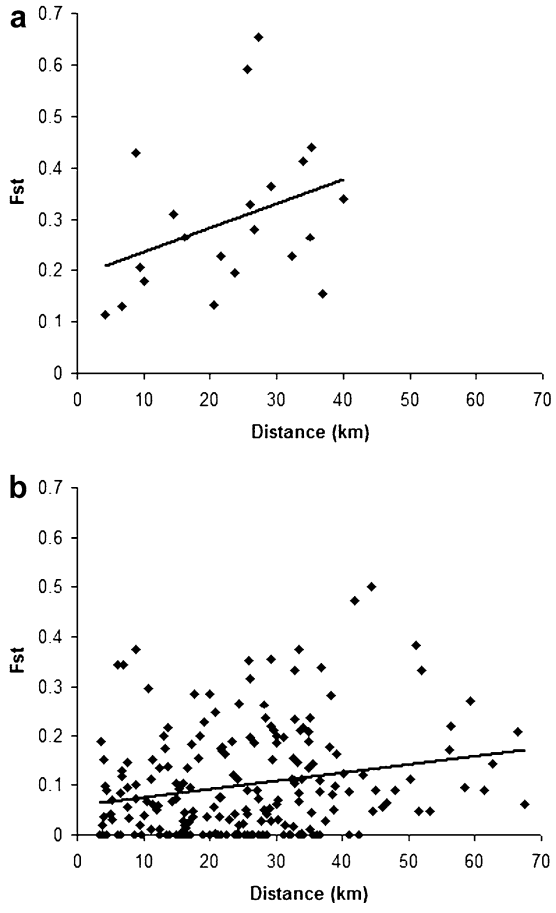

Fig. 2 For clarity the value of F_{ST} was plotted against the geographic distance (in kilometres) for pairs of *C. melitaearum* (**a**) and *H. horticola* (**b**) samples from different SINs in the Åland Islands. Both relationships are significant according to the Mantel test while regressing $F_{ST}/(1-F_{ST})$ with the natural logarithm of distance (*Cotesia*, $r_S = 0.193$, $P = 0.022$, $n = 7$; *Hyposoter*, $r_S = 0.043$, $P = 0.028$, $n = 21$)

Clustering of multilocus genotypes

Clustering of multilocus genotypes placed *C. melitaearum* and *H. horticola* individuals into several clusters. The models that explained the data best ($P \sim 1.000$) partitioned *C. melitaearum* into seven clusters and *H. horticola* into three clusters. All the other models were inadequate ($P < 0.001$). There was a clear spatial pattern in the occurrence of *C. melitaearum* clusters, with individuals from the southern part of the main Åland Island placed in clusters 1, 5, and 6, and individuals from the western part of Åland belonging to clusters 2, 3, 4, and 7 (Fig. 3a). There is also temporal consistency in the spatial occurrence of the clusters. For example, individuals belonging to cluster 2 were sampled from the populations shown in Fig. 3a in years from 1998 to 2002, whereas individuals belonging to cluster 4 were identified from the local host populations indicated in Fig. 3a in samples taken from 1997 until 2001. Unfortunately, because of the small sample sizes, limited by the smallness of the populations themselves, a more refined analysis cannot be conducted.

The spatial pattern of multilocus clusters is very different for *H. horticola* (Fig. 3b). Here individuals belonging to the three different clusters occur across the entire Åland Islands. It is noteworthy that whereas in *C. melitaearum* a large fraction of individuals (70%) was assigned to a particular cluster with a high probability($P > 0.9$; Fig 4a), only 20% of *H. horticola* individuals were assigned to one cluster with the corresponding probability ($P > 0.9$; Fig. 4b).

Discussion

The key finding of this study is that *C. melitaearum* has a more distinct spatial genetic structure than *H. horticola* in the Åland Islands, in spite of the fact that the two parasitoids share a single host species, the Glanville fritillary butterfly. The overall F_{ST} value of 0.378 for *C. melitaearum* was substantially greater than the respective value for *Hyposoter* ($F_{ST} = 0.063$). None of the *H. horticola* "metapopulations" within SINs showed significant genetic structure (deviations from Hardy–Weinberg equilibrium), whereas *C. melitaearum* in three SINs showed such structure, even though the statistical power of the test is low due to very small sample sizes. There was a significant isolation by distance relationship in both parasitoid species when studied at the level of SINs, but the strength of this relationship was greater for *C. melitaearum*. Another way of gauging the degree of mixing that exists in a region is by looking at the proportion of individuals that are assigned to a given cluster of multilocus genotypes. This proportion was much higher for *C. melitaearum* than for *H. horticola* (Fig. 4), and the clusters into which individuals were assigned corresponded to geographical structure in *C. melitaearum* but not in *H. horticola*.

