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Phylogenetics and population genetics of the Eurasian parasitoid *Macrocentrus cingulum* based on mitochondrial and nuclear loci

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Abstract

Specifying species boundaries is often tricky, because advanced biomolecular analyses can reveal that morphologically similar individuals in fact belong to distinct species. This is frequently the case when populations previously considered as a single polyphagous taxon prove to consist of several genetically distinct taxa using different resources, e.g., among insect parasitoids. *Macrocentrus cingulum* Brischke (Hymenoptera: Braconidae), a parasitoid of the genus *Ostrinia* (Lepidoptera: Crambidae) feeding on various host plants across the world, is one of them. In Western Europe, *M. cingulum* has never been found in *Ostrinia nubilalis* (Hübner) populations feeding on maize, although it heavily parasitizes sympatric *Ostrinia scapulalis* Walker populations feeding on mugwort. In contrast, it contributes to pest control of *Ostrinia furnacalis* Guenée feeding on maize in Asia and *O. nubilalis* feeding on maize in America, suggesting that European and Asian *M. cingulum* populations might form two distinct taxa. We tested this hypothesis by conducting phylogenetic and population genetic analyses based on two mitochondrial and two nuclear genes, on 97 *M. cingulum* individuals sampled in Asia, USA, and Europe. Our analyses not only suggest that all sampled *M. cingulum* probably belong to the same species, but also show a significant genetic differentiation between individuals originating from Europe on the one hand and Asia/USA on the other, which correlates with infestation patterns. Moreover, they show that American specimens are closely related to Asian ones, consistent with historical records about *M. cingulum* introductions into the USA in the 1920s and 1930s to control expanding *O. nubilalis* populations. Combining these results with what is known about the evolutionary history within the genus *Ostrinia*, we offer a candidate evolutionary scenario that is amenable to future empirical testing.

Introduction

Under the scrutiny of advanced genetic and taxonomic tools, many taxa initially described as a single species turn out to actually consist of distinct genetic entities (Bickford et al., 2007). Examples can be found in many taxa (e.g., for herpetofauna, Speybroeck & Crochet, 2007; for algae, Leliaert et al., 2009; for birds, Speybroeck et al., 2010), including arthropods, for instance Hymenoptera (Smith et al., 2006, 2008) and Lepidoptera (Hebert et al., 2004; Burns et al., 2008). More specifically, many species that live in close association with a host – such as phytophagous insects (Berlocher & Feder, 2002; Drès & Mallet, 2002; Dyer et al., 2007; Feder & Forbes, 2010; Matsubayashi et al., 2010) or parasites (De Meeûs et al., 1998; Jousson et al., 2000; McKoy et al., 2005) – were first considered as generalist feeders and later split into several specialized taxa using distinct resources. Strong and highly specific selection pressures from their hosts and/or coevolutionary processes may have favoured their diversification. As a result, even though parasitic insects already

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represent almost half of all described species (Feder & Forbes, 2010), their diversity is probably still largely underestimated at both specific and infra-specific level (Novotny et al., 2002).

Hymenoptera is one of the most species-rich insect orders (Daly et al., 1998; Feder & Forbes, 2010). They include many species that parasitize a wide array of host species and are thereby submitted to an even wider diversity of selection pressures. They are often small and sometimes show very little and hardly detectable morphological variation, which increases the probability of cryptic species remaining unnoticed and makes them a particularly tricky group from a taxonomic point of view. The initial suspicion that a number of species are actually complexes of several cryptic species has been confirmed in several studies on parasitic wasps (e.g., Atanassova et al., 1998; Kankare et al., 2005; Samara et al., 2008; Smith et al., 2008) – though not all (Cronin & Abrahamson, 2001; Baer et al., 2004; Althoff, 2008).

Species belonging to the genus *Macrocentrus* (Hymenoptera: Braconidae) live as obligatory parasites of Lepidoptera (van Achterberg, 1993) and are actual or candidate biological control agents against stem or twig borers (Crambidae, Gelechiidae, Pyralidae) and leaf rollers (Tortricidae). Among them, *Macrocentrus cingulum* Brischke – also known as *Macrocentrus abdominalis* Fabricius (Baker et al., 1949), *Macrocentrus grandii* Goidanich, or *Macrocentrus gifuensis* Ashmead (van Achterberg & Haeselbarth, 1983) – naturally occurs in the Palearctic region (from Western Europe to Japan: van Achterberg, 1993) and parasitizes two important maize pests of the genus *Ostrinia* (Lepidoptera: Crambidae) (Baker et al., 1949). Some records also mention lepidopteran hosts other than *Ostrinia* spp. (Shenefelt, 1969; He et al., 2000), but a large-scale literature survey found no record of these host species or genera ever being infested by *M. cingulum* in field studies conducted over more than 30 years in Europe and North America, and, to a lesser extent, in Asia (De Nardo & Hopper, 2004). This survey included 25 Crambidae, 17 Pyralidae, 7 Lymantriidae, 43 Noctuidae, and 17 Nymphalidae species (De Nardo & Hopper, 2004). Therefore, *M. cingulum* is considered as a specialist of the genus *Ostrinia* (Thompson & Parker, 1928; van Achterberg, 1993; He et al., 2000; De Nardo & Hopper, 2004). Although considered as a single species distributed worldwide, *M. cingulum* displays strong variations in parasitism success within the genus *Ostrinia* across its geographical range (Thompson & Parker, 1928; Baker et al., 1949; Frolov et al., 2007; Pélissié et al., 2010), and more specifically within and between three species: *Ostrinia nubilalis* (Hübner), *Ostrinia scapulalis* Walker, both sensu Frolov et al. (2007), and *Ostrinia furnacalis* Guenée.

