

PHYLOGENY AND HOST ASSOCIATIONS OF *COTESIA*
PARASITOIDS ATTACKING CHECKERSPOT
BUTTERFLIES

Maaria Kankare

Metapopulation Research Group
Department of Biological and Environmental Sciences
Division of Population Biology
University of Helsinki
Finland

Academic dissertation
To be presented, with permission of the Faculty of Biosciences
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of Viikki Biocenter 2 (Viikinkaari 5)
on May 28th 2004 at 12 o'clock noon.
Helsinki 2004

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Cover illustration: *Cotesia melitaeorum* by Zdravko Kolev

Author's address:

Department of Biological and Environmental Sciences
Division of Population Biology
P.O. Box 65 (Viikinkaari 1)
00014 University of Helsinki
Finland
e-mail: maaria.kankare@helsinki.fi

ISBN 952-91-7125-0 (Paperback)
ISBN 952-10-1820-8 (PDF, <http://ethesis.helsinki.fi>)

Yliopistopaino
Helsinki 2004

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The thesis is based on the following articles and manuscripts, which are referred to in the text by their Roman numerals:

- I** Kankare, M. and Shaw, M.R. (2004) Molecular phylogeny of *Cotesia* (Hymenoptera: Braconidae: Microgastrinae) parasitoids associated with Melitaeini butterflies (Lepidoptera: Nymphalidae: Melitaeini). *Molecular Phylogenetics and Evolution*. In press.
- II** Kankare, M., Stefanescu, C., van Nouhuys, S. and Shaw, M. R. Host specialization by *Cotesia* wasps (Hymenoptera: Braconidae) parasitising species-rich Melitaeini (Lepidoptera: Nymphalidae) communities in north-eastern Spain. Submitted.
- III** Kankare, M., van Nouhuys, S. and Hanski, I. Genetic divergence among host-specific cryptic species in *Cotesia melitaeorum*, a parasitoid of checkerspot butterflies. Submitted.
- IV** Kankare, M., van Nouhuys, S., Gaggiotti, O. and Hanski, I. Metapopulation genetic structure of coexisting primary parasitoids of the Glanville fritillary butterfly. Submitted.
- V** Kankare, M., Jensen, M. K., Kester, K. M. and Saccheri, I. J. Characterization of microsatellite loci in two primary parasitoids of the butterfly *Melitaea cinxia*, *Cotesia melitaeorum* and *Hyposoter horticola* (Hymenoptera). *Molecular Ecology Notes*. In press.

CONTRIBUTIONS

The following table shows the major contributions of authors to the original articles or manuscripts

	I	II	III	IV	V
Original idea	IH	IH, MK	IH, MK	IH	MK
Study design	MK	MK	MK	MK	MK
Biological Data gathering	*	CS	*	GL, SvN	*
Analyses	MK	MK, SvN	MK, SvN	MK	MK
Manucript preparation	MK, MRS	MK, SvN, CS, MRS	MK, SvN, IH	MK, SvN, OG, IH	MK

CS = Constantí Stefanescu, GL = Guangchun Lei, IH = Ilkka Hanski, MRS = Mark R. Shaw,
OG = Oscar Gaggiotti, SvN = Saskya van Nouhuys,

* = numerous researchers in different countries

Supervised by Prof. Ilkka Hanski
University of Helsinki
Finland

Reviewed by Doc. Craig Primmer
University of Helsinki
Finland

Doc. Heikki Roininen
Finnish Forest Research Institute
Finland

Examined by Prof. Pekka Pamilo
University of Oulu
Finland

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1. INTRODUCTION

1.1 Host associations and cryptic species in parasitoid wasps

Parasitoids are insect species whose larvae develop by feeding on the bodies of other arthropods, usually insect (Godfray, 1994). Parasitoids are abundant and diverse insects that are present in nearly all terrestrial ecosystems, and it has been suggested that parasitoids may constitute 20-25% of all insect species (Godfray, 1994). Most parasitoids are either wasps (Hymenoptera) or flies (Diptera) (Godfray, 1994). The hymenopterans parasitoids studied in this thesis belong to the families Braconidae and Ichneumonidae, in the superfamily Ichneumonoidea. Braconidae in particular are mostly relatively host-specific, being adapted to attack and feed as larvae on a narrow range of host species. As they have such an intimate relationship with their host, they have been widely used in biological control of insect pests.

Parasitoids are categorised by developmental characteristics that directly or indirectly influence certain ecological parameters, such as the potential for a wide host range. Koinobiont parasitoids let their host continue to develop after the female parasitoid has oviposited into it, whereas idiobionts feed on corpses or paralysed hosts. Koinobionts have an intricate physiological relationship with their host and consequently tend to have relatively narrow host ranges (Haeselbarth, 1979; Askew and Shaw, 1986),

while idiobionts have potentially broader host ranges than koinobionts. However, in practice many idiobionts are so niche-specific that there is only a narrow range of host species available to them.

It is typically difficult to judge parasitoid host ranges based on literature records (Shaw, 1994), because these records are full of errors and because closely related species (especially koinobionts) can have quite a spread of host ranges, from extreme specialists to species that have much wider host ranges. As far as is known, few parasitoids are absolutely host specific (have a single host species), though many parasitoid species that have a fairly narrow host range may be entirely host-specific locally.

Cospeciation refers to joint speciation (parallel cladogenesis) of unrelated taxa that are ecologically associated (Page, 2003). Most studies of cospeciation have been focused on host-parasite complexies. Though host-parasitoid relationships are exceedingly common in nature, relatively few studies have investigated coevolution and cospeciation in these interactions. For example, Stiremann and Singer (2003) studied host use of parasitoid flies in the family Tachinidae. They found that host abundance, gregariousness, food-plant type, and morphology were all important determinants of tachinid host use, but they found little concordance between the phylogenetic relationships of the tachinid parasitoids and their hosts. A common view among parasitoid taxonomists appears to be that parallel cladogenesis has not played an important role in parasitoid radiations (Godfray, 1994), but certainly molecular studies will be helpful in critically testing these questions.