The spatial genetic structures in the two parasitoid species are consistent with expectations based on their biologies. *C. melitaearum* is a poor disperser, with individuals typically dispersing < 1 km, and this species is only present in well-connected host populations (Lei and Hanski 1997; van Nouhuys and Hanski 2002a). In contrast, *H. horticola* is very mobile, potentially dispersing distances up to at least 5 km (van Nouhuys and Hanski 2002a). This parasitoid is present in virtually all host populations regardless of their age (years since colonization) or connectivity (van Nouhuys and Hanski 2002a,b; van Nouhuys and Ehrnsten, 2004). Gene flow due to high migration rate is apparently preventing significant genetic differentiation among populations of *H. horticola*. We note that the global F_{ST} value among local populations of the host butterfly is 0.1 (Saccheri et al . 2004), which is intermediate between the values for the two parasitoids (0.063 and 0.378). This result agrees nicely with the observed dispersal distances in the three species (van Nouhuys and Hanski 2002a): up to 1 km in

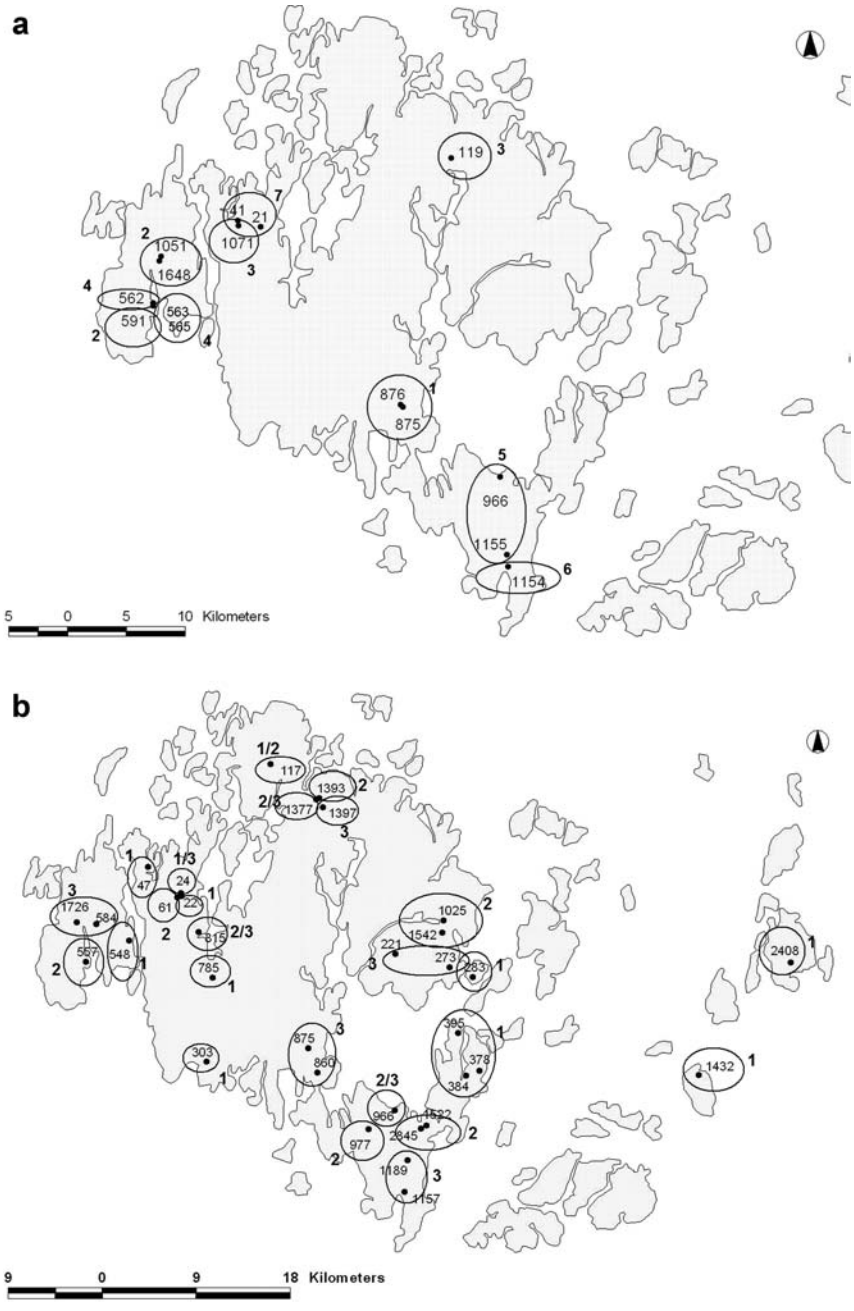

Fig. 3 A map of the Åland Islands showing the locations of individuals belonging to different clusters of multilocus genotypes in *Cotesia* (**a**) and in *Hyposoter* (**b**)

C. melitaearum, 3–4 km in the host butterfly, and >5 km in *H. horticola*.

The present analysis of spatial genetic structures in the two parasitoids is limited by the sample sizes that are available from the mostly very small local populations. Nonetheless, comparison of the two species produced informative results in spite of the low statistical power of the analyses. The good correspondence between previous ecological results and the present genetic results points to the conclusion that the two species exhibit markedly different spatial dynamics with significant consequences for the maintenance of spatial genetic structure and ultimately for their evolutionary dynamics in the spatial context.

