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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;

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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 evaluating their dispersal and parasitism level following releases, and for quality control assessment of mass rearings.

Recently, the unreliability of morphological characters in separating closely related species of *Encarsia* prompted the use of new identification methodologies such as sequencing (Babcock *et al*., 2001; Manzari *et al*., 2002; Polaszek *et al*., 2004) and polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) (Babcock & Heraty, 2000), randomly amplified polymorphic DNA–polymerase chain reaction (RAPD–PCR) techniques (Legaspi *et al*., 1996; Goolsby *et al*., 1998; Baldanza *et al*., 2001), chromosome analysis (Baldanza *et al*., 1999; Baldanza & Giorgini, 2001) and protein analysis (Baldanza *et al*., 2001; Caprio & Viggiani, 2001).

Particular attention has been focused on the characterization of *Encarsia* species parasitic on whiteflies of economic importance, such as the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae), and the sweet potato whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). These pests seriously damage agronomic, horticultural and ornamental crops throughout the world (Gerling, 1990; Kirk *et al*., 2000). Their control by chemical insecticides is very difficult because of the high level of resistance readily developed in frequently sprayed populations (Dittrich *et al*., 1990; Prabhaker *et al*., 1997; Castle *et al*., 2002). Consequently, integrated management has been considered as the most effective approach for their control, with natural enemies playing a key role within this strategy.

Species of *Encarsia* in the *luteola* and *strenua* groups are common parasitoids of *T. vaporariorum* and *B. tabaci* (Polaszek *et al*., 1992; Schauff *et al*., 1996) and are among the most effective biocontrol agents of the whitefly populations in several areas of the world (Gerling, 1967, 1983; Chermiti *et al*., 1997; Goolsby *et al*., 1998; Kirk *et al*., 2000; Onillon & Maignet, 2000).

Encarsia formosa Gahan and *Encarsia luteola* Howard (both Hymenoptera: Aphelinidae) are closely related species belonging to *luteola* group. They are difficult to distinguish on the basis of morphological characters (Polaszek *et al*., 1992; Pedata *et al*., 1994) and for this reason species-specific molecular markers have been developed by restriction analysis of the D2 region of 28S rDNA (Babcock & Heraty, 2000). *Encarsia formosa* is a cosmopolitan species (Polaszek *et al*., 1992) which reproduces by microbe-induced thelytoky (Zchori-Fein *et al*., 1992; van Meer *et al*., 1995) and is successfully used in biological control of *T. vaporariorum* on greenhouse crops in several countries (van Lenteren *et al*., 1997; Hoddle *et al*., 1998). *Encarsia luteola* is an arrhenotokous species (Gerling & Rivnay, 1984) distributed in North, Central and South America (Polaszek *et al*., 1992; Schauff *et al*., 1996) where it contributes to the control of whitefly populations (Castineiras, 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 *strenua* group, *Encarsia sophia* (Girault & Dodd) (Aphelinidae) is one of the more cosmopolitan species (Heraty & Polaszek, 2000) found worldwide to be a dominant parasitoid of *B. 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 reproductive incompatibility, 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 (*strenua* 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 Biolab (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 *Parabemisia myricae* (Kuwana) (Aleyrodidae) 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 $25 \pm 0.5°C$, $60 \pm 5\%$ R.H. and L14:D10 photoperiod. *Coccophagoides 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*.

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

Primer	Sequence	Position[c]
C1-J-2183[a]	5′ CAACATTTATTTTGATTTTTTGG 3′	2481
C1-J-2195[a]	5′ TTGATTTTTTGGTCATCCAGAAGT 3′	2492
TL2-N-3014[a]	5′ TCCAATGCACTAATCTGCCATATTA 3′	3382
C1-f2[b]	5′ TTATTTACTATAGGAGGTTTAACAGG 3′	2826
C1-prof3[b]	5′ ATTAAAATTTTTAGATGATTGGC 3′	2739
C1-rev2[b]	5′ ATATTATAGGATACCAATAAATAAATC 3′	2988

[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-J-2183 or C1-J-2195 in combination with TL2-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 $MgCl_2$, 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 amplicons 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 tRNA-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%).