1.2 Molecular markers in studies of cryptic species

Molecular markers are increasingly used in studies of insect population structure, genetic divergence, and species richness (e.g. 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; Stone *et al.*, 2001; Alvarez and Hoy, 2002; Chen *et al.*, 2002; Abrahamson *et al.*, 2003; Rokas *et al.*, 2003). Molecular markers are often the only practical tools to discriminate between morphologically and ecologically very similar species, which may be common in parasitoids. Knowledge of possible cryptic species is naturally essential for any ecological or evolutionary study of the taxa, including the study of their host associations but also, host-parasitoid community structure and dynamics (Althoff and Thompson, 1999; van Nouhuys and Hanski, 2002b), or the effect of local mate-competition for the coexistence of species (Zhang *et al.*, 2004). When ecologically similar species coexist there is potential for direct and indirect interactions among them, including direct and apparent competition that can affect the community structure (Holt, 1977; Holt and Lawton, 1994; Bonsall and Hassell, 1997).

Several recent studies using molecular data have revealed unexpectedly great host specificity, ecological diversity, and the presence of cryptic species in parasitoids. For example, Hoy *et al.* (2000) showed with conserved coding genes (Actin 1 and 2) that the introduced parasitoids *Ageniaspis* imported into the US from Australia and Taiwan were actually two cryptic species. In another study, Jager and Menken (1994) used RAPD-

PCR to report presence of cryptic species in the parasitoid wasp *Ageniaspis fuscicollis* reared from different host species from one locality in Europe.

Extensive molecular phylogenies for various taxa have been used to study the status of species in complex species assemblages. For example, Gómez *et al.* (2002) investigated the species complex of passively dispersing rotifer *Brachionus plicatis* (Rotifera: Monogononta) by constructing phylogenies based on mtDNA and nuclear genes. They found several genetically divergent lineages of which many were sympatric, and they found no evidence for hybridization or introgression, suggesting a reproductive isolation between the lineages. These researches concluded based on their own study and on other published results that each of the lineages represented a distinct biological species or species group. In another phylogenetic study, Sharpe *et al.* (2000) studied intra- and interspecific molecular variation in the *minimus* group of *Anopheles* (Diptera: Culicidae) with mtDNA and nuclear genes. Based on their results, they were able to confirm the presence of two previously recognized cryptic species and they found evidence for yet another cryptic species.

More recently it has been shown that microsatellite markers can be useful in studies of closely related species (Takezagi and Nei, 1996) and for testing present or recent gene flow among populations. For example, Molbo *et al.* (2003) used microsatellites and mtDNA to demonstrate the coexistence of several unrecognized cryptic fig wasp species. They concluded that some cryptic species pairs sharing the same host seemed to be sister taxa while others did not. They suggested that existence of cryptic species

may be a common feature of fig wasps worldwide. In another study using microsatellites, McCoy *et al.* (2001) found evidence of sympatric tick races infesting different seabird host species. High genetic variation was found among ticks collected from different sympatric hosts but little differentiation among ticks gathered from the same host species. These researchers concluded that host-related selection pressure has led to specialization in this species and that host race formation may be an important mechanism for explaining the rich diversity in parasites in general.

1.3 *Cotesia* species attacking checkerspot butterflies

The genus *Cotesia* (Hymenoptera: Braconidae: Microgastrinae) is a large group of primary parasitoids of Lepidoptera, with about 1500-2000 species worldwide (Mason, 1981). It is entirely associated with Lepidopteran hosts (Shaw and Huddleston, 1991), records from other insects almost certainly being erroneous (M. Shaw, pers. comm.). Many *Cotesia* species are important natural enemies of agricultural and forestry pests, and several species have been used as biocontrol agents. For example, *C. glomerata* (Linnaeus) is a common parasitoid of the Eurasian cabbage white butterflies (*Pieris*).

The biology of the *Cotesia* species is diverse and the taxonomy is controversial with relatively recent usage of the generic name *Cotesia* (Mason, 1981). Previously the traditional name *Apanteles* Foerster, was used in the literature (Mason, 1981). All *Cotesia* species are koinobiont endoparasitoids, meaning that their host continues to develop af-

ter the female parasitoid has oviposited into it. Many *Cotesia* are specialist parasitoids and hence their population dynamics must be strongly coupled with the dynamics of their hosts (Porter, 1981; Lei and Hanski, 1997; van Nouhuys and Hanski, 2004). The potential impact of many *Cotesia* species on their host populations is unusually large, partly because they may have several generations during a single host generation, and because they have large broods (clutches) when parasitizing large (older) host larvae.

The *Cotesia* species parasitizing checkerspot butterflies (Melitaeini) are not known to parasitize any other species of butterflies (van Nouhuys and Hanski, 2004). Several species of *Cotesia* are key parasitoids of Melitaeini in Eurasia and North America (van Nouhuys and Hanski, 2004). There are seven described species of *Cotesia* that are known to parasitize Melitaeini. *Cotesia acuminata* (Reinhard), *C. bignellii* (Marshall), *C. cynthiae* (Nixon), *C. lycophron* (Nixon) and *C. melitaeorum* (Wilkinson) occur in Europe and Asia, and *C. euphydryidis* (Muesebeck) and *C. koebelei* (Riley) occur in North America. Some of these species are recorded from a single host species throughout their range, while others have been recorded from several host species. The traditional morphological treatment of European *Cotesia* (as the *Apanteles glomeratus*-group) by e.g. Nixon (1974) and Papp (1986, 1987) strongly suggests that two separate monophyletic clades are associated with Melitaeini. One of the clades includes *C. acuminata* and *C. bignellii*, and is morphologically isolated with respect to other *Cotesia*, whereas the other clade (*C. melitaeorum*, *C. cynthiae* and the enigmatic *C. lycophron*) is more similar to some other *Cotesia* species.

1.4 The host butterflies

Melitaeini is a distinct group of butterflies in the family Nymphalidae, consisting of ca 250 species widely distributed in the Holarctic and Neotropic regions (Higgins, 1981; Wahlberg and Zimmermann, 2000). Several Melitaeini species may co-occur in the same habitat (Murphy *et al.*, 2004), for example, in northern Spain, which has an exceptionally high diversity of butterflies (Stefanescu *et al.*, 2004), up to eight species of Melitaeini may co-occur. Adults of most Melitaeini species are relatively sedentary and females usually lay eggs in clusters.