Ostrinia nubilalis feeds on maize, *Zea mays* L. (Poaceae), occurs in Europe, and has been introduced accidentally into North America (see below) at the beginning of the 20th century (Mutuura & Munroe, 1970). *Ostrinia furnacalis* also feeds on maize and ranges over Eastern Asia (China, Japan, Korea, Philippines, Vietnam) and Australia (Mutuura & Munroe, 1970), with no or very little overlap with *O. nubilalis*. Finally, *O. scapulalis* feeds on a variety of host plants including mugwort, *Artemisia vulgaris* L. (Asteraceae), hop, *Humulus lupulus* L., and hemp, *Cannabis sativa* L. (both Cannabaceae). Its geographical range spans all northern Eurasia, so that it is largely sympatric with *O. nubilalis* in Europe and *O. furnacalis* in Asia (Frolov et al., 2007).

In Europe, *M. cingulum* heavily parasitizes (up to 75%) populations of *O. scapulalis* (Thompson & Parker, 1928; Thomas et al., 2003; Pélissié et al., 2010). In contrast, it has never been found emerging from *O. nubilalis*, even in areas of sympatry with *O. scapulalis* populations that were heavily parasitized (Pélissié et al., 2010) and/or in populations in which it has been molecularly detected within *O. nubilalis* larvae (Pélissié et al., 2010), suggesting that both oviposition and larval development may be affected. Not a single adult emerged from 7 500 *O. nubilalis* larvae from 64 populations collected on maize over four successive years in France (Pélissié et al., 2010) or from 1 700 *O. nubilalis* larvae collected in four localities in Spain (Monetti et al., 2003). Moreover, despite a quite extensive search of the European literature about natural enemies of *O. nubilalis* in the last few decades, we could find no record of *M. cingulum* emerging from larvae collected on maize in this area (former Yugoslavia: Manojlovic, 1984a,b, 1989; France: Grenier et al., 1990; Hungary: Dolinka, 1974; Italy: Maini, 1973; Platia & Maini, 1973; Romania: Perju, 2005; western Russia: Frolov et al., 1982).

In contrast, *M. cingulum* does parasitize *O. furnacalis* feeding on maize (Baker et al., 1949; He et al., 2000). It also emerges from *Ostrinia* larvae collected on various host plants – including mugwort – in China and in Japan (J Tabata, D Bourguet & S Ponsard, unpubl.). Although in those cases the exact *Ostrinia* host species could not be determined, the host plant species, as well as population genetics studies conducted on *Ostrinia* larvae collected from the same location and the same plants on the same date (D Bourguet, R Streiff & S Ponsard, unpubl.) strongly suggest that they were *O. scapulalis*, *O. orientalis*, or *O. narynensis*, all of which Frolov et al. (2007) propose to synonymize with *O. scapulalis*.

Ostrinia nubilalis, as well as *M. cingulum*, have only recently been introduced into North America (in the 1910s and 1920s, respectively; Thompson & Parker, 1928), the former accidentally and the latter willingly to control

it. In this area, maize is infested by *O. nubilalis*, whereas *O. scapulalis* is absent, and only two other, phylogenetically more distant, *Ostrinia* species are present: *Ostrinia penitalis* (Grote) and *Ostrinia obumbratalis* (Lederer) (Mutuura & Munroe, 1970). At least *O. nubilalis* and *O. penitalis* are parasitized by *M. cingulum* in USA (Baker et al., 1949), no study having dealt with parasitism on *O. obumbratalis* to our knowledge.

Thus, western Europe is the only known area where *M. cingulum* is present but unable to develop in an *Ostrinia* species feeding on maize – in this case *O. nubilalis* – even though it is locally abundant. There is little chance of this being due to scarcity of observations, as *O. nubilalis* natural enemies have been thoroughly studied over the past few decades, due notably to its pest status. An appealing hypothesis to explain this intriguing pattern is that European, Asian, and American populations of *M. cingulum*, although morphologically similar (Baker et al., 1949; van Achterberg & Haeselbarth, 1983; He et al., 2000), belong to distinct taxa differing in their abilities to complete their life cycle in *Ostrinia* populations feeding on maize. To explore this hypothesis, we assessed and compared the molecular diversity of *M. cingulum* populations sampled in Europe, Asia, and USA, using mitochondrial and nuclear DNA markers.

Materials and methods

Taxon sampling

Macrocentrus cingulum – pupae or adults – were reared from *O. nubilalis* larvae sampled in the field and preserved in 90% ethanol before DNA extraction. As *M. cingulum* is polyembryonic, we either extracted the DNA from one single larva, or pooled several adults of the same sex in a single DNA extract. The geographical origin, host plant on which *Ostrinia* larva were collected, putative *Ostrinia* host species, date of sampling, and method for DNA extraction of the 97 *M. cingulum* samples analysed in this study, are given in Table 1.

As an outgroup for building phylogenetic trees, we used a specimen from a congeneric species, *Macrocentrus sylvestrellae* (van Achterberg, 2001) (kindly provided by H Jactel, INRA, France) reared from a larva of the pine stem borer *Dioryctria sylvestrella* (Ratzeburg) collected in south-western France.

DNA sequencing

Genomic DNA was extracted using the DNeasyTissue Kit (Qiagen,Veulo,The Netherlands). For each sample, we amplified sequences of two mitochondrial coding regions: part of the cytochrome b (Cyt b) gene, using primers CB-J-10933 and CB-N-11367 (Simon et al., 1994), and part of the cytochrome oxidase subunit I (COI) gene, using primers Lco1490 and Hco2198 (Folmer et al., 1994). In addition, we amplified (by direct amplification) partial sequences of two nuclear genes: the internal transcribed spacer 2 (ITS2), using primers FcM and B1D (Ji et al., 2003), and a region of the elongation factor 1 subunit alpha F2 (EF1a-F2), using primers EF1A1F and EF1A1R (Belshaw & Quicke, 1997). For EF1a-F2, both coding (exons) and non-coding (introns) regions were sequenced. Standard cycling conditions were 5 min at 96 °C followed by 35 cycles of 1 min at 96 °C, 1 min at 50 °C for Cyt b and ITS2, 47 °C for COI, and 47–52 °C for EF1a-F2, 90 s at 72 °C, and a final step at 72 °C for 7 min. The PCR products were directly sequenced in both directions using BigDye v3.1 sequencing kits and Applied 3730xl sequencers. The new sequence data generated in this study were deposited in GenBank, under accession numbers HQ177097 to HQ178683.

