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RESEARCH ARTICLE

DNA-based confirmation that the parasitic wasp *Cotesia glomerata* (Braconidae, Hymenoptera) is a new threat to endemic butterflies of the Canary Islands

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Abstract Island-endemic species can be particularly vulnerable to alien invasion. There are many examples of introduced insect parasitoids having a serious impact on endemic butterflies and moths. In 2006, a population of parasitic wasps was reared from larvae of the Canary Island Large White butterfly (*Pieris cheiranthi*), an endemic inhabitant of laurel forests unique to the Canary Islands of Macaronesia. Parasitoids were tentatively identified as *Cotesia glomerata* (Braconidae, Hymenoptera), a widely introduced agricultural bioagent. To corroborate this finding we sequenced 632 bp of mitochondrial *cox1* from parasitoids and hosts from La Palma and from the native range of *C. glomerata* in continental Europe. These were combined with GenBank sequences and a character-based, phylogenetic approach was used to assess the species status of parasites and hosts. The La Palma parasitoid could unambiguously be assigned to *C. glomerata* under the criterion of diagnosibility with corroboration from multiple lines of evidence (DNA, morphology). We suggest that this opportunistic, non-native parasitoid wasp will be a threat to *P. cheiranthi* and other endemic Canarian butterflies. Parasitoid populations were recorded from *P. cheiranthi* in marginal forest habitats but not in central forest areas, suggesting that comprehensive habitat conservation of the Canarian laurel forests could prevent penetration of the alien parasitoid wasps and subsequent mortality of endemic butterfly populations.

Keywords Alien parasitoids · Threatened butterflies · Laurel forests · Island endemics · Mitochondrial DNA

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Introduction

Insect parasitoids have been introduced into a variety of ecosystems as bioregulators and there have been many successful cases of their being used to control harmful native arthropods (DeBach and Schlinger 1964; DeBach 1974). Species introduced to control agricultural pests have also infiltrated fragile insect communities of many oceanic ("Darwinian") islands where they can cause significant mortality and even local extinction of endemic fauna. The most severe impacts of alien parasitoids on island-endemic populations of butterflies and moths (Lepidoptera) have been recorded from the Hawaiian Islands and Guam (Nafus 1993; Asquith 1995; Henneman and Memmott 2001; Stokstad 2001; Gillespie and Roderick 2002). Alien parasitoids have also been found in the Galapagos and other Pacific islands, perhaps after accidental introduction (Peck et al. 1998).

In May 2006, the authors discovered a small population of the parasitoid wasp *Cotesia glomerata* (Braconidae, Hymenoptera) on La Palma Island in the western Canary Islands of Macaronesia. This microgastrine wasp is a

gregarious koinobiont endoparasitoid, primarily of *Pieris* spp. butterflies associated with mustard oil crucifer plants (Brassicaceae). Nonetheless, its host range includes several lepidopteran families including Pieridae (*Pieris brassicae*, *P. rapae*, *P. napi*, and *Aporia crataegi*) and Lymantriidae (*Lymantria dispar*) (Tobias et al. 1986; Balevski 1999; Ohsaki and Sato 1994; Jiang ShuangLin 2001). *C. glomerata* have been released in the United States, New Zealand, and Chile and have since been reported from Argentina and Brazil (Sharkey et al. 2000; Scaglia et al. 2003). Records of *C. glomerata* parasitism on non-target, native insect hosts can be subject to mis-identification and require confirmation using alternative methods such as genetic analysis (Sands and Van Driesche 2004). There are no records of parasitoids or predators being introduced to the Canary Islands for the biological control of agricultural pests. Nonetheless, the widespread introduction of *C. glomerata* against *P. rapae* may well have resulted in exposure of the former to other hosts, causing significant mortality in non-target species (Benson et al. 2003; Sands and Van Driesche 2003; Hoddle 2004). Accidental introduction could also cause problems and result in threats to local fauna of Lepidoptera.

The Canary Islands Large White butterfly *Pieris cheiranthi*, like many other canarian Lepidoptera, is strictly associated with the local endemic ecosystems of the relict laurel forests ("laurisilva"; see Wiemers 1995, Bramwell and Bramwell 2001). These ancient natural forests and their biota represent one of the highest nature conservation priorities of the Canary Islands, not least because they are part of a unique laurel forest formation known only from Macaronesia (Kunkel 1976; Bramwell and Bramwell 2001). Most of the laurel forest butterflies and other native insects are endemics to particular Canarian and other Macaronesian islands (Jones et al. 1987; Wiemers 1995; Tolman 1997; Brunton and Hurst 1998; Juan et al. 2000; Borges et al. 2005; Tennent 2005). *P. cheiranthi* occurs on the islands of Tenerife, Gomera (although probably now extinct) and La Palma where it is most abundant locally.