Melitaeini larvae tend to live gregariously in silken nests for at least the first few instars and diapause during climatic extremes (summer or winter; Kuussaari *et al.*, 2004). A single butterfly species may use many plant species in several genera over its geographic range, but locally larvae are generally restricted to one or a few host plant species (Singer, 2004). Melitaeini have been widely used in ecological and evolutionary studies for more than four decades (e.g. Ehrlich *et al.*, 1975; Thomas and Singer, 1998; Hanski, 1999; Ehrlich and Hanski, 2004). There are records of egg, larval and pupal parasitoids of Melitaeini, which involve species from both Hymenoptera

and Diptera (van Nouhuys and Hanski, 2004). The interactions between several species of Melitaeini and their parasitoids have been studied in detail. For example, host-parasitoid interactions involving *Euphydryas editha* (Boisduval) (White, 1973; Moore, 1989a,b) and *E. phaeton* (Drury) (Stamp, 1981a,b, 1982) have been studied in North America, and *E. aurinia* (Rottemburg) (Ford and Ford, 1930; Porter, 1981, 1983; Eliasson and Shaw, 2003), *E. maturna* (Linnaeus) (Eliasson and Shaw, 2003) and *Melitaea cinxia* (Linnaeus) (Lei *et al.*, 1997; van Nouhuys and Hanski, 2004) have been studied in Europe.

To date, most of the host-parasitoid studies involving Melitaeini have been conducted in areas where only one or a few hosts co-occur (but see Eliasson and Shaw, 2003). However, there are many localities in southern Europe and Asia where more than five Melitaeini species may co-occur (e.g. Komonen, 1998; Wahlberg *et al.*, 2001; Stefanescu *et al.*, 2004). In these communities there is the potential for the population dynamics of Melitaeini species to be linked with one another through shared host plants and shared parasitoids (van Nouhuys and Hanski, 2005). The degree to which this occurs may offer clues to the ecological and evolutionary processes involved in host specialization.

2. OVERVIEW OF THIS THESIS

During the several years of study the contents of this thesis changed substantially. The original idea was that this thesis would consist of two parts. The aim of the first part was to build a molecular phylogeny of *Cotesia* parasitoids associated with Melitaeini hosts. In the second part, I planned to study the spatial genetic structure of the parasitoids of *Melitaea cinxia* in the Åland Islands, including the two primary and two secondary parasitoids in the system. Owing to difficulties in marker development for these four species I decided to put the main effort on microsatellite isolation for the two primary parasitoids and to limit the comparisons to this trophic level.

In the construction of the molecular phylogeny for *Cotesia*, great effort was made to get samples from as many host butterfly species and localities as possible. To achieve this goal, several researchers in Europe, Asia and in North America contributed *Cotesia* samples. Below, I briefly summarize the aims and key achievements of each of the chapters in this thesis.

In the first Chapter (I) we analysed the broad phylogenetic relationships among the *Cotesia* species parasitizing Melitaeini and reared from a large number of populations of several host species in Europe, Asia, and North America, using DNA sequence and microsatellite data. In the course of this work, it became evident that there exist several cryptic, previously unrecognized species of *Cotesia*. This work set the stage

for the more detailed investigations that followed.

In the second Chapter (II), we studied the ecological and genetic structure of the Melitaeini-*Cotesia* community in Catalonia, northern Spain, within a relatively small geographical area that should not pose any physical barriers to gene flow. We collected information on the natural history of most of the host butterflies, including data on their larval host plants and phenology, as well as on the composition of the local parasitoid communities. This large sample turned out to be particularly informative, and it provided a great opportunity to study the structure of an entire community of Melitaeini-associated *Cotesia*. The study region has one of the richest butterfly fauna in Europe (Martín and Gurrea, 1990; Dennis and Williams, 1995; Stefanescu *et al.*, 2004), and 10 out of the 20 European species of Melitaeini occurs here (Tolman and Lewington, 1997). Remarkably, this study revealed the presence of no less than seven biologically distinct species of *Cotesia* within a relatively small geographic area. In particular, *Cotesia melitaeorum* agg. and *C. acuminata* agg. were each found to represent a series of cryptic species with narrow host associations. This finding led us to study the *C. melitaeorum* aggregate in a greater detail with samples from several host species gathered throughout Europe and Asia.

The pattern of genetic variation and evolutionary divergence among *C. melitaeorum* agg. populations from several host species is presented in the third Chapter (III). Molecular results for *C. melitaeorum* agg. reared from two host

species, *Melitaea cinxia* and *M. athalia*, in the Åland Islands, were supplemented with intensive field observations on the rate of parasitism and laboratory experiments on the behaviour of parasitoids encountering host larvae. The results of this study are in agreement with those in Chapter II, and we now confirmed that a single *Cotesia* species may parasitize two closely related host species. We also found a more complex pattern of divergence between the *Cotesia* species attacking the two widely distributed and common host species, *Melitaea cinxia* and *Euphydryas aurinia*.

In the fourth Chapter (IV) we compared the small scale spatial genetic structures of *C. melitaeorum* agg. from one host species, *Melitaea cinxia*, in the Åland Islands. In this study we also compared the genetic structure of *C. melitaeorum* agg. with that of *Hyposoter horticola* (Gravenhost) (Ichneumonidae: Campopleginae), the other primary parasitoid attacking *M. cinxia* in the Åland. We also extended the comparison of the

two species to a larger scale in Europe. This study confirmed the tendency of *C. melitaeorum* to exhibit genetic differentiation even at relatively small spatial scales, in contrast to *H. horticola*. These results agree with the strikingly different mobility of the two parasitoids, *C. melitaeorum* having a shorter but *H. horticola* a longer range of dispersal than the host butterfly (van Nouhuys and Hanski, 2002a).