All sequence alignments were performed using ClustalX v1.83 (Thompson et al., 1997) with default settings. ITS2 sequences proved strictly invariable across all samples and were thus not used in further analyses. Cyt b and COI alignments revealed no gaps. One gap corresponding to a supplementary position only found in the outgroup species *M. sylvestrellae* was present in the EF1a-F2 gene. Alignment of EF1a-F2 sequences also showed eight heterozygous positions, which were coded using the IUPAC nucleotide ambiguity codes (e.g., Sota & Vogler, 2003). After alignment, the combined sequence was 1 694 bp long: the COI region (668 characters), the Cyt b region (386 characters), and the intron region of the EF1a-F2 gene (640 characters). For the latter, the two coding regions were situated between positions 1–119 and 337–615, respectively.

Genetic diversity and genetic differentiation

Genetic diversity was assessed using DNASP v5.10 (Librado & Rozas, 2009) to estimate haplotype (H) and nucleotide (π) diversity. The outgroup species *M. sylvestrellae* was excluded from these analyses. To take into account the information carried by heterozygous sites in the EF1a-F2 gene, phase at linked loci was inferred using the PHASE algorithm (Stephens et al., 2001; Stephens & Donnelly, 2003) implemented in DNASP, which uses a coalescence-based Bayesian method to reconstruct haplotypes from genotypes (Stephens et al., 2001). In addition, the software PAUP* v4.0b10 (Swofford, 2003) was used to estimate mean sequence divergence among populations.

The level of genetic differentiation between taxa of distinct geographical origins (American, Asian, or European) was assessed for each gene and for the two mitochondrial genes together (mitochondrial compartment)

Table 1 Characteristics of the 97 *Macrocentrus cingulum* used in this study. All specimens originated from diapausing *Ostrinia* spec. larvae collected on their host plants. The host plants are given in the last column: maize (*Zea mays*), rumex (*Rumex* spec.), xanthium (*Xanthium* spec.), mugwort (*Artemisia vulgaris*), foxtail bristlegrass (*Setaria italica*), and sunflower (*Helianthus annuus*)

Name	Life cycle stage	Extraction method[1]	Sampling				
			Date	Continent	Country	Location	Host plant
USA751-6, USA771-6	Female adult	Q	na	America	USA	Rosemount, MN	*Z. mays*
USA761-3, USA781-4	Male adult	Q	na	America	USA	Rosemount, MN	*Z. mays*
ASI31	Pupa	Q	2005	Asia	China	Beijing (Langfang)	*Z. mays*
ASI33	Pupa	Q	2005	Asia	China	Beijing (Rian Shan Jian)	*Z. mays*
ASI20-4	Adult	Q	2005	Asia	China	Shandung	*Z. mays*
ASI30, ASI32, ASI34, ASI36, ASI37, ASI39	Pupa	Q	2005	Asia	China	Shanghaï	*Z. mays*
ASI11, ASI15	Pupa	Q	2005	Asia	Japan	Akigawa, Tokyo Pref.	*A. vulgaris*
ASI13	Pupa	Q	2005	Asia	Japan	Akigawa, Tokyo Pref.	*Rumex* spec.
ASI14	Pupa	Q	2005	Asia	Japan	Akigawa, Tokyo Pref.	*Xanthium* spec.
ASI16, ASI17	Pupa	Q	2005	Asia	Japan	Akigawa, Tokyo Pref.	*Z. mays*
ASI47-60	Adult	Q	2001	Asia	Japan	Higashi, Tokyo Pref.	*Z. mays*
ASI1-7	Adult	Q	2005–2006	Asia	Japan	Kannondai, Ibaraki Pref.	*S. italica*
ASI67	Adult	Q	2006	Asia	Japan	Moriya, Ibaraki Pref.	*H. annuus*
ASI8-10	Pupa	Q	2005	Asia	Japan	Yawara, Ibaraki Pref.	*Xanthium* spec.
ASI68	Adult	Q	2006	Asia	Japan	Akigawa, Tokyo Pref.	*Ambrosia* spec.
EUR27-9	Pupa	Q	2006	Europe	France	Glisy	*A. vulgaris*
EUR45, EUR46	Pupa	Q	2005–2006	Europe	France	Glisy	*A. vulgaris*
EUR128, EUR134-6, EUR138, EUR139	Pupa	βme	2005–2006	Europe	France	Houdan	*A. vulgaris*
EUR15-7, EUR19, EUR66	Pupa	Q	2004–2005	Europe	France	Ile de France	*A. vulgaris*
EUR25	Pupa	Q	2004–2005	Europe	France	Lille	*A. vulgaris*
EUR4, EUR5, EUR7, EUR8, EUR25, EUR41, EUR43, EUR44	Pupa	Q	2004–2005	Europe	France	Lille	*A. vulgaris*
EUR11-4, EUR61-5	Pupa	Q	2004–2005	Europe	Germany	Kropp	*A. vulgaris*

Individuals who shared the same information have been pooled in one row.
na = not available.
[1]Q, Qiagen; βme, β-mercapto-ethanol.

by estimating three distinct test statistics (F_{ST}, K_{ST^*}, and S_{nn}) with DNASP. F_{ST} is a statistic that measures the diversity of randomly chosen alleles within the same population relative to what is found in the entire geographical sample. The K_{ST^*} is a test statistic that takes account of the number of nucleotide differences between different haplotypes but does not give much weighting to large numbers of differences (Hudson et al., 1992). S_{nn} is usually referred to as the nearest-neighbour statistic and is a measure of how often the nearest neighbours (in the matrix) of sequences are from the same population in geographical space (Hudson, 2000). Because these three indices are known to be more or less sensitive to specific data set features (such as a low level of genetic diversity or a low sample size), they

were used in combination to ensure robust detection of differentiation (Morales-Hojas et al., 2008). For each index, a permutation test of 1 000 replicates was performed under DNASP to assess the significance of the subdivision parameters.

