Molecular characterization of closely related species in the parasitic genus Encarsia (Hymenoptera: Aphelinidae) based on the mitochondrial cytochrome oxidase subunit I gene

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Abstract

The genus Encarsia Förster includes parasitoid species that are effective natural enemies of whitefly and armoured scale insect agricultural pests. Within this genus, several species groups have been recognized on the basis of morphological similarity, although their monophyly appears uncertain. It is often difficult to separate morphologically similar species, and there is evidence that some species could in fact be complexes of cryptic species. Their correct identification is fundamental for biological control purposes. Recently, due to unreliability of morphological characters, molecular techniques have been investigated to identify markers that differentiate closely related species. In this study, DNA variation in an ~900 bp segment of the mitochondrial cytochrome oxidase subunit I (COI) gene was examined by both sequencing and PCR–RFLP. Two pairs of species that are difficult to distinguish morphologically were analysed: Encarsia formosa Gahan and Encarsia luteola Howard, belonging to the luteola group, and two populations of Encarsia sophia (Girault & Dodd) from Pakistan and Spain, belonging to the strenua group, recently characterized as cryptic species. High sequence divergence and species-specific restriction patterns clearly differentiate both species pairs. Parsimony analysis of the nucleotide sequences was also performed, including Encarsia hispida De Santis (luteola group) and Encarsia protransvena Viggiani (strenua group). Two monophyletic clades supporting the two groups of species considered were resolved. The results of this study support the use of the COI gene as a useful marker in separating species of Encarsia, for which morphological differences are subtle. Moreover, the COI gene appears potentially useful for understanding phylogenetic relationships in this genus.

Keywords: Encarsia, Aphelinidae, Hymenoptera, COI gene, molecular characterization, phylogeny, cryptic species

Introduction

The genus Encarsia Förster (Hymenoptera: Aphelinidae) includes to date 275 valid species (Heraty & Wolley, 2002; Noyes, 2002), mostly parasitoids of whiteflies and armoured scale insects (Hemiptera: Aleyrodidae and Diaspididae) (Huang & Polaszek, 1998), with some species known from aphids (Hemiptera: Aphidoidea) (Evans et al., 1995) and eggs of Lepidoptera (Polaszek, 1991). Since many species of Encarsia are considered effective natural enemies of agricultural pests, in recent years the systematics of this genus has begun to be studied thoroughly (Polaszek et al., 1992, 1999; Schauff et al., 1996; Huang & Polaszek, 1998;
Schmidt et al., 2001; Pedata & Polaszek, 2003). Within this genus, several groups of species have been recognized on the basis of morphological similarity (Viggiani & Mazzone, 1979; Hayat, 1998), although the phylogenetic value of these groups appears uncertain because of unknown polarity and homoplasy of characters utilized (Babcock et al., 2001). In some species groups it is sometimes difficult to separate species with similar morphology due to the scarcity of diagnostic characters and morphological variability within species (Polaszek et al., 1992; Pedata et al., 1994; Babcock & Heraty, 2000; Heraty & Polaszek, 2000). Moreover, there is evidence that some species could in fact be complexes of cryptic species (Polaszek et al., 1999; Giorgini, 2001; Manzari et al., 2002; Giorgini & Baldanza, 2004; Polaszek et al., 2004). In biological control, characterization of natural enemies is essential because cryptic species may have different biological performances leading to a variable capacity to control a specific pest (De Bach & Rosen, 1991). In addition, the correct identification of parasitoid populations is required for the control of whitfly populations (Casineiras, 1995; Riley & Ciomperlik, 1997). It was introduced in Israel in a classic biological control programme of B. tabaci (Rivnay & Gerling, 1987) and was used for the control of B. tabaci on Poinsettia in greenhouses (Heinz & Parrella, 1994).

In the stenura group, Encarsia sophia (Girault & Dodd) (Aphelinidae) is one of the more cosmopolitan species (Heraty & Polaszek, 2000) found worldwide to be a dominant parasitoid of T. tabaci (Kapadia & Puri, 1990; Osborne et al., 1990; Kajita et al., 1992; Ozawa et al., 1992; McAuslane et al., 1993; Stansly et al., 1997) and T. vaporariorum (Gerling, 1983). It was studied as a potential biocontrol agent of B. tabaci (Hoelmer et al., 1994; Goolsby et al., 1996, 1998). Recently, three populations, one of E. luteola from California, and two of E. sophia from Pakistan and Spain, respectively, were chosen as potential candidates for mass rearing and field release attempts, and imported in Italy in a programme of biological control of T. vaporariorum. The difficulty in discriminating E. luteola from E. formosa, and the impossibility in separating the two populations of E. sophia on the basis of single morphological characters prompted a thorough study of discriminatory methods. In a previous work (Giorgini & Baldanza, 2004), the Pakistani and Spanish populations of E. sophia have been distinguished on the basis of reproductively incompatible, karyological characters and morphometric analysis, suggesting their status of cryptic species. In this study, DNA variation in a fragment of the mitochondrial COI gene was examined by sequencing and PCR-RFLP in order to identify a reliable molecular marker that clearly differentiates E. formosa from E. luteola and the two populations of E. sophia. Also, Encarsia hispida De Santis (luteola group) and Encarsia protransvena Viggiani (stenura group) (Aphelinidae) were included in the sequence analysis to test the support for the species groups considered.