Here we examined reared parasitoids (*C. glomerata*) and their hosts (*P. cheiranthi*) from La Palma, along with comparative material of *P. brassicae* hosts and their reared parasitoids (*C. glomerata*) from their native range in continental Europe (Czech Republic). Although it was straightforward to morphologically assign the reared Canary Island parasitoids to *C. glomerata* s.l. when compared with other reared European material (from Romania and Moldova, see Costea et al. 2002), we used DNA sequence data to test whether the reared specimens could be unambiguously matched to European *C. glomerata* or whether they constitute a diagnosible, endemic population (or "geographic race") of *C. glomerata* that has perhaps coevolved with endemic butterflies. Cryptic species are known to occur among other *Cotesia*, where biologically distinct species have narrow host associations (Kankare et al. 2005a, b).

We sequenced 630 bp of mitochondrial *cox*1 from recently collected specimens and we used publicly available sequence data from species of *Cotesia* to build a preliminary DNA taxonomy of the group (sensu Ahrens et al. 2007). We used a character-based phylogenetic approach to assess the species status of parasites and hosts using the criterion of diagnosibility with corroboration from multiple lines of evidence (DNA, morphology, geography sensu DeSalle et al. 2005).

Material and methods

We sampled two colonies of *P. cheiranthi* from La Palma Island. Larvae of both colonies were found feeding on the endemic food plant *Crambe santosii* Bramwell (Brassicaceae, see Bramwell and Bramwell 2001). One group of larvae ($n = 30$) was collected in marginal parts of the laurel forest near La Galga and 50% ($n = 15$) of the larvae were parasitized by *C. glomerata*. The second group of larvae was collected in the center of a laurel forest near Los Tilos and no larvae ($n = 25$) were attacked by parasitoids. The food plant *C. santosii* and threatened butterfly *P. cheiranthi* are endemic to these laurel forests and the vegetation of both localities in La Palma is described by Bramwell and Bramwell (2001).

Approximately 600 specimens of *C. glomerata* were reared from *P. cheiranthi* larvae from La Palma (May 2006). Three exemplars of *C. glomerata* and two *P. cheiranthi* were chosen for mitochondrial DNA analysis. European *C. glomerata* (ca. 700 specimens) were reared from larvae of the Large White butterfly, *P. brassicae*. These were collected near České Budějovice, Czech Republic, from several abundant colonies (July 2006). Two *C. glomerata* and one *P. brassicae* specimens from the Czech Republic were also used for genetic analysis.

Live specimens were either dried or preserved in 100% ethanol. Total DNA was extracted from individual parasitoids (whole body) or butterflies (middle legs) using a DNeasy tissue kit (Qiagen Inc.) according to the manufacturer's protocol for animal tissues. PCR amplification was carried out using the TaKaRa Ex Taq system (TaKaRa Bio Inc.) and using universal primers LCO1490 and HCO2198 (Folmer et al. 1994) to amplify 658 bp of the 5′ end of mitochondrial cytochrome oxidase I (*cox1*). Reactions (50 µl) consisted of 5 µl of template DNA (not quantified), 5 µl of 10× reaction buffer, 4 µl of dNTP mixture (2.5 mM each), 0.5 mM of each primer and 1.25 unit of ExTaq polymerase. Reactions were conducted on an Eppendorf Mastercycler gradient S thermocycler with

the following profile: 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 47°C for 45 s, 72°C for 1 min, and final extension in 72°C for 3 min. PCR products were cleaned with the QIAquick PCR Purification Kit (Qiagen Inc.).

Sequencing was performed on both strands using the above primers in a BigDye v. 3.1 sequencing reaction and using an ABI 310 automated sequencer (Applied Biosystems, Inc.) at the sequencing facility of the Institute of Entomology (Biological Centre of the Czech Academy of Sciences). Forward and reverse sequencing reads were assembled and edited in Sequencher v. 4.6 (Gene Codes Corporation) and ClustalW (align.genome.jp) was used to align *Cotesia* and *Pieris* sequences separately. There was no length variation in the fragment analysed and so sequences could be aligned unambiguously in each case using the default parameters. All newly generated sequences have been deposited to GenBank (accession numbers EU143657-EU143664, see Fig. 1) and morphological vouchers are held within the collection of the Institute of Entomology, Biological Centre of the Czech Academy of Sciences.