Haplotype networks

The phylogenetic reconstructions showed a low number of haplotypes and the genetic analyses indicated a low level of genetic differentiation between populations (see Results). Hence, we might be dealing with genetic diversity at intraspecific, rather than interspecific, level. We therefore also investigated the relationships between the different haplotypes using statistical parsimony networks (Posada & Crandall, 2001), which are appropriate for data exhibiting low genetic divergence (Zhang & Hewitt, 2003). Such networks help inferring spatial and historical patterns within and among populations. Haplotype networks were reconstructed separately for the EF1a-F2 gene and for the mitochondrial gene compartment. To take into consideration the information contained in heterozygous sites of the EF1a-F2 gene, the corresponding haplotype network was reconstructed using the haplotypes inferred by the PHASE algorithm (see above). All networks were estimated with a 95% parsimony connection limit (without the outgroup taxon), using TCS v1.21 (Clement et al., 2000).

Phylogenetic analyses

To explore a possible conflict between information carried by the mitochondrial and nuclear genes in our data set, we conducted several incongruence length difference (ILD) tests (Farris et al., 1994), as implemented in PAUP*, with 1 000 replicates and all invariant characters excluded (Cunningham, 1997).

To estimate the phylogenetic relationships between taxa, we carried out Bayesian inference (BI) analyses using MrBayes v3.12 (Ronquist & Huelsenbeck, 2003). In all analyses, the sole gap event was treated as missing data whereas heterozygous positions were taken into account. Separate Bayesian analyses were conducted for each gene, as well as for the combined data set.

For BI analyses on the combined data set, five distinct partitioning strategies were used: P_1, 'one partition strategy': one single partition for the whole combined data set; P_2, 'two partition strategy': one partition for the mitochondrial compartment and one for the nuclear gene; P_3, 'three partition strategy': one partition per gene; P_4, 'four partition strategy': one partition per codon position for the two mitochondrial genes (Cyt b and COI) plus one partition for the nuclear gene; and P_5, 'seven partition strategy': one partition per codon position for Cyt b, one partition per codon position for COI, plus one partition for the nuclear gene. Evolutionary models for each gene or partition were selected using Modeltest v3.06 (Posada & Crandall, 1998), based on the corrected Akaike's Information Criterion (AIC). Allowing subsets of the data to evolve under different models is expected to increase both phylogenetic accuracy and posterior probabilities estimates (Nylander et al., 2004), although, on the other hand, estimating model parameters on smaller data sets increases the risk of random error (Brandley et al., 2005). In this study, we used the Bayes factor (B_F) as an objective criterion to choose between pairs of alternative strategies. The traditional cut-off criterion of $2 \ln B_F \geq 10$ was used to choose among competing strategies (Brandley et al., 2005). B_F values were estimated based on the ratio of the harmonic mean of the likelihoods (sampled from the posterior using the 'sump' option in MrBayes with a specified burn-in period). For all analyses (i.e., separate analyses of each gene and analyses of the combined data set using different partitioning strategies), two independent runs of 10 000 000 generations were performed. For each run, we used eight Metropolis-coupled chains, with high heating values (T = 0.8), random starting trees, and default priors. Distinct parameters were estimated for each partition under the best-fit substitution models (determined according to the AIC). During the run, the trees and branch lengths were saved every 100 generations – 100 000 trees were thus saved at the end of each Monte Carlo Markov chain (MCMC) run. We applied a conservative burn-in period of 1 000 000 generations, so we kept the topologies sampled in the last 9 000 000 generations. For each analysis, results were generated using the pooled tree samples from the stationary phases of the independent runs – reached in all cases before generation 1 000 000 – and the support for each node of this tree was given by clade posterior probability (CPP) estimates. Because Bayesian posterior probabilities are probably less conservative than non-parametric bootstrap values, especially for short internodes (Alfaro et al., 2003; Erixon et al., 2003), only clades with posterior probabilities >0.9 were considered as well-supported ones in BI analyses.

Additional analyses were also conducted under parsimony with the software TNT v.1.1 (Goloboff et al., 2008). Initial heuristic searches were carried out using the tree bisection reconnection (TBR) algorithm ('traditional search' option in TNT), with random starting trees, 100 random-addition replicates, and a 'MaxTrees' value of 1 000. Additional analyses were conducted using sectorial searches with random sectorial searches (RSS) and consensus-based sectorial searches (CSS) (Goloboff, 1999), with the options for tree ratchet, tree drifting, and tree fusing (Goloboff, 1999) selected ('new technology search' option in TNT), 100 random-addition replicates and a

'MaxTrees' value of 1 000. For each analysis, 1 000 non-parametric bootstrap replications were performed (standard sample with replacement).