Materials and methods

Specimens

Encarsia formosa was supplied by a commercial rearing from Biolan (now Bioplanet), Italy. Encarsia hispida was collected in Portici, Napoli, Italy from B. tabaci on Cistus monspeliensis L. (Cistaceae). Encarsia luteola came from Holtville, Imperial County, California, USA from B. tabaci on rose. Encarsia protransvena was collected in Baia, Napoli, Italy from Parthenium hysteresis (Kujawa) (Aphelinidae) on citrus, plant. Individuals of E. sophia, native to Multan, Pakistan from Parthenium hysteresis (Kujawa) (Aphelinidae) on citrus plant. Individuals of E. sophia, native to Multan, Pakistan (identification number M95107) and Murcia, Spain (identification number M93003) were received from the USDA-APHIS-PPQ, Mission Biological Control Center, Texas. For all species, molecular analysis was performed on individuals reared on T. vaporariorum on bean plants Phaseolus vulgaris L. cv. ‘Borlotto nano’ (Fabaceae) at 25 ± 0.5°C, 60 ± 5% R.H. and L14:D10 photoperiod. Coccosphagoides moeris (Walker) (Hymenoptera: Aphelinidae), collected in Matera, Italy by Malaise traps, was chosen as an outgroup for a phylogenetic analysis of the six populations of Encarsia.
Molecular characterization of Encarsia species

Table 1. Primers used for amplification and sequencing of the COI gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-f-2183</td>
<td>5’ CAACATTTATTGTGGAGTTTTTTG 3’</td>
<td>2481</td>
</tr>
<tr>
<td>C1-f-2195</td>
<td>5’ TTTTGTGTTTGGTGTCACTCCAGAT 3’</td>
<td>2492</td>
</tr>
<tr>
<td>T2-N-3014</td>
<td>5’ TCCAAATGCTAAATCTCCATATTAT 3’</td>
<td>3382</td>
</tr>
<tr>
<td>C1-f2</td>
<td>5’ TTAATTACATAGGAGTTACCGG 3’</td>
<td>2826</td>
</tr>
<tr>
<td>C1-prof3</td>
<td>5’ ATTTATATTGTGTTTGGTGT 3’</td>
<td>2739</td>
</tr>
<tr>
<td>C1-rev2</td>
<td>5’ ATATTATAGGACATACATTAAAAT 3’</td>
<td>2988</td>
</tr>
</tbody>
</table>

a Simon et al., 1994.
b Designed by the authors.
c Position refers to the nucleotide position of the 5’ end of primers in the mitochondrial DNA in Apis mellifera (Crozier & Crozier, 1993).

DNA extraction and sequencing

Adult females were killed directly in 95% ethanol and stored at −20°C; specimens were air-dried for about 15 min before DNA extraction. DNA was extracted from single insects according to Roehrdanz et al. (1993) with the outlined modifications. Individual insects were ground with a flame-sealed Pasteur pipette in a 1.5 ml microcentrifuge tube containing 10 μl of homogenization buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Nonidet N-P40, 100 μg ml⁻¹ proteinase K). The pestle was rinsed with 40 μl more of homogenization buffer, and the sample was first incubated 20 min at 65°C, and then 10 min in boiling water. Finally, samples were centrifuged 5 min at 17,000 g.

A region of the COI gene was amplified using either primer C1-f-2183 or C1-f-2195 in combination with T2-N-3014 respectively (Simon et al., 1994) (table 1). Polymerase chain reactions were carried out in 40 μl reaction volumes by using a Techne thermocycler, 4 μl (10 ×) PCR buffer (Sigma-Aldrich), 5 μl 25 mM MgCl₂, 3.2 μl dNTPs (2.5 mM each), 2 μl forward and reverse primer (50 ng μl⁻¹), 0.6 units REDTaq DNA polymerase (Sigma-Aldrich) and 4 μl DNA template. The cycling programme was 3 min at 94°C followed by 35 cycles of 45 s at 94°C, 1 min at 50°C, 2 min at 72°C, with a final extension of 2 min at 72°C. Amplicons were first checked on a 1.5% agarose gel stained with ethidium bromide, then purified with the GenElute PCR DNA purification kit (Sigma-Aldrich), directly sequenced with ABI Prism Dye Terminator cycle sequencing kit (Perkin Elmer) and analysed on ABI Prism 310 Genetic Analyzer (Applied Biosystems) sequencer, according to manufacturer’s procedures. To obtain overlapping sequences and to solve ambiguities, shorter fragments were amplified with the nested primers C1-f2, C1-prof3 and C1-rev2 (table 1) and processed as described above. Annealing temperatures were optimized for each primer-template reaction.