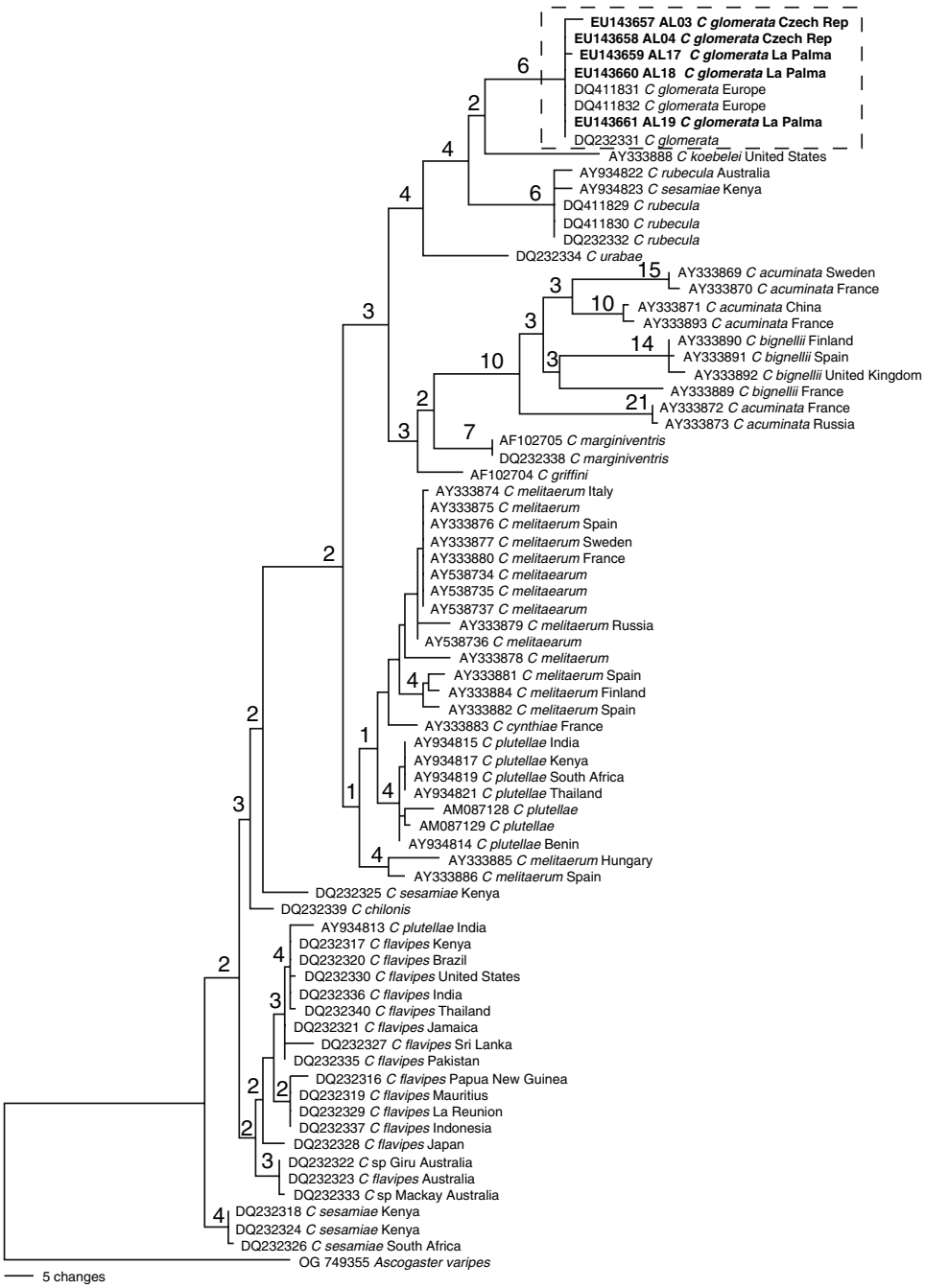

Fig. 1 Rooted maximum parsimony phylogram of *cox1* data showing newly sequenced (dashed box, terminals coded AL) and GenBank *Cotesia* spp. Terminals are labeled with GenBank accession number, Linnean binomial and geographical origin where known. Bremer support values are given above branches