Phylogenetic hypotheses testing

To know whether or not European, Asian, and American populations of *M. cingulum* should be considered as sister groups, we tested for both the possible monophyly of populations from each continent and for the possible reciprocal monophyly of some of them using the Shimodaira Hasegawa (SH) test (Shimodaira & Hasegawa, 1999). Following Goldman et al. (2000), we tested for the difference between an optimal tree (resulting from an unconstrained analysis based on maximum likelihood) and a constrained tree (in which specific individuals are constrained to be monophyletic). Three distinct tests were performed: (1) all American individuals constrained to monophyly; (2) all Asian individuals constrained to monophyly; and (3) all American and Asian individuals constrained to monophyly on the one hand and all European individuals constrained to monophyly on the other (test for reciprocal monophyly). All SH tests were carried out with PAUP* (RELL method; 1 000 replicates).

Results

Genetic diversity and genetic differentiation

The ILD tests revealed no significant incongruence between COI and Cyt b, therefore we also performed supplementary analyses considering them as a single evolutionary unit (mitochondrial compartment). The number of distinct haplotypes was 14 for the EF1a-F2 gene and 32 for the mitochondrial compartment (Table 2).

On average, the distance between haplotypes was low, especially for EF1a-F2. Nevertheless, none of the mitochondrial haplotypes found in European populations were ever found in Asian or American populations, and three and two EF1a-F2 haplotypes found in European populations were also found in Asian and American populations, respectively. The number of shared haplotypes was higher or equal between American and Asian populations (two for the mitochondrial compartment, three for EF1a-F2; Table 3). The American population displayed both the highest haplotype diversity (up to 0.83, depending on gene, vs. 0.71 and 0.72 for the European and Asian populations, respectively; Table 2) and the highest nucleotide diversity (up to 0.0072 vs. 0.0022 and 0.0031 for the European and Asian populations, respectively; Table 2).

A strong and highly significant ($P<0.0001$ for all comparisons) genetic structure between European populations and their Asian or American counterparts was consistently found for mitochondrial genes as well as for EF1a-F2 (F_{ST}, K_{ST^*}, and S_{nn} values ranged 0.45–0.79, 0.28–0.55, and 0.82–1, respectively; Table 3). We also found a significant genetic structure for both the mitochondrial compartment and the nuclear gene between the Asian and American populations, although the genetic differentiation was lower between these two populations than between any of them and the European population (F_{ST}, K_{ST^*}, and S_{nn} equalled 0.40, 0.20, and 0.88 for the mitochondrial compartment and 0.05, 0.03, and 0.69 for EF1a-F2; Table 3).

Haplotype networks

For clarity and because both COI and Cyt b reflect the history of the same evolutionary unit (as confirmed by the ILD test, see below), we display only two networks, one based on the combination of both mitochondrial genes and one based only on EF1a-F2 sequences (Figure 1). Due to the lower number of haplotypes (n = 14), the network based on nuclear sequences was less reticulated than that based on mitochondrial sequences. Nevertheless, both networks exhibited essentially the same patterns. First, most European haplotypes were grouped together. Second, the Asian population contained the most frequently sampled and best-connected haplotype (Crandall & Templeton, 1993), suggesting that it might represent the ancestral population. Third, American individuals were not completely clustered, some of them sharing Asian haplotypes.

Table 2 Genetic diversity estimates for each set of populations for each gene

	European populations				Asian populations				American population			
Gene	N	n	H	π	N	n	H	π	N	n	H	π
CO1	33	8	0.69	0.0017	45	11	0.52	0.0032	19	7	0.66	0.0056
Cyt b	33	4	0.53	0.0022	45	7	0.48	0.0032	19	7	0.74	0.0073
Mitochondrial compartment	33	9	0.71	0.0019	45	14	0.72	0.0032	19	11	0.83	0.0062
EF1a-F2	66	3	0.52	0.0008	90	10	0.51	0.0012	38	7	0.74	0.0025

Note that for the EF1a-F2 gene, the N values take into account haplotype reconstructions using the PHASE algorithm. N, no. individuals/alleles analysed; n, no. haplotypes; H and π, haplotype and nucleotide diversity, respectively.

Table 3 Genetic differentiation values estimated for each pair of continents of origin and for each locus, and number of shared haplotypes (s.h.)

	European vs. Asian populations				Asian vs. American populations				European vs. American populations			
Gene	s.h.	F_{ST}	K_{ST*}	S_{nn}	s.h.	F_{ST}	K_{ST*}	S_{nn}	s.h.	F_{ST}	K_{ST*}	S_{nn}
CO1	0	0.716***	0.460***	1.0***	2	0.364***	0.226***	0.848***	0	0.505***	0.314***	0.981***
Cyt b	0	0.792***	0.549***	1.0***	2	0.454***	0.264***	0.854***	0	0.677***	0.434***	1.0***
Mitochondrial compartment	0	0.752***	0.437***	1.0***	2	0.404***	0.204***	0.875***	0	0.598***	0.324***	1.0***
EF1a-F2	3	0.544***	0.331***	0.83***	3	0.052**	0.031*	0.691***	2	0.447***	0.277***	0.903***

P-values for each of these analyses were calculated using 1 000 permutations.
*0.01<P<0.05; **0.001<P<0.01; ***P<0.001.

Phylogenetic analyses and phylogenetic hypotheses testing

There were 1 054 alignment positions in the mitochondrial data set, 27 of which were parsimony informative (24 when excluding the outgroup taxon). Only 7 of 640 alignment positions were parsimony informative in the nuclear data set (six when excluding the outgroup taxon). Unsurprisingly, the ILD test found no significant incongruence between the two mitochondrial genes (P>0.05), as they belong to the same evolutionary unit. In contrast, a significant incongruence was detected between mitochondrial and nuclear compartments (P = 0.01).