The fifth Chapter (V) describes the development of the microsatellite markers used in all the other chapters. We isolated 5 microsatellite loci for *C. melitaeorum* and 3 for *H. horticola*. We quantified the allelic diversity and heterozygosity in samples from the Åland Islands as well as from other localities around Europe. The microsatellite loci for *C. melitaeorum* agg. from Åland have been amplified in many other *Cotesia* species from several different host species in Europe, Asia and North America

3. MATERIAL AND METHODS

3.1 Samples

Cotesia were sampled as cocoons in the field or (mostly) reared from field-collected host larvae collected by numerous researchers. Some samples were only available as dried, pinned museum specimens. Samples for the broad phylogenetic study (I) were obtained from a large number of populations of altogether 16 host species in Europe, Asia, and North America (Appendix A in Chapter I). For the second Chapter (II), host larvae were collected from 17 sites in Catalonia, NE Spain. In this case, seven out of nine Melitaeini species sampled were parasitized by *Cotesia*. In addition, other parasitoid species apart from *Cotesia* were collected and are detailed in Chapter II (Table 2). For the third Chapter (III), *Cotesia melitaeorum* agg. were collected from the two common Palaearctic host species, *Melitaea cinxia* and *Euphydryas aurinia*, across many localities in Europe and Asia, as well as from four more narrowly distributed European host species. For the fourth Chapter (IV), *C. melitaeorum* agg. were collected from 30, and *Hyposoter horticola* from 123, local host populations in the Åland Islands in Finland. In addition, *H. horticola* samples were collected from one locality in Sweden (10 individuals) and Spain (7 individuals) and from 5 different localities in Estonia (41 individuals).

3.2 Molecular analyses

A general overview of the molecular methods and analyses used in this thesis is given below. Detailed laboratory pro-

cedures and statistical methods are presented in the original papers.

3.2.1 DNA sequence analyses

DNA fragments from mitochondrial cytochrome oxidase subunit I (COI) and NADH1 dehydrogenase (ND1) genes and from nuclear ribosomal DNA internal transcribed spacer region (ITS2) (altogether 2200 bp) were sequenced to study the phylogenetic relationships among *Cotesia* (I). In Chapters II and III, mtDNA analyses were based on sequences of the COI gene (1500 bp). Phylogenetic analyses were performed using the maximum parsimony (MP) and maximum likelihood (ML) criteria in the first Chapter (I) and MP criteria in the other two Chapters (II and III), with the program PAUP version 4.0b10 (Swofford, 2002). For the ML analysis, the most appropriate substitution model was selected with Hierarchical Likelihood Ratio Tests (hLRTs), implemented in MODELTEST program, version 3.06 PPC (Posada and Crandall, 1998).

3.2.2 Microsatellites

Altogether 12 microsatellite loci for *Cotesia* were used in this thesis. Five of these loci, *Cme1*, *Cme3*, *Cme4*, *Cme15* and *Cme17* were isolated from *Cotesia melitaeorum* as detailed in Chapter V. Seven microsatellite loci, *Cco1A*, *Cco5A*, *Cco27*, *Cco42*, *Cco65A*, *Cco65B* and *Cco68*, were originally isolated from *Cotesia congregata* (Say) (Jensen *et al.*, 2002). All the 12 microsatellite loci were used to study the phylogenetic relationships among *Cotesia* (I) and to examine the genetic structure of *C. melitaeorum* agg. from several host species in

Europe and Asia (III). Ten of the twelve loci (as two *Cme* loci did not amplify constantly) were used in the analysis of a smaller group of co-occurring *Cotesia* in northern Spain (II) and nine loci (as three of the twelve loci were monomorphic in this scale, V) in the study of metapopulation genetic structure of *C. melitaearum* agg. in the Åland Islands (IV). Four microsatellite loci were used for *H. horticola*: *Hho1*, *Hho3*, and *Hho5* isolated from *H. horticola* (V) and one, *VGT1*, originally isolated from *Venturia canescens* (Gravenhorst) (Butcher, Kankare, Hubbard and Whitfield, in prep.).

3.3 Experiments on host acceptance behaviour

In the second Chapter (II) we observed the behaviour of adult female *C. melitaearum* agg. from three different host species when provided with the original host species as well as with nine other host species from several localities (II, Table 3). In the third Chapter (III) the oviposition behaviour of *C. melitaearum* agg. females reared from *Melitaea athalia* was observed when provided with *M. athalia* and *M. cinxia* larvae. Behaviour of *C. melitaearum* agg. reared from *M. cinxia* was also recorded with all instars of *M. athalia* larvae in the laboratory.

3.4 Methods of data analysis

Interspecific and intraspecific genetic distances were estimated with Cavalli-Sforza and Edward's (1967) chord distance (D_{CE}) using microsatellite data. The genetic distance matrices were used to construct Neighbor-Joining (N-J) and consensus trees.

The genetic samples were characterized with several measures. We calculated Nei's expected gene diversity (H_e ; Nei, 1987), observed heterozygosity (H_o), mean number of alleles (MNA) over all loci and allele ranges for samples from each host species in each Chapter (I–V). In addition, we estimated deviations from the Hardy-Weinberg (HW) equilibrium (assessed by F_{IS}) and from the genotypic linkage equilibrium using log-likelihood tests in FSTAT 2.9.3.1 (Goudet, 2001) in each sampling locality (II–V). The Bonferroni correction was applied for multiple tests. FSTAT was also used to test for variance in allele frequencies (F_{ST} ; Weir and Cockerham, 1984) and pairwise genetic differentiation in each population (II–IV). Correlations between genetic ($F_{ST}/(1-F_{ST})$) and geographic distances (III–IV) were tested using Spearman rank correlation coefficient (r_s). The significance of the correlation was assessed with a Mantel test (2000 permutations; Mantel, 1967) using GENETPOP (<http://wbiomed.curtin.edu.au/genepop>; Raymond and Rousset, 1995). Because of the haplodiploid nature of inheritance in Hymenoptera, only data for females were used in most of the analyses.

Genetic population structure within species was studied with a model-based clustering method using the program STRUCTURE (Pritchard *et al.*, 2000). This method identifies clusters of genetically similar diploid individual based on their multilocus genotypes without prior knowledge of their population affinities (III, IV).

4. RESULTS AND DISCUSSION

4.1 Molecular phylogeny of checkerspot-parasitizing *Cotesia* wasps

The phylogenetic study (I) based on sequence and microsatellite data showed that two major clades of checkerspot-associated *Cotesia* exist in Europe (Fig. 1). The first clade (A) includes the notional species *C. acuminata* and *C. bignelli*, and the second clade (B) includes the notional species *C. melitaearum* and *C. cynthiae*. When the distinction between the two clades was first observed (I), the most striking difference between them appeared to be the host specificity of the parasitoids. *Cotesia* in the clade A (the “*acuminata*-group”) had narrow and non-overlapping host ranges, while *Cotesia* in clade B (the “*melitaearum*-group”) appeared to parasitize several broadly sympatric host species (though see below).