Analysis of molecular data

Sequences were assembled by using SeqMan in the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Analysis of sequence composition was performed using EditSeq and alignments realized with ClustalW method of MegAlign in the same package. Two individuals for each species were sequenced, except for one individual for E. protransvena and C. moeris.

Phylogenetic and molecular analysis was conducted using MEGA version 2.1 (Kumar et al., 2001). The amount of sequence diversity within and between species was measured by calculating the uncorrected P, the proportion of nucleotide sites at which two compared sequences differ, obtained by dividing the number of nucleotide differences by the total number of nucleotides compared. Maximum parsimony analysis was performed by using the branch and bound search method with equally weighted characters. Because of the near identity of multiple representatives of each species of Encarsia, a reduced data set that included only one sequence for each species was used. To assess branch support, the data set was bootstrapped 1000 times. Nucleotide sequences used for parsimony analysis have been deposited in GenBank database, with accession numbers AY264337, AY264338, AY264339, AY264340, AY264341, AY264342 and AY264343.

Restriction analysis

Sequences from E. formosa and E. luteola, and the Pakistani and Spanish populations of E. sophia were analysed with MapDraw program (Lasergene software package DNASTAR, Madison, Wisconsin, USA), in order to identify restriction enzymes that would result in species-specific banding patterns. Digestion of the COI amplicon was performed on 10 μl of previously ethanol precipitated PCR products, by using the restriction enzymes Dra I and Taq I for E. luteola and E. formosa, and Dra I and Xho I for the two populations of E. sophia, according to manufacturer’s suggestions (Roche). The fragments were separated on high resolution TBE agarose gel 2%. The molecular weight of the restriction fragments was estimated from the standard marker 100 bp DNA ladder (Promega). Ten specimens for each Encarsia were analysed.

Results

Analysis of molecular data

The amplified region of the COI gene was about 900 bp in all species of Encarsia except E. protransvena. This latter gave a single band slightly higher, due to a non-transcribed region between the COI and rRNA-leu genes, as has been found in some other insects (Zhang & Hewitt, 1996; Stauffer, 1997).

The sequenced region of the amplicon was 762 bp. Alignments were unambiguous and no insertion or deletion mutations were detected. Overall, the sequences showed strong A/T bias with an average of 77.6% of either A or T, with the strongest A/T bias in the third position (94.8%) and the least in the second (66.8%).
Nucleotide sequence divergence (table 2), expressed as uncorrected P values, ranged from 0 to 0.9% at the intraspecific level and 6.5–16.3% at the interspecific level. Within the strenua group, uncorrected P values ranged from 10 to 13.5% and the sequence divergence between the Pakistani and Spanish populations of E. sophia (10%) was greater than that found among the species in the luteola group (6.5–8.7%).

Many of the nucleotide polymorphisms within the genus Encarsia were due to synonymous changes (60–82%), whilst polymorphisms between C. moeris and species of Encarsia were reflected in altered amino acid sequences (synonymous changes 49–55%). In fact, compared to nucleotides, amino acid sequences showed a reduction of divergence in pairwise comparisons between species of Encarsia and an increase between C. moeris and species of Encarsia (table 2).

Parsimony analysis based on the sequenced COI gene region yielded a single most parsimonious tree of length 337 (fig. 1) with a consistency index (CI) of 0.73 and a retention index (RI) of 0.43. Of 762 aligned bp, 112 were parsimony-informative characters. Species of the luteola and strenua group clustered into separate clades.

**Table 2. Nucleotide and amino acid sequence divergence within and between species of Encarsia and the outgroup Coccophagoides moeris expressed as uncorrected P (the proportion of sites at which two compared sequences differ).**