To each alignment we added sequences from GenBank by searching the nucleotide database using nominal queries for *Cotesia* and *Pieris*. We verified our database coverage for *Cotesia* by using BLASTn 2.2.16 (Altschul et al. 1997) to find the top 100 sequences matching one newly sequenced individual (AL03). Outgroups were either sequenced as above (*Ascogaster varipes*, leg. Kuheman, Germany, collection of Imperial College, UK) or downloaded from GenBank (*A. crataegi*). Complete matrices were aligned as above and then subjected to parsimony-based tree searches using PAUP v 4b10 (Swofford 2003). We ran full heuristic searches using TBR branch swapping and 200 random addition sequences. Branches were collapsed where minimum length was zero ("amb-"). We calculated Bremer support with PAUP v. 4b10 using constraint files written with TreeRot v. 2c (Sorenson M.D., 1999, Boston University). The number of diagnostic nucleotides (fixed differences) for morphological species and sequence clusters were calculated with DNAsp v 4.00.2 (Rozas et al. 2003).

Results

A total of 74 and 30 ingroup sequences were included in the *Cotesia* and *Pieris* analyses, respectively. The BLASTn query of our newly sequenced *Cotesia* produced identical results to the taxonomic (nominal) query in that 76 *Cotesia* sequences were recovered. These were monophyletic on a *cox1* gene tree that included the remaining 24 BLASTn hits from other taxa (not shown). We removed two GenBank sequences of *Cotesia plutellae* (AY934816, AY934820) which contained indels that disrupted the reading frame. There were three database sequences ascribed to *C. glomerata* and three sequences ascribed to *P. brassicae* from published sources. Aligned matrices each contained 632 characters although the extent of missing data within matrices varied. Twenty eight of the *Cotesia* sequences from GenBank (belonging to *Cotesia griffini*, *C. marginiventris, C. plutellae*, and *C. flavipes*) were missing 29–38% of nucleotides in the matrix because they only partially overlapped the rest of the *cox1* data. The *Pieris* matrix had the complete sequence for only six individuals from GenBank and was missing 47% of nucleotides for the other 20 individuals, thus for *Pieris* we used a shortened matrix (333 bp) for the analysis whereby all individuals had <5% missing data.

There were 19 shortest trees under maximum parsimony (length = 481, HI = 0.4241, RI = 0.8564) for *Cotesia*. Bremer support (total = 138) was largely distributed among the nodes subtending tip clusters (sum = 95) rather than at deeper nodes (sum = 43, Fig. 1). Sequences from the three reared *Cotesia* from La Palma fell clearly within a well-supported cluster of sequences containing reared *C. glomerata* from the Czech Republic and all GenBank database sequences ascribed to the species (Fig. 1). La Palma, the Czech Republic and the database all had a shared haplotype, thus making these individuals genetically indistinguishable. As a result, the La Palma sequences had no characters with which they could be diagnosed as a geographical group (i.e. using PAA, Davis and Nixon 1992). There were three fixed nucleotide differences between *C. glomerata* and all remaining ingroup sequences, at nucleotide positions 268 (G), 562 (A) and 631 (G) of the aligned matrix. Several additional character combinations contributed to the branch support of this cluster (Fig. 1). There were 21 fixed nucleotide differences between all sequences in the *C. glomerata* cluster and the single sequence of *Cotesia koebelei*. The cluster containing *C. rubecula* from three sources (Muirhead et al. 2006; Traugott et al. 2006; Rattan et al. unpublished) as well as an individual ascribed to *Cotesia sesamiae* had 17 fixed differences from *C. glomerata*. It is unclear whether this cluster constitutes a single morphological group because other sequences ascribed to *C. sesamiae* appear throughout the tree.

Other species and species groups represented in the *Cotesia* tree included members of the *Cotesia flavipes* complex (*C. flavipes*, *C. sesamiae*, *C. chilonis*). These clustered into several distinct groups and the *cox1* topology of the complex, including the paraphyly of *C. sesamiae*, broadly reflected that of the original study that combined *cox1* and 16S (Muirhead et al. 2006). *Cotesia acuminata* and *Cotesia bignelli* clustered into several groups that Kankare and Shaw (2004) also recognised as having host specificity and distinctive morphology. *Cotesia melitaearum* occurred within a highly diverse cluster that included *Cotesia cynthiae* as found in the original study (Kankare and Shaw 2004). Interestingly, this cluster also included individuals of *Cotesia plutellae* deposited to GenBank more recently from two different sources (Rattan et al., unpublished; Wagener, unpublished).