As mentioned before, there is evidence that clade A and clade B do not constitute a monophyletic group, with the implication that the host group Melitaeini has probably been colonised twice by *Cotesia*. It is interesting to ask which clade colonized Melitaeini first. Previous studies have demonstrated that in some closely related groups of koinobiont parasitic wasps, taxa have radically different breadths of host ranges (Shaw, 1994, 2002; Shaw and Horstmann, 1997). Those with the broadest host ranges often parasitize an array of phylogenetically unrelated but morphologically or behaviourally similar hosts occurring in the same microhabitat (Shaw, 1994). Clade A has narrower host ranges, as

well as a greater degree of morphological differentiation among subclades (M. Shaw, pers. comm.). It may therefore be that clade A was the first one to colonize Melitaeini, and has subsequently radiated into relatively isolated specialist taxa. This radiation may have involved competitive exclusion of sister taxa on shared host species. Alternatively, the rate of evolution has been higher in clade A than in clade B. Studies reported in Chapters II and III show that clade B also consists of host-specific cryptic species, but they appear to be less differentiated than taxa in clade A. Interestingly, when taxa from these two clades use the same host species (*M. didyma*, *E. matura*, and *E. aurinia*), the clade A species seem to be more successful (I). Clade B appears not to have been able to colonize *M. phoebe* at all, possibly because of the presence of a clade A specialist.

4.2 Host specialization

Substantial to very high genetic differentiation between *Cotesia* populations reared from different host species was found in a small geographic area in northern Spain (II). Differentiation among *Cotesia* populations reared from the same host species was substantially smaller and different *Cotesia* populations largely clustered according to their host species in the microsatellite distance tree. Indicative host-associated microsatellite allele variation was observed in all *Cotesia* taxa, with at least one unique allele that was not observed in other populations, suggesting limited gene flow. Quite unexpectedly, there were as many as seven biologically distinct species of *Cotesia* parasitising the Melitaeini community within a relatively small geographic area. In contrast to the inference

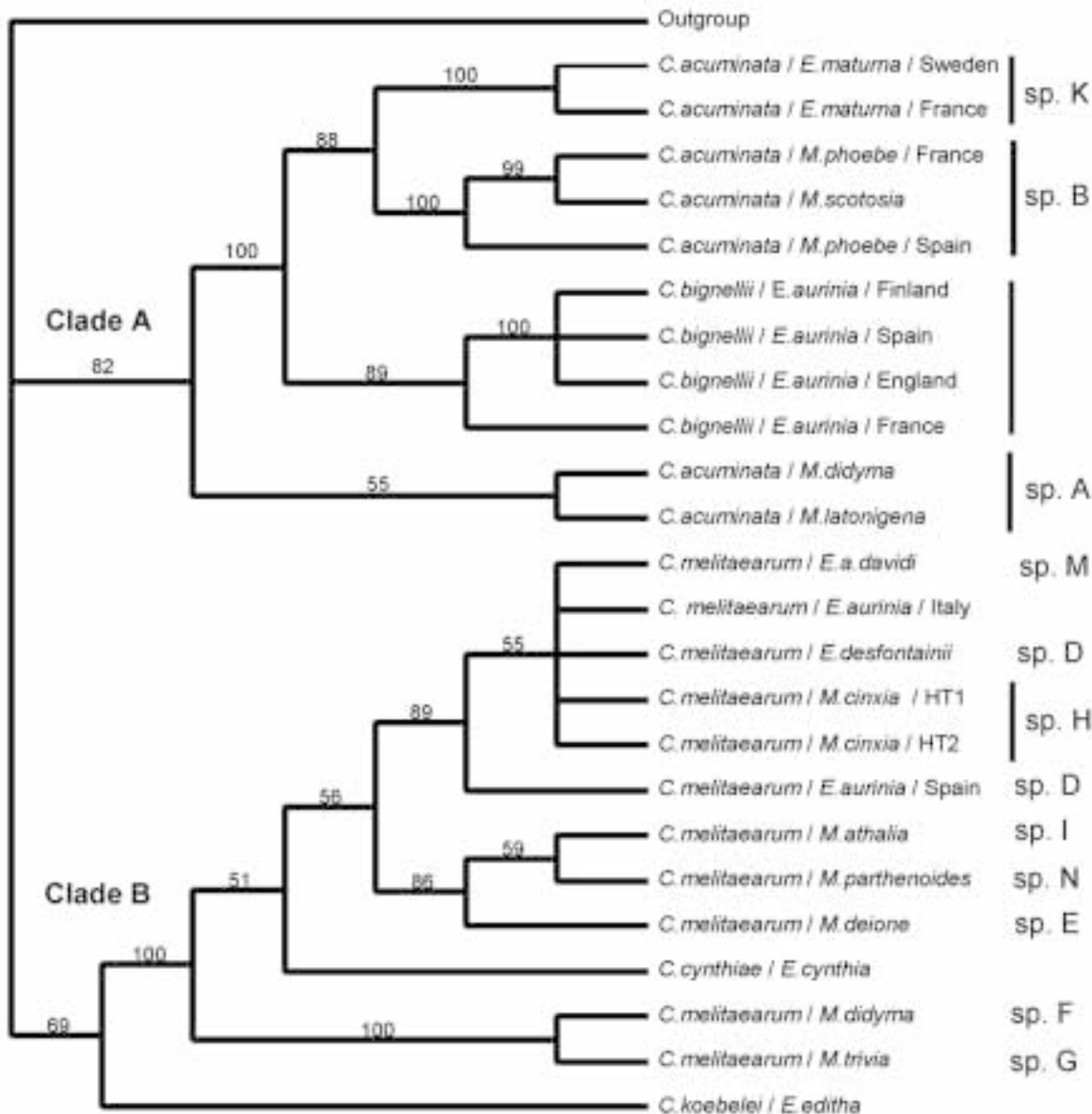


Fig. 1. Maximum parsimony tree of *Cotesia* species from different Melitaeini host butterflies based on the combined mtDNA and nuclear data (from Chapter I), with identification of the cryptic species in Table 1. Bootstrap support estimates (100 replicates) are indicated for statistically supported groupings ($\geq 50\%$).

from the broad phylogeny (I), the small-scale study (II) strongly indicates that clade B also mostly consists of specialist taxa, each associated with just one host species. Nonetheless, cryptic species in clade A appear to be more genetically differentiated, and hence “less cryptic”, than the taxa in clade B.