(Meta) population structures

Many insect species with limited dispersal capacity live in fragmented landscapes, in which they have a metapopulation structure with high population turnover (Hanski et al. 2004). The genetic structure of such metapopulations is strongly affected by local extinctions, recolonizations, and spatially structured dispersal (for reviews see Pannell and Charlesworth 2000; Whitlock 2004). The spatial distribution of specialized parasitoids such as *C. melitaearum* and *H. horticola*, which are entirely dependent on a single host species, can only be more fragmented than that of their host, and the habitat for the parasitoid (consisting of local host pop-

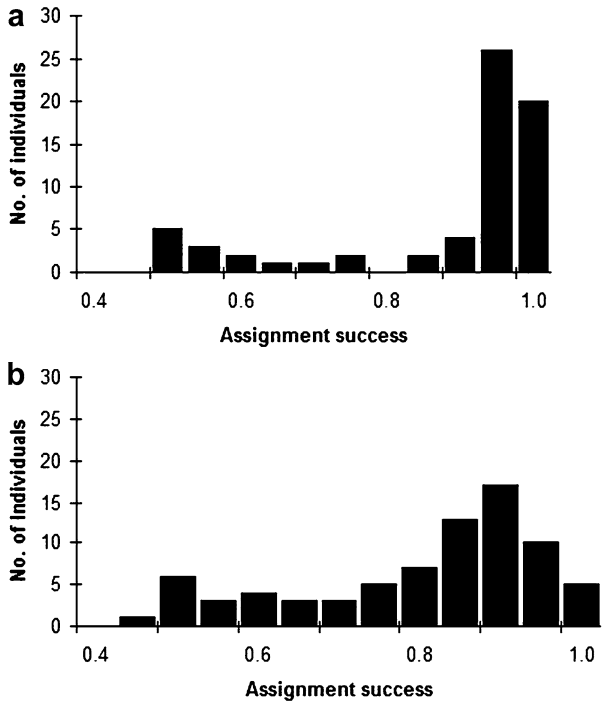

Fig. 4 Frequency distributions of the maximum assignment probability to a particular cluster in *Cotesia* (**a**) and in *Hyposoter* (**b**)

ulations) is necessarily more dynamic than the habitat for the host.

There is ample ecological evidence of metapopulation structure both for the host butterfly *M. cinxia* (Hanski 1999) and for *C. melitaearum* (Lei and Hanski 1997; van Nouhuys and Hanski 1999) but not for *H. horticola* (van Nouhuys and Hanski 2002a). In agreement with high rates of population turnover but relatively little gene flow among existing populations, the host butterfly shows spatial genetic structuring among local populations within patch networks, among patch networks, and regionally (Saccheri et al . 2004). *C. melitaearum* disperses shorter distances than its host and experiences a more fragmented and less stable habitat, and our results show that genetic structuring in the parasitoid occurs at similar spatial scales to those in the host. *H. horticola* on the other hand maintains an overall population size proportional to that of the host butterfly at any spatial scale within Åland (van Nouhuys and Ehrnsten 2004). This can only happen because individuals move frequently among local host populations and patch networks, as indicated by the lack of clustering of *H. horticola* into spatially distinct genetic units.

The spatial genetic structure of *C. melitaearum* indicates that individuals inhabiting different local host populations within a patch network do not mix freely and hence comprise a metapopulation at the patch network level. Genetic differentiation is likely to be enhanced by the generally very small local population sizes, often as few as ten individuals in the early spring generation (van Nouhuys and Tay 2001). The number of colonists to new populations must also be very small and their relatedness is likely to be high. In contrast, *H. horticola* has a relatively large population size, parasitizing roughly one-third of the host individuals in local host populations. *H. horticola* populations were in Hardy–Weinberg equilibrium within patch networks (SINs), suggesting that individuals inhabiting such a network, even if they come from several distinct local host populations, are from a single panmictic parasitoid population.

With increasing loss and fragmentation of the habitat for the host, a specialist parasitoid will reach the landscape-level threshold for long-term persistence sooner than the host (van Nouhuys and Hanski 2002b, 2004). *C. melitaearum* is in fact an example of a species on the brink of regional extinction due to the relatively sparse occurrence of the host populations in recent years. Currently, there are only 11 tiny local populations of the parasitoid in the entire Åland Islands (S. van Nouhuys, personal observation). The decline in metapopulation size and distribution of the parasitoid is reflected in the genetic population structure with low average heterozygosity, high overall F_{ST} value, and clear spatial aggregation of the populations.

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