For each gene (or molecular compartment), the general time reversible model (GTR; Yang, 1994) was identified as the most appropriate substitution model according to the corrected AIC. The comparison of B_F values identified P_4 (i.e., one partition per codon position for the two mitochondrial genes plus one partition for the nuclear gene) as the optimal partitioning strategy. Overall, the topologies resulting from the separate BI analyses of the three genes were unresolved and weakly supported (only three nodes were supported by CPP values >0.9). However, most sampled individuals were genetically distinct and clustered by geographical origin, especially in the trees based on COI and Cyt b. In those topologies, one clade including European specimens and one or several clades including Asian and/or American specimens were consistently recovered.

Analyses of the combined data set under BI yielded similar topologies (with slight variations in branching order). Compared with those based on single genes, the topology (Figure 2) based on the combined data was much more resolved and better supported (15 nodes with CPP values >0.9). In the resulting tree, almost all specimens were clustered by geographical origin. This was especially true for the European specimens, which constituted a monophyletic group nested within a larger clade including all the Asian and American specimens. The latter appeared paraphyletic, as the monophyly hypothesis was rejected both for American and for Asian populations (SH tests: P = 0.044 and P = 0.002, respectively). The SH test in which European specimens were constrained into a sister group position with another including both the American and the Asian specimens also resulted in rejection of the null hypothesis (P = 0.023), strongly suggesting that these two groups do not exhibit reciprocal monophyly.

In contrast with BI, under parsimony, not only the topologies resulting from the separate analyses but also the topologies resulting from the analysis of the combined data set were mostly unresolved and weakly supported. For the combined data set, 'Traditional search' analyses generated 20 equiparsimonious trees ('MaxTrees' value was fixed to 1 000 trees), whereas 'New Technology search' analyses recovered one equiparsimonious tree of the same size. All trees exhibited the following characteristics: length = 257, consistency index = 0.852, and retention index = 0.933. In agreement with BI analyses, the European group was the only monophyletic group recovered when analysing either the combined data set or the Cyt b data set (although weakly supported in this case; bootstrap values <30%).

Discussion

Macrocentrus cingulum populations probably belong to a single species worldwide

Altogether, our results suggest that all *M. cingulum* specimens from Europe, America, and Asia are closely related to each other, and probably part of the same species. First, molecular divergence between European and Asian populations was weak, especially for the nuclear loci. The ITS proved to be monomorphic across all specimens, although it is known to evolve rapidly and to be clearly differentiated between well-established species (Stouthamer et al., 1999), sibling species (Allemand et al., 2002; Alvarez & Hoy, 2002), and even conspecific populations (Alvarez & Hoy, 2002) of various hymenopteran parasitoids. Similarly, the EF1a-F2 gene showed a relatively low

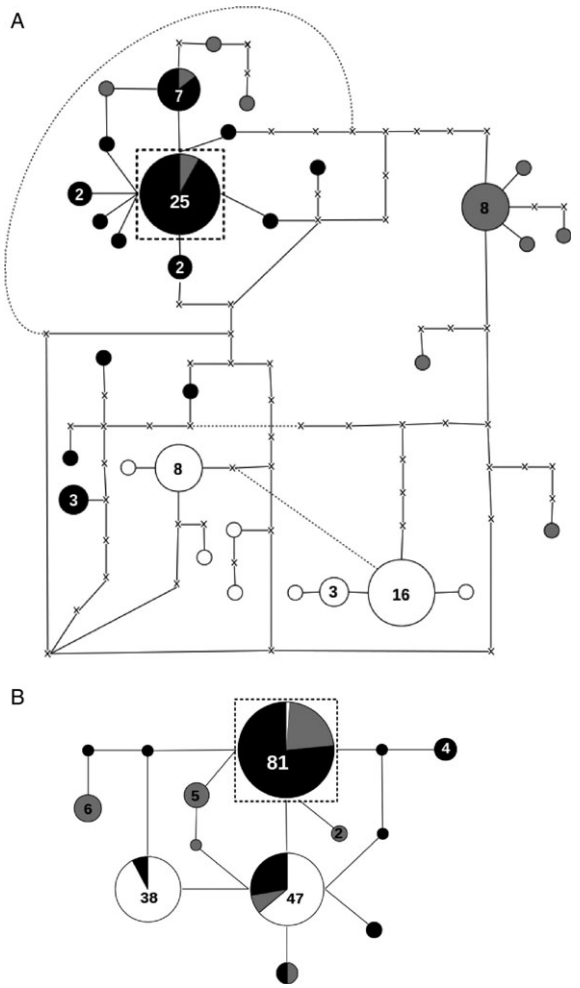

Figure 1 Statistical parsimony haplotype networks based on (A) mitochondrial (COI and Cyt b genes) and (B) nuclear (EF1a-2F gene) compartments. Each circle represents a sampled haplotype and its geographical origin (black: Asia; grey: USA; white: Europe). Black crosses represent hypothetical, non-sampled haplotypes. Circles and black crosses are separated by one mutational step (loops are represented by dotted lines). Circular areas are proportional to the number of sampled individuals sharing the haplotype (specified when >1). The haplotype most likely to be ancestral (i.e., both the most frequently sampled and the most connected to others) is surrounded by a dotted square.

number of haplotypes (n = 14), two of which were shared among European, Asian, and American populations. In the mitochondrial compartment, we found no shared haplotype between Europe and any of the two other continents, but we still found <0.5% mean divergence (i.e., the number of nucleotidic substitutions) between the COI sequences in Asian and European populations. As a comparison, for the same gene, Danforth et al. (1998) found a minimum of 4% divergence between two cryptic species, *Halictus ligatus* Say and *Halictus poeyi* Lepeletier, and Kruse & Sperling (2001) found 1.5–2.5% divergence between two closely related species, *Archips argyrospila* Walker and *Archips goyerana* Kruse.