There were nine shortest trees (length = 118, HI = 0.2034, RI = 0.9406) for *Pieris* and the *cox1* haplotype of the Czech specimen of *P brassicae* from which the parasitoids were reared was identical to three database sequences (Fig. 2) from published sources (Traugott et al. 2006; Chew and Watt 2006). The two La Palma *P. cheiranthi* specimens were genetically identical, with strong node support for them as a cluster (Fig. 2). There were six fixed differences between La Palma *Pieris* and all other ingroup sequences, and nine fixed differences between this haplotype and the *P. brassicae* haplotype.

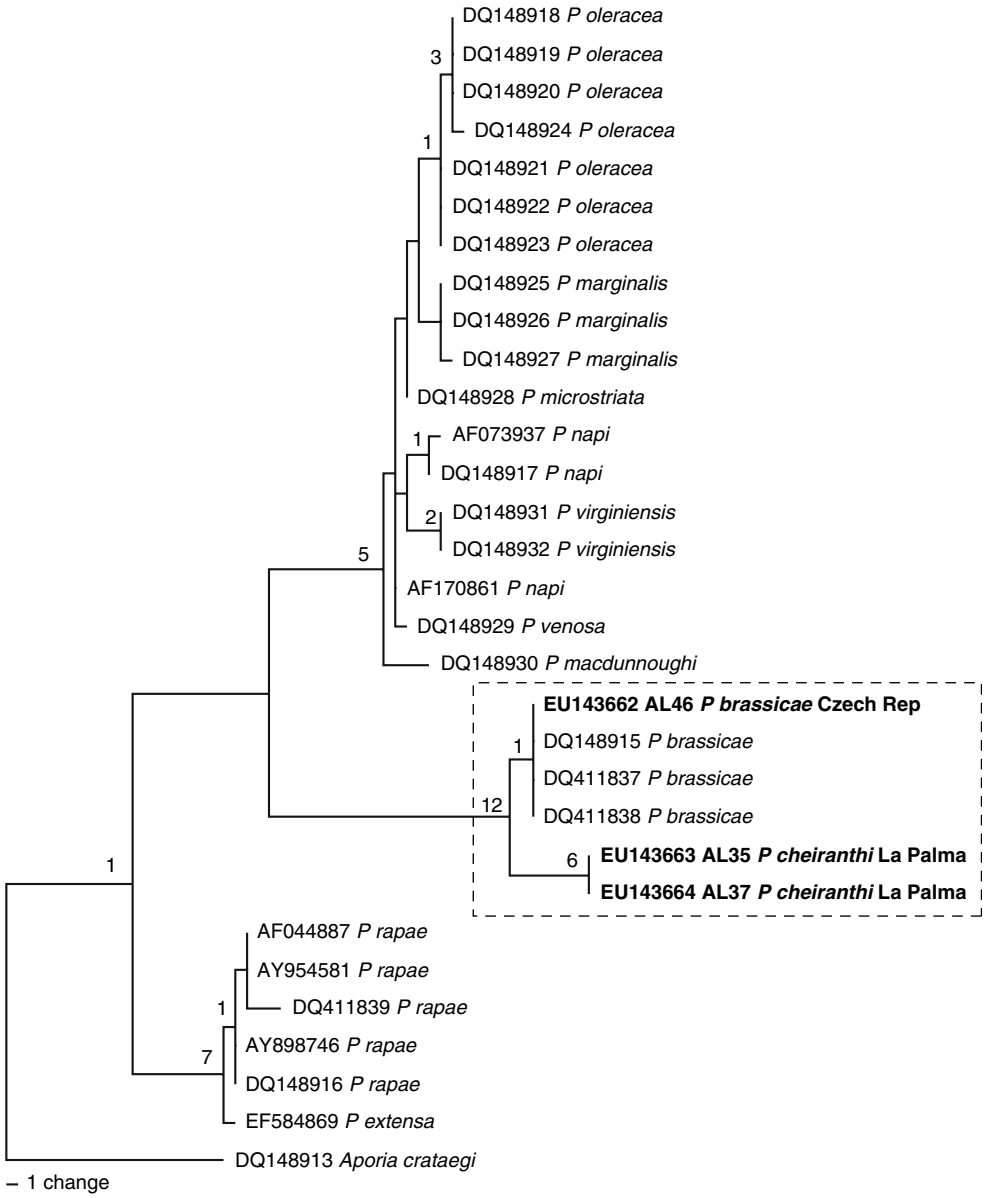

Fig. 2 Rooted maximum parsimony phylogram of *cox1* data showing newly sequenced (dashed box, terminals coded AL) and GenBank *Pieris* spp. Terminals and branches are labeled as in Fig. 1