It is worth emphasizing that with the exception of *C. melitaearum* sp. D (from *E. aurinia* and *E. desfontainii*), we found no hosts sharing the same *Cotesia* species in the study area in northern Spain, and with two exceptions, butterflies did not host more than one *Cotesia* species in any single location. The exceptions were *Cotesia* sp. A and sp. F, which were both reared from *M. didyma* larvae from two local communities, and *C. bignellii* and *Cotesia* sp. D, which were reared from *E. aurinia* at one site (II). These results indicate that at least presently and locally there is little direct interspecific competition among different *Cotesia* species, and no current indirect interaction (apparent competition) among butterfly species due to shared *Cotesia*. This leads to two interesting questions. First, what drives specialization in *Cotesia* parasitizing Melitaeini? And second, to what extent can the observed lack of coexistence be attributed to past competition or competitive exclusion (Connell, 1980; Hawkins, 2000)?

As mentioned earlier, for the most part our data provided little evidence for apparent competition, indirect interaction among the host species mediated by shared parasitoids (Holt and Lawton, 1993, 1994). The exception is *E. aurinia* and *E. desfontainii*, which co-occur at the same sites and are parasitised by *Cotesia* sp. D. The abundances of these two butterfly species are temporally correlat-

ed, which might be due to the interaction with the shared parasitoid (*C. Stefanescu* and *S. van Nouhuys*, in prep.). Though at present this is an exceptional case, similar situations in the past have conceivably resulted in local elimination of host populations due to apparent competition via shared *Cotesia*.

4.3 Genetic divergence among host-specific cryptic species in *Cotesia melitaearum* agg.

Mitochondrial DNA yielded a complex geographic pattern in the haplotypes of *C. melitaearum* agg. reared from *M. cinxia* and *E. aurinia* in Europe and Asia (III). We found only two distinct *C. melitaearum* agg. haplotypes from *M. cinxia* hosts but a cluster of eight rather similar haplotypes from *E. aurinia* (plus one more from the closely related *E. desfontainii*) (III, Fig 2a, b). An important detail in these results is that one of the *M. cinxia*-associated *C. melitaearum* agg. haplotypes is similar to the *E. aurinia*-associated haplotypes. The microsatellite data indicated the existence of two host-associated clades that include populations across Europe. This result suggests that currently there is no gene flow among the *Cotesia* populations attacking these two host species. The taxa have been designated as *Cotesia* sp. D (from *E. aurinia*) and *Cotesia* sp. H (from *M. cinxia*). In addition, combined molecular and behavioural data demonstrated that *Cotesia* reared from two closely related host species, *E. aurinia* and *E. desfontainii*, are conspecific. In contrast, definite evidence was obtained for the *Cotesia* species attacking *Melitaea cinxia* and *M. athalia* in the Åland Islands in Finland in fact con-

sisting of two species, sp. H and sp. I. It is noteworthy that while *E. aurinia* and *E. desfontainii* are two closely related sister species, *M. cinxia* and *M. athalia* are not so closely related (Fig. 1).

Post-glacial history could explain the dissimilar geographic distributions of the two haplotypes reared from *M. cinxia*. Both haplotypes occur in Asia, but only one of them 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) following the last glacial maximum (Saccheri *et al.*, 2004). It is possible that the specialist wasp migrated to northern Europe from the same refugium. There is of course no need for the host and the parasitoid to exhibit identical post-glacial migration patterns, but the relationship here between host and parasitoid range expansions is interesting (see Hochberg and Ives, 1999) and worthy of further study. How the *M. cinxia*-associated *C. melitaearum* agg. (sp. H) acquired the haplotype that is similar to the haplotypes from *E. aurinia*/*E. desfontainii* hosts remains another open question.

4.4 Metapopulation genetic structure of coexisting primary parasitoids of *Melitaea cinxia*

Chapter IV is focused on a comparison of the spatial genetic structures in the two primary parasitoids that attack *Melitaea cinxia* in the Ålands Islands, *Cotesia melitaearum* agg. (sp. H) and the ichneumonid *Hyposoter horticola*. We found more spatial genetic structure in the *C. meli-*

taearum metapopulation than in the *H. horticola* (meta)population (IV). This finding is consistent with expectations based on the biology of the two species. *Cotesia melitaearum* agg. (sp. H) is a poor disperser, with individuals typically dispersing less than 1 km (van Nouhuys and Hanski, 2002a), and this species is only present in well-connected host populations (Lei and Hanski, 1997). In contrast, *H. horticola* is very mobile, dispersing to distances greater than 5 km, and to longer distances than the host butterfly (van Nouhuys and Hanski, 2002a). *Hyposoter horticola* is present in virtually all host populations regardless of their isolation (van Nouhuys and Hanski, 2002a). Gene flow due to high migration rate is apparently preventing significant genetic differentiation among populations of *H. horticola*.

Genetic differentiation among the *C. melitaearum* populations is likely to be enhanced by the generally very small sizes of local populations. The number of colonists in new populations is probably very small and their relatedness high, which is expected to increase genetic differentiation among local populations in a metapopulation (Whitlock, 2004). In contrast, local populations of *H. horticola* are relatively large, roughly one third of the host populations. All the *H. horticola* populations were found to be in the Hardy-Weinberg equilibrium across habitat patch networks, suggesting that individuals of this species inhabiting individual patch network in fact constitute single panmictic populations. In contrast, our analyses showed that *C. melitaearum* inhabiting different local host populations within a patch networks cannot be considered as panmictic populations.

4.5 Cryptic species in *Cotesia* and their relationship with host phylogeny

Genetic differentiation among the *Cotesia* populations reared from different Melitaeini host species across Europe and Asia presents a complex pattern that is not consistent with the previous knowledge of just few species. Rather, it is clear that this group of parasitoids includes many cryptic species. Table 1 summarizes the results based on the broad phylogeny of Melitaeini-associated *Cotesia* (I), on the study of the Melitaeini-*Cotesia* community in a relatively smaller area in northern Spain (II), and on the patterns in the genetic differentiation of *C. melitaeorum* agg. from many host species across Europe and Asia (III).