Second, both haplotype networks were well connected and haplotypes were separated from each other by only few mutations, suggesting that they all belong to the same or very closely related taxa. If individuals belonged to more distant taxa, haplotypes would rather be expected to be scattered in loosely or even disconnected networks (Crandall & Templeton, 1993). Third, the hypothesis of reciprocal monophyly between, on the one hand, European populations (which formed a well-supported monophyletic group in the selected topologies), and, on the other, Asian/American populations (which were paraphyletic), was rejected. This pattern points to the existence of either one single taxon or two weakly differentiated taxa, rather than two clearly differentiated ones.

Nevertheless, European *Macrocentrus cingulum* form a distinct clade

Our results also show that a European clade can be clearly distinguished at both mitochondrial and nuclear loci. This clade only contained European individuals, all of which emerged from *Ostrinia* larvae collected on mugwort. The European populations also exhibited the strongest indices of genetic differentiation vs. both American and Asian populations, the latter being the most genetically distant. American and Asian populations were also significantly differentiated, but less so than they both were from European populations.

Asian populations appear as ancestral compared with the other *M. cingulum* populations in our data set, as, in both mitochondrial and nuclear haplotype networks, the most frequently sampled and best-connected haplotype was mainly carried by Asian individuals (Figure 1). Moreover, Asian haplotypes belong to the more 'basal' cluster of the phylogenetic tree (Figure 2) – although the support of the deeper nodes needs to be improved to confirm this conclusion. The American population appeared paraphyletic and mixed with Asian individuals, both in the phylogenetic reconstructions (Figure 2) and in the haplotype networks (Figure 1). In contrast, the monophyletic cluster of European populations remained nested in the 'basal' Asian/American cluster, suggesting that it also derived from the Asian populations, although probably earlier in time than the Rosemount (USA) population did. Therefore, although our study argues – until contradicting evidence – in favour of *M. cingulum* populations being considered as a single species worldwide, it also shows that, within this species, European populations form a group

Figure 2 Phylogenetic trees of 97 *Macrocentrus cingulum* individuals corresponding to the result of the partitioned Bayesian inferences. The largest tree corresponds to the analysis carried out using the best partitioning strategy (P_4). Nodes supported by CPP values >0.9 are highlighted with grey circles and represent well-supported clades in the BI analysis. European and American individuals are figured using italic and bold characters, respectively. In addition, results from the analyses of separate data sets are figured for the three genes. In the corresponding trees, individuals from the USA are represented using thick lines, whereas European specimens are indicated by blurred lines (normal lines are used for Asian specimens).

that is somewhat differentiated from the American and Asian group.

Genetic differentiation correlates with parasitization patterns

Despite extensive investigations, *M. cingulum* has never been observed emerging from *O. nubilalis* feeding on maize in Europe (Maini, 1973; Platia & Maini, 1973; Dolinka, 1974; Frolov et al., 1982; Manojlovic, 1984a,b, 1989; Grenier et al., 1990; Monetti et al., 2003; Thomas et al., 2003; Perju, 2005; Pélissié et al., 2010), although it is found sometimes at high rates in *O. scapulalis* larvae feeding on mugwort and hop (Thomas et al., 2003; Pélissié et al., 2010). In Asia, *M. cingulum* can parasitize (possibly several species of) *Ostrinia* developing on a variety of host plants including mugwort and maize – as well as *Xanthium* spec., *Ambrosia* spec., *Helianthus* spec., *Setaria italica* (L.)

P. Beauv., *Rumex* spec., and possibly others –, and we found no evidence for the existence of a separate, host plant-related *M. cingulum* taxon (analyses not shown). Indeed, our reconstructions did not cluster individuals collected in Asia on mugwort or other non-maize plants any closer to the European specimens (also collected on mugwort) than to Asian specimens collected on maize. In contrast, we did find evidence for genetic divergence between European and Asian *M. cingulum* populations (see above). Isolation by distance may contribute to this divergence, as the closest pair of European and Asian specimens was sampled at a distance of ca. 9 000 km. Isolation by distance has been documented in other hymenopteran parasitoids at similarly large (e.g., Grillenberger et al., 2009) or even smaller geographical scale (e.g., Anton et al., 2007; Althoff, 2008). However, the influence of geographical distances on genetic diversity must be moderate here, as we did not detect any differentiation between specimens sampled in China and Japan, which are separated by some 1 000–1 500 km: all Asian individuals were mixed in the phylogenetic reconstructions regardless of geographical origin (data not shown). The genetic differentiation between our Asian and European samples could thus be correlated with a difference between Asian and European populations of *M. cingulum* in their ability to parasitize *Ostrinia* populations feeding on maize, rather than or in addition to isolation by distance.

An evolutionary scenario with three testable predictions

Our results, along with historical records, allow us to build an evolutionary scenario that could explain the intriguing differences between *M. cingulum* infestation patterns in *Ostrinia* populations feeding on maize vs. mugwort in Europe, and *Ostrinia* populations on maize across the world. At this stage, this scenario is simply compatible with our data. Nevertheless, it is falsifiable in the Popperian sense, as it implies at least two predictions that can be tested in future experiments.

The centre of highest diversity – and probably the centre of origin – of the genus *Ostrinia* is thought to be eastern Asia (eastern China and Japan; Mutuura & Munroe, 1970), where a variety of *Ostrinia* species are found on a variety of host plants. *Ostrinia scapulalis* and *O. nubilalis* are the two main species present in Europe, and, according to Kim et al.'s (1999) phylogeny, they are the two most recently diverged species within the genus *Ostrinia*. The fact that *O. nubilalis* is not documented eastwards of Xinjiang (Wang et al., 1995; Xu et al., 1998) suggests that the divergence between *O. scapulalis* and *O. nubilalis* may have occurred in central/western Asia or Europe, rather than in eastern Asia. Given that the major current host plant of *O. scapulalis* – mugwort – is native from Eurasia, whereas *O. nubilalis*' main host – maize – is not, it is parsimonious to assume that the ancestral *Ostrinia* species used to feed on mugwort, a common weed widely distributed across the entire continent. The divergence between *O. nubilalis* and *O. scapulalis* may then have been either triggered or followed by a host shift of *O. nubilalis* on maize (Malausa et al., 2005, 2007). Meanwhile, in eastern Asia, *O. furnacalis* must have shifted or differentiated on maize after this crop was introduced in its distribution range, around 500 years ago.