<table>
<thead>
<tr>
<th></th>
<th>E. formosa</th>
<th>E. luteola</th>
<th>E. hispida</th>
<th>E. protransvena</th>
<th>E. sophia Spain</th>
<th>E. sophia Pakistan</th>
<th>C. moeris</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. formosa</td>
<td>0.009/0.012</td>
<td>0.029</td>
<td>0.042</td>
<td>0.085</td>
<td>0.069</td>
<td>0.077</td>
<td>0.135</td>
</tr>
<tr>
<td>E. luteola</td>
<td>0.065</td>
<td>0.007/0.008</td>
<td>0.034</td>
<td>0.089</td>
<td>0.079</td>
<td>0.083</td>
<td>0.124</td>
</tr>
<tr>
<td>E. hispida</td>
<td>0.087</td>
<td>0.072</td>
<td>0.001/0.004</td>
<td>0.109</td>
<td>0.097</td>
<td>0.093</td>
<td>0.135</td>
</tr>
<tr>
<td>E. protransvena</td>
<td>0.135</td>
<td>0.140</td>
<td>0.139</td>
<td>–</td>
<td>0.071</td>
<td>0.067</td>
<td>0.165</td>
</tr>
<tr>
<td>E. sophia Spain</td>
<td>0.124</td>
<td>0.110</td>
<td>0.119</td>
<td>0.135</td>
<td>0.000/0.000</td>
<td>0.016</td>
<td>0.157</td>
</tr>
<tr>
<td>E. sophia Pakistan</td>
<td>0.118</td>
<td>0.128</td>
<td>0.122</td>
<td>0.127</td>
<td>0.100</td>
<td>0.000/0.000</td>
<td>0.154</td>
</tr>
<tr>
<td>C. moeris</td>
<td>0.116</td>
<td>0.109</td>
<td>0.110</td>
<td>0.163</td>
<td>0.146</td>
<td>0.152</td>
<td>–</td>
</tr>
</tbody>
</table>

Values below the diagonal represent nucleotide divergences between species, and above the diagonal represent amino acid divergences between species. Values along the diagonal in bold type represent nucleotide divergences within species, in italic type represent amino acid divergences within species; –, data unavailable.

**Discussion**

The mitochondrial COI gene appears to show substantial sequence diversity to discriminate between closely related species in almost all animal phyla, and has been promoted as a useful marker for a DNA barcoding identification system for the animal kingdom (Hebert et al., 2003). In insects, the genetic divergence of closely related species and populations has been investigated at genes characterized by fast rates of evolution (Hoy, 1994; Caterino et al., 2000). In particular, the COI gene has shown high interspecific and low intraspecific
variability in many insect genera (Vogler et al., 1993), and has been widely utilized to discriminate between cryptic species and in phylogenetic analysis of morphologically similar taxa (Sperling & Hickey, 1994; Danforth et al., 1998; Szalanski et al., 2000; Kruse & Sperling, 2001; Lin & Wood, 2002; Linton et al., 2002).

Our results on the composition of the COI gene sequence and its variability in the genus Encarsia revealed a high A/T content, in agreement with previous studies on insect mitochondrial DNA (Simon et al., 1994), with the strongest A/T bias in the third position (Danforth et al., 1998). High genetic divergence was found between the species analysed, with a mean value of 11.98%, in accordance to the mean value known for the Hymenoptera (11.5%) (Hebert et al., 2003). A similar sequence variation was found in the genus Encarsia for the D2 region of 28S rDNA (average 11.5%, range 2.0–19.2%) (Babcock et al., 2001). However, this molecular marker revealed no significant difference between the Pakistani (M95107) and Spanish (M93003) populations of E. sophia, which showed instead a consistent genetic divergence based on the COI gene (10%). This value was higher than that found between different species in the luteola group (6.5–8.7%), supporting the status of cryptic species for the two E. sophia populations, as suggested by reproductive incompatibility, karyological differentiation and morphometric analysis (Giorgini & Baldanza, 2004). Since in recently derived species the genes that evolve rapidly could show differences, the COI gene is probably a better marker for diagnosis of Encarsia cryptic species.

The high interspecific sequence variation of the COI gene found in the genus Encarsia, coupled with a low intraspecific variation, supports the use of PCR–RFLP as a simple and effective technique for molecular diagnosis of species, as has been shown in other insects (Loxdale & Lushai, 1998; Clark et al., 2001; Otranto et al., 2003). In this study, species-specific restriction patterns characterized all the specimens tested belonging to the closely related E. formosa and E. luteola as well as to the two cryptic species referred to E. sophia.

Phylogenetic analysis of the genus Encarsia based on combined morphological characters and nuclear sequences of the D2 region of 28S rDNA (Babcock et al., 2001) revealed the luteola and strenua species groups to be monophyletic. The analysis based on mitochondrial sequences of the COI gene confirmed monophyly of these two groups of species.

In conclusion, the results of this study support the use of the mitochondrial COI gene as a useful marker in the genus Encarsia to separate species for which morphological differences are extremely subtle, and to characterize natural enemies that may be useful in biological control programmes. Moreover, the COI gene appears potentially useful, together with other molecular markers, for understanding phylogenetic relationships in the genus Encarsia and supporting groups of species defined on the basis of morphological characters.

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