Molecular phylogeny including all known taxa revealed two distinct clades (A and B; Fig. 1). Two of the subclades in clade A, consisting of *C. acuminata* agg. reared from *M. didyma* and *M. latonigena*, and *C. acuminata* agg. reared from *M. phoebe* and *M. scotosia*, differ substantially from the other taxa in clade A, suggesting that they represent distinct *C. acuminata* agg. sp. A and sp. B, respectively (I). *Cotesia acuminata* agg. from *E. maturna* at several localities represents a single haplotype, and the cryptic species designated as *C. acuminata* sp. K (I). Morphological data support the distinctions among these subclades (I). Though there is no doubt about the species status of *Cotesia bignellii* from *E. aurinia* in clade A, individuals from all other localities apart from France have almost identical haplotypes, whereas those collected from France show a substantial divergence from the others

(I). The resolution of *C. bignellii* from France requires further study with additional samples from multiple localities. The evidence for cryptic species in *C. melitaeorum* agg. is detailed in the original papers and summarized in Table 1. It is important to note that in this case the conclusions are critically based on the geographical patterns, namely that the different cryptic species occur sympatrically, but on different host species, across many localities in Europe. It seems probable that *C. lycophron*, which has been reared only from a single brood of *M. didyma* from France, is conspecific with *C. melitaeorum* sp. F from *M. didyma* (I; M. Shaw, in prep.).

Our results suggest that the radiation of Melitaeini-associated *Cotesia* has to a large extent been driven by host phylogeny (Fig. 2). In cases when two host species are parasitized by the same *Cotesia* species, the host species are very closely related: *M. didyma* and *M. latonigena*, *M. phoebe* and *M. scotosia*, and *E. aurinia* and *E. desfontainii* (Fig. 2). On the other hand, the reverse is not true, two closely related *Cotesia* species may use host species that are not closely related. For example, *C. melitaeorum* agg. sp. D and sp. H, which are clearly closely related, use *E. aurinia*/*E. desfontainii* and *M. cinxia*, respectively. In two cases, the same host species is used by two different *Cotesia* species at the same site, in which case the two parasitoids are not closely related. Thus *C. acuminata* agg. sp. A and *C. melitaeorum* agg. sp. F, as well as *C. bignellii* and *C. melitaeorum* agg. sp. D, were both reared from a single host species, *M. didyma* and *E. aurinia*, respectively.

We do not have *Cotesia* material from any other host genera than *Melitaea* and *Euphydryas*. According to the

Table 1. Summary of cryptic species documented in this thesis.

Parasitoid	Cryptic sp.	Host butterfly	Host plant species	Geographic area	References*	Chapter
<i>Cotesia acuminata</i> agg.	sp. A [§]	<i>Melitaea didyma</i>	<i>Plantago</i> spp., <i>Veronica</i> spp., <i>Valeriana</i> spp. ^a	Europe	6	II
	sp. B [§]	<i>Melitaea latonigena</i>	<i>Veronica incana</i> ^a	Siberia	6, 11	I
		<i>Melitaea phoebe</i>	<i>Plantago? Centaurea</i> spp., <i>Stemmacantha uniflora</i> ^a	Europe, Siberia	5, 11	II
		<i>Melitaea scotosia</i>	?	China	12	I
sp. K	<i>Euphydryas maturna</i>	<i>Fraxinus excelsior</i> , <i>Plantago lanceolata</i> , N Europe <i>Melanopyrum pratense</i> , <i>Viburnum</i> spp. ^{a, c}	Europe	1, 2, 9	I	
sp. L	<i>Melitaea athalia</i>	<i>Veronica</i> spp., <i>P. lanceolata</i> , <i>Melanopyrum</i> spp.	Europe	11	I	
<i>Cotesia bignellii</i>		<i>Euphydryas aurinia</i>	<i>Succisa pratensis</i> , <i>Scabiosa comosa</i> ^{a, b}	Europe	2, 4	II
		<i>Euphydryas cynthiae</i>	<i>Plantago alpina</i> , <i>Viola calcarata</i> ^a	C Europe	5	I
<i>Cotesia euphydryidis</i>		<i>Euphydryas phaeton</i>	<i>Chelone glabra</i> ^f	NE America	13	I
<i>Cotesia koebelei</i>		<i>Euphydryas editha</i>	?	NW America	13, 14, 15	I, II
<i>Cotesia lycophron</i>	sp. F [#]	<i>Melitaea didyma</i>	<i>Plantago</i> spp., <i>Veronica</i> spp., <i>Valeriana</i> spp. ^a	Europe	11	II
<i>Cotesia melitaearum</i> agg.	sp. D	<i>Euphydryas aurinia</i>	<i>Succisa pratensis</i> , <i>Scabiosa comosa</i> ^{a, b}	Europe	4, 6, 10	II
		<i>Euphydryas desfontainii</i>	<i>Cephalria leucantha</i> , <i>Dipsacus fullonum</i> , <i>Knautia arvensis</i> ^a	Europe SW Europe	1	II
	sp. M	<i>Euphydryas a. davidi</i>	<i>Scabiosa comosa</i> ^a	Siberia	6	I, III
	sp. I	<i>Melitaea athalia</i>	<i>Veronica</i> spp., <i>P. lanceolata</i> , <i>Melanopyrum</i> spp. ^{a, d, e}	Europe	3, 6, 12	III
	sp. H	<i>Melitaea cinxia</i>	<i>Plantago</i> spp., <i>Veronica</i> spp., <i>Centaureae</i> spp. ^a	Europe, China	11	II, III
	sp. E	<i>Melitaea deione</i>	<i>Linaria</i> spp., <i>Antirrhium</i> spp. ^a	SW Europe	11	II, III
	sp. F	<i>Melitaea didyma</i>	<i>Plantago</i> spp., <i>Veronica</i> spp., <i>Valeriana</i> spp. ^a	Europe	11	II
	sp. N	<i>Melitaea parthenoides</i>	<i>Plantago</i> spp. ^a	SW Europe	2, 7, 8	II, III
	sp. G	<i>Melitaea trivialis</i>	<i>Verbascum</i> spp. ^a	S Europe	9, 11	II

[§] *Cotesia acuminata* from *M. didyma* and *M. latonigena* are morphologically distinctive from the other *C. acuminata* species (M. Shaw, pers. comm., chapter I) and most probably both belong to *C. acuminata* sp. A.