Discussion

We employed standard morphological identification, *cox1* sequence data and geographical location to diagnose (sensu DeSalle et al. 2005) species of *Cotesia* and *Pieris* on La Palma and in continental Europe. The La Palma *Cotesia* parasitoids could unambiguously be assigned to *C. glomerata* under the criterion of diagnosibility using corroboration from DNA and morphology. This is, to our knowledge, a newly recorded host of the microgastrine wasp *C. glomerata*. There was no evidence for the populations of *C. glomerata* from La Palma being a genetically distinct population or ("Canarian") geographic race of *Cotesia* in historical coevolution with endemic butterflies. Canarian endemic hosts and parasitoids (the totricid moth *Cydia alazon* and its braconid parasitoid *Apanteles alazoni*) have been observed before (Spitzer and Jaroš 2006; Lozan 2007) and had this been the case here, then the threat may not be as great because presumably populations of the native hosts would be more resistant to native parasites than to recently introduced parasitoids.

The host *Pieris* was distinct from European *P. brassicae* using DNA and geographic evidence, as well as using morphological variation by which it was assigned to The Canary Islands Large White butterfly, *P. cheiranthi*. Thus although their introduction to the Canary Islands was probably accidental, this parasitoid appears to have adapted to at least one native and endemic host. Other Canarian butterflies, such as *Gonepteryx cleobule* (Wiemers 1995; Brunton and Hurst 1998) also may be at risk. We propose

that the Canary Islands Large White is exposed to potential risk of significant mortality and population extinction. *C. glomerata* is believed to have reduced the geographical range of native butterflies elsewhere (e.g., *Pieris virginiensis* in USA, see Hoddle 2004), although Benson et al. (2003) concluded that *P. virginiensis* is not threatened by these parasitoids even though its larvae are successfully attacked in the laboratory by *C. glomerata* and related *C. rubecula*.

Oceanic islands are fragile ecosystems, often having low resistance against invasive biota (Elton 1958), and the endangerment of island-endemic butterflies and moths following the introduction of biocontrol agents (e.g. parasitoids) has been observed on other areas (Henneman and Memmott 2001; Gillespie and Roderick 2002). The Large White *Pieris wollastoni*, closely related to *P. brassicae* and endemic to the Madeira Archipelago (Macaronesia) is now probably extinct. A possible explanation for extinction of this species is the introduction of unknown bioagents such as disease (Gardiner 2003; Aguiar and Karsholt 2006); however, the introduction of an alien parasitoid should not be excluded.

The host *P. cheiranthi* is ecologically different from many of its relatives (e.g., *P. brassicae, P. rapae*). It is primarily associated with native forest, rather than agricultural areas, in the Canary Islands. *C. glomerata* is associated with sunny, exposed habitats typical of agriculture (gardens, meadows) and here we observed it to parasitize *P. cheiranthi* only in marginal areas of laurel forest and not in central, intact areas. Increased parasitism at habitat margins has been observed elsewhere and provides further evidence of the importance of spatial configuration of habitat (e.g., Tscharntke et al. 2002). This is an important consideration when assessing butterfly habitat requirements and further monitoring is needed to determine whether parasitoids penetrate into more protected forest or remain at the margins of these laurel forests.

DNA provides an alternative and complementary character system with which to test morphological and geographical hypotheses and to diagnose entities on the basis of fixed character differences (DeSalle et al. 2005; Vogler and Monaghan 2007). The broad geographical distribution of *Cotesia* species, in part because of their intentional introduction as bioagents, probably limits the utility of geographical criteria in delimiting groups (i.e., populations, Davis and Nixon 1992); however, morphology, host specificity, and behaviour (e.g., Muirhead et al. 2006; Kankare and Shaw 2004) all may be invoked as diagnostic characteristics to support or refute species delineation with DNA characters. Pons et al. (2006) used DNA sequences to delineate species groups using diagnostic criteria and using statistical analyses of branching patterns in an ultrametric tree. While the sampling conducted in our study was not sufficient to study branching patterns within populations and among species, an increased effort to sequence morphological *Cotesia* spp. from a broad geographical range would lead to a DNA taxonomy of the group, thus enabling the regular testing of species hypotheses and, in future, the incorporation of all life stages using a single character set (e.g., Ahrens et al. in press). This could eliminate the rearing step and perhaps lead to more rapid diagnosis of potentially harmful introductions.

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