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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).

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

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.

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.

Restriction analysis

Digestion patterns (table 3) showed diagnostic differences for both species pairs analysed. Restriction enzymes *Dra* I and *Taq* I were able to distinguish *E. luteola* and *E. formosa* (fig. 2), while *Dra* I and *Xho* I distinguished the Pakistani and Spanish populations of *E. sophia* (fig. 3). No intraspecific variation for restriction patterns was observed in the examined samples.

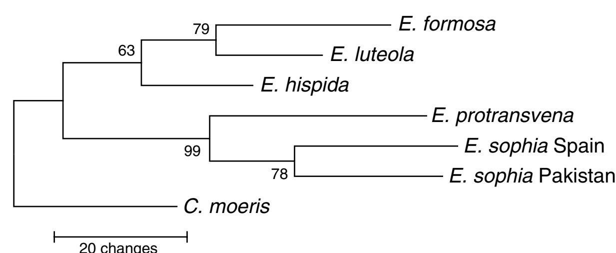

Fig. 1. Single most parsimonious tree based on 112 informative characters in the COI gene region studied. Tree length=337; CI=0.73; RI=0.43. Numbers above branches are bootstrap percentage of 1000 replications (bootstrap values <50% not shown).

Table 3. Restriction fragment lengths in bp generated by digestion of the COI amplicons.

	Dra I	*Taq* I	*Xho* I
Encarsia formosa	470, 310, 120	680, 220	–
E. luteola	780, 120	620, 150, 130	–
E. sophia Spain	290, 250, 240, 120	–	900
E. sophia Pakistan	440, 260, 110, 90	–	680, 220

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

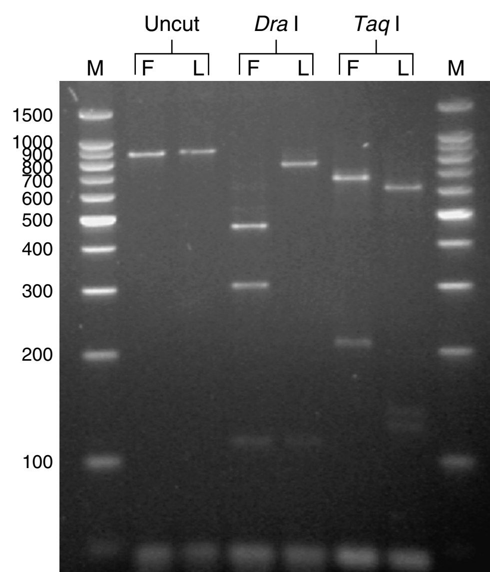

Fig. 2. *Dra* I and *Taq* I restriction fragments of the amplified COI gene region for *Encarsia formosa* (F) and *Encarsia luteola* (L). M=marker.

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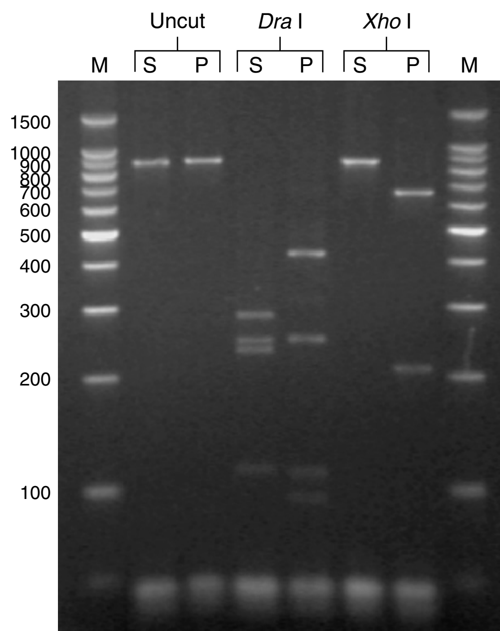

Fig. 3. *Dra* I and *Xho* I restriction fragments of the amplified COI gene region for the Spanish (S) and Pakistani (P) populations of *Encarsia sophia*. M=marker.

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 only 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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