In this framework, *M. cingulum* as a species, and/or its close association with the genus *Ostrinia*, might have originated in eastern Asia. Later, the ancestral species from which *O. scapulalis* and *O. nubilalis* diverged must have extended westwards where other *Ostrinia* spp. were rare or absent. *Macrocentrus cingulum* may have followed it, while losing certain 'generalist traits' that allow it to parasitize various *Ostrinia* species on various host plants in Asia, as it was exposed to selection pressures exerted by only one particular host. When *O. nubilalis* diverged from *O. scapulalis* and shifted on maize, *M. cingulum* populations that had evolved only or mostly on *O. scapulalis* for many generations may no longer have been 'generalist enough' to parasitize this new host. In contrast, more generalist and diverse East-Asian *M. cingulum* populations were able to 'follow' *O. furnacalis* when it started feeding on maize.

The fact that the American *M. cingulum* population analysed here is closer to the Asian than to the European ones fits well into this scenario. Indeed, several attempts were made to introduce *M. cingulum* into various regions of the USA between 1926 and 1940 (Thompson & Parker, 1928; Baker et al., 1949; Hudon et al., 1989; Sked & Calvin, 2005). These introductions were conducted both with individuals collected in Asia – probably from *O. furnacalis* larvae – and in Europe – probably from *O. scapulalis* larvae. Unfortunately, the origin of the released parasitoids in each locality was not always precisely recorded, and not all introductions were subjected to a local follow-up to see if self-sustaining populations had established. Nevertheless, there does seem to be a tendency in Baker et al.'s (1949) report for introductions carried out with Asian populations to be the most or possibly even the only successful ones. As American *O. nubilalis* populations are of recent European origin (Hudon et al., 1989), it would not be surprising if European *M. cingulum*, which appear unable to develop on *O. nubilalis* in Europe, were also unable to do so in America. In contrast, the Asian *M. cingulum* parasitizing a variety of *Ostrinia* spp. on a variety of host plants including maize in Asia may have retained generalist traits that enabled them to parasitize *O. nubilalis* on maize when introduced into America.

The high haplotype diversity of the unique American population we analysed (comparable to that of our entire Asian or entire European samples; Table 2) is rather unexpected in this scenario, as introductions usually rather cause genetic bottlenecks. Moreover, most Asian *M. cingulum* introduced into America seem to have originated from Korea and Japan, a relatively small geographical area (Baker et al., 1949). This high diversity might partly be due to a mixture of Asian and European haplotypes: although our American population did not share a single haplotype with our European populations – whereas it did with Asian ones –, additional analyses may reveal the existence of shared European-American haplotypes in Rosemount or in other American *M. cingulum* populations. On the other hand, historical records show that releases of *M. cingulum* into the USA were massive, well spread over the country, and repeated in time: from 1926 to 1940, a total of 416 125 *M. cingulum* individuals were released in a total of 80 localities across nine states, from which at least 229 423 came from Europe and 85 650 came from Asia (Baker et al., 1949). The intensity of the biocontrol effort during 14 years may have been sufficient to account for high genetic diversity in American populations even if they are all of Asian origin.

Three testable predictions emerge from our results. First, most if not all current *M. cingulum* populations in North America must be recently derived from Asian ancestors. This can be checked by analysing further American *M. cingulum* populations, sampled in localities where this species was introduced in the 1920s and 1930s. Second, Asian *M. cingulum*, contrary to European populations of the parasitoid, should be able to develop in *O. nubilalis* feeding on maize in Europe, as they are in America. This can be verified by comparing the ability of *M. cingulum* from Asia and Europe to oviposit and to develop in European *O. nubilalis* on maize, possibly in semi-natural conditions to avoid unwanted introductions. Note that *O. furnacalis* is known to be able, to some degree, to prevent Asian *M. cingulum* eggs from developing until adult stage by means of specialized haemocytes that encapsulate the egg or early embryo (Hu et al., 2003, 2008). Therefore, knowing that Pélissié et al. (2010) found that European *M. cingulum* does emerge from *O. scapulalis* but not from *O. nubilalis* larvae, it seems that it may lack the ability to escape encapsulation by *Ostrinia* feeding on maize, contrary to Asian and American ones. Another hypothesis (non-reciprocally exclusive with the latter) is that European *O. nubilalis* may have a stronger ability to encapsulate than *O. furnacalis* and American *O. nubilalis*. Both hypotheses can be tested by studying the encapsulation efficiency of American vs. European *O. nubilalis* larvae of eggs from American (or Asian) vs. European *M. cingulum*.

Third, whether or not *M. cingulum* from all three continents belong to a single species in the biological sense, as suggested by their low genetic differentiation, is currently being investigated by testing their ability to interbreed. Note that our scenario does not depend crucially on this result, as differences in parasitization patterns in Europe and Asia are possible with or without reproductive isolation. However, in addition to further clarifying the taxonomic status of Asian and European *M. cingulum*, it will indicate whether or not populations of both origins may have interbred in America over the last century, and whether or not they might do so in Europe in the event of a release for biological control.

Beyond this special case, the recent evolutionary history of the maize/*Ostrinia* spp./*M. cingulum* system could not only become an interesting model to study the effects and evolution of tritrophic interactions during range expansions or after species introductions, but also provide important insights into the theory and practise of biological control.

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