[§] *Cotesia acuminata* from *M. phoebe* and *M. scotosia* are morphologically distinctive from the other *C. acuminata* species (M. Shaw, pers. comm., chapter I) and most probably both belong to *C. acuminata* sp. B.

[#] *C. lycophron* is conspecific with *C. melitaearum* sp. F from *M. didyma* (M. Shaw, pers. comm., chapter I).

^a Tolman and Lewington, 1997; ^b Klemetti and Wahlberg, 1997; ^c Wahlberg, 1998; ^d Selonen, 1997; ^e Wahlberg, 1997; ^f Stamp, 1982.

* references for host butterfly and geographical area: 1. Eliasson, 1991; 2. Komonen, 1997; 3. Lei et al., 1997; 4. Porter, 1981; 5. Williams et al., 1984; 6. Wahlberg et al., 2001; 7. S. van Nouhuys, pers. comm.; 8. Warren, 1987; 9. Komonen, 1998; 10. Ford and Ford, 1930; 11. Nixon, 1974; 12. I. Hanski, pers. comm.; 13. Marsh, 1979; 14. White, 1973; 15. Moore, 1989a.

literature data, *Cotesia euphydryidis* has been reared from *Closyne harrisii* and *C. koebeleri* from two different *Closyne* species (Table 1 in Chapter I). Both of these North American *Cotesia* species were only available from *Euphydryas* in our material (Table 1, Fig. 2.). In general, *Phyciodes* and *Chlosyne* species have been studied relatively little (N. Wahlberg, pers. comm.). It is worth noting that *Euphydryas* species are parasitized by a wide range of clearly distinct *Cotesia* species, whereas those parasitizing *Melitaea* involve only species from *C. acuminata* agg. and *C. melitaeorum* agg. (assuming that *C. lycopron* is actually conspecific with *C. melitaeorum* sp. F, see above). *Euphydryas* is the basal group in the host phylogeny (Wahlberg and Zimmermann, 2000), and hence probably older than *Melitaea*. *Euphydryas* may thus have had more time than

Melitaea to accumulate a diverse set of *Cotesia*. Furthermore, *Euphydryas* species collectively use a larger number of host plant families than any other genus of Melitaeini, which may be significant, though there is no simple pattern in the host plant use by the host butterflies of different species of *Cotesia* (Table 1).

Wahlberg *et al.* (2004) concluded that the radiation of Melitaeini butterflies has been affected by host plant taxonomy, but they found no evidence for the reciprocal influence, which was expected as the host plant taxa are much older than the butterflies. Similarly, our results suggest that the radiation of Melitaeini-associated *Cotesia* has to a large extent been driven by host phylogeny. To what extent Melitaeini butterflies and their *Cotesia* parasitoids have possibly coevolved remains an interesting open question for further research.

5. ACKNOWLEDGEMENTS

First of all, I would like to express my warmest gratitude to my supervisor Ilkka Hanski for his extreme flexibility and patience during the years I have been working with this thesis. Particularly during the time when I had “two successive complications” (read: had two babies) and tried to remember what DNA and parasitoid mean after using mostly words like diapers and breastmilk for almost two years. His contribution to the thesis has also been crucial on the very final stages of the project when I was trying to put all the data gathered during several years to some kind of order.

I sincerely thank all my co-authors for their invaluable contribution to this thesis. I have been really lucky to have such experienced people to work with. Especially Saskya van Nouhuys has been more like my second supervisor during these years. I greatly appreciate her knowledge of the biology of the parasitoid species, experience for doing the parasitizing experiments and willingness to correct my English in the manuscripts.

Past and present members of the MRG group have offered a fruitful working atmosphere and we have definitely shared many memorable moments in our annual meetings and other “outdoor activities”. I wish to thank Leena Suvanto in particular for all kind of small favours she did for me while I was working at home. Thanks Leena for also being such a good friend!

I also wish to thank Craig Primmer and Heikki Roininen for pre-examining this thesis with pretty tight schedule. I wish to express my warmest thanks to Toshka Nyman who has prepared huge amount of sequencing and microsatellite samples and Anne Aronta for running

them. The atmosphere in the MES laboratory has always been very relaxing, already in the “old days” in Arkadiankatu when just a few people were working there. Thanks Ilik, Pia, Gunilla, Jarmo, Jukka, Jouni, Tytti and Hannu (and also all the others who have joined us later)! Ilik Saccheri taught me all the relevant molecular techniques and Jodie Painter helped especially with microsatellite analyses.

Many researchers in several countries have collected *Cotesia* samples (detailed list is provided in Chapter one) used in this study. In particular I wish to mention Mark R. Shaw in UK and Constanti Stefanescu in Spain who provided so many samples and also worked as my co-authors giving their expertise (wasp morphology and butterfly biology, respectively) for my use.

Väitöskirjan tekeminen on aina iso urakka ja sitä se on myös kahden pienen lapsen äidille. Työn loppuunsaattaminen ei koskaan olisi ollut mahdollista ilman aviomieheni Markon täydellistä tukea. Kiitos Makke kaikesta henkisestä tuesta, rakkaudestasi ja siitä, että olet huolehtinut jokapäiväisistä askareista vähintään 50%:sti. Unohtamatta sitä, että mahdollistit kotona työskentelemiseni tarjoamalla laitteet ja yhteydet. Kiitos Samppu ja Anniina arkielämän pienistä mutta sitäkin tärkeämmistä hetkistä. Tämä ei ole ollut helppoa teillekään – kysymys ”Äiti, taasko sinä teet töitä?” jää muistiini ikuisiksi ajoiksi. Kiitän vanhempiani, sisartani Annikaa ja ystäväämme Markettaa yhteisistä hetkistämme Kuikkarannassa, omassa paratiisissamme. Erityiskiitos äidille vastoista!

Parhaat ystävämme Virva, Vekku, Heidi ja kaikki karvakorvat ovat useiden vuosien aikana tarjonneet monia unohtumattomia hetkiä kauniissa saaristossa ja maaseudulla. Nuo yhdessäolon hetket ovat saaneet minut unohtamaan työhuolet edes hetkeksi.

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