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Diagnostic molecular markers and the genetic relationships among three species of the *Cheilosia canicularis* group (Diptera: Syrphidae)

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Abstract. To re-evaluate the taxonomic status of *Cheilosia canicularis* (Panzer, 1801), *C. himantopus* (Panzer, 1798) and *C. orthotricha* Vujić & Claussen, 1994, variation in the mitochondrial DNA (mtDNA) sequence of the cytochrome *c* oxidase subunit I (COI) gene and 18 nuclear allozyme genes were surveyed in allopatric and sympatric populations from Serbia and Montenegro. Genetic relationships among five populations of these species from the Fruška Gora (Serbia), Kopaonik (Serbia) and Durmitor (Montenegro) mountains were analyzed. Seven allozyme loci (*Aat, Aco, Fum, Idh-1, Idh-2, Mdh*-2 and *Sdh*) were diagnostic for delineating *C. orthotricha* from the other two species, while only a low, but consistent, genetic differentiation was observed between *C. canicularis* and *C. himantopus*. Differentiating all three species was possible based solely on the species-specific alleles at the *Est-?* locus. Sequence comparisons of 738 bp of the COI gene from eleven specimens was consistent with the variability in nuclear allozymes. Sequence data revealed variation in 5% of the nucleotide sites among *C. orthotricha* and the *C. canicularis*/*C. himantopus* pair, while less variation (0.68%) was observed within the pair *C. canicularis/C. himantopus.* However, the presence of one diagnostic allozyme locus and five consistently variable nucleotide sites in sympatric populations of *C. canicularis* and *C. himantopus* (Durmitor, Montenegro) suggest that these two species have separate gene pools.

INTRODUCTION

Cheilosia s. str. Meigen, 1822 (Diptera: Syrphidae) includes a recently defined group of five taxa closely related to the species *Cheilosia canicularis* (Barkalov, 2002). The *C. canicularis* complex of species comprises *C. canicularis* (Panzer, 1801), *C. himantopus* (Panzer, 1798), *C. orthotricha* Vujić & Claussen, 1994, *C. japonica* Herve-Bazin, 1914, and *C. yesonica* Matsumura, 1905, and was formed in 1994 after the description of *C. orthotricha* (Vujić & Claussen, 1994). *Cheilosia himantopus* was re-established as a valid species by Stuke & Claussen (2000) after detailed study of the morphological variability and ecology of both adult and immature specimens. The species *C. japonica* and *C. yesonica* occur in Japan, while the other three have wider distributions in the Palaearctic region (Peck, 1988). Adults inhabit deciduous and evergreen forest habitats and clearings along streams and rivers (Stuke & Claussen, 2000; Speight, 2003; Vujić & Šikoparija, 2001). The immature stages of *C. canicularis* and *C. himantopus* develop in stems, leaves and rhizomes of *Petasites* spp. (Stuke & Claussen, 2000; Speight, 2003; Vujić & Šikoparija, 2001). Females of *C. canicularis* have been observed to oviposit on leaves surrounding flower buds or on leaf buds in late summer or early autumn and those of *C. himantopus* on leaf buds in late spring (Vujić & Šikoparija, 2001). The larvae of *C. orthotricha* develop in flower stems of *Petasites hybridus* (Stuke & Claussen, 2000) and pupate in the rhizome or in the vicinity of the host plant.

The taxonomic status of *C. canicularis* has been a subject of debate ever since its description. This resulted in a number of synonyms (Vujić & Claussen, 1994; Stuke & Claussen, 2000) and discussions about its systematic relationship with *C. himantopus*. After defining *C. orthotricha* (Vujić & Claussen, 1994) as a species, the study of the *canicularis* group intensified (Stuke, 1995, 2000; Stuke & Brückner, 2001; Stuke & Claussen, 2000; Vujić, 1996). A study of the phylogenetic relationships of 57 *Cheilosia* species, using a large 1128 bp fragment of the COI gene in a separate parsimony analysis, combined with a sequence fragment of the nuclear 28S rRNA gene, always revealed a monophyletic clade (*C. orthotricha* + (*C. canicularis* + *C. himantopus*)) (Ståhls et al., 2004). Diagnostic characters of adults (length of aristal hairs, specific hypandrium structure and distribution of pilosity on tergites III and IV in females; Stuke & Claussen, 2000) and larvae and pupae (Stuke, 2000) indicated that the separation of the closely related species *C. himantopus* and *C. canicularis* and the species *C. orthotricha* was defendable, even if the exhibited levels of variation were low. In order to obtain data about the levels of variation in mtDNA, Stuke & Brückner (2001) sequenced the mitochondrial cytochrome *c* oxidase subunit II (COII) gene and parts of tRNA genes tLeu and tLys of a few specimens of allopatric *C. canicularis* and *C. himantopus* from Germany. However, the sample was too small to determine conclusively whether the two species were independent. Still, there are difficulties in discriminating between certain individuals of *C. canicularis* or *C. himantopus* and there are specimens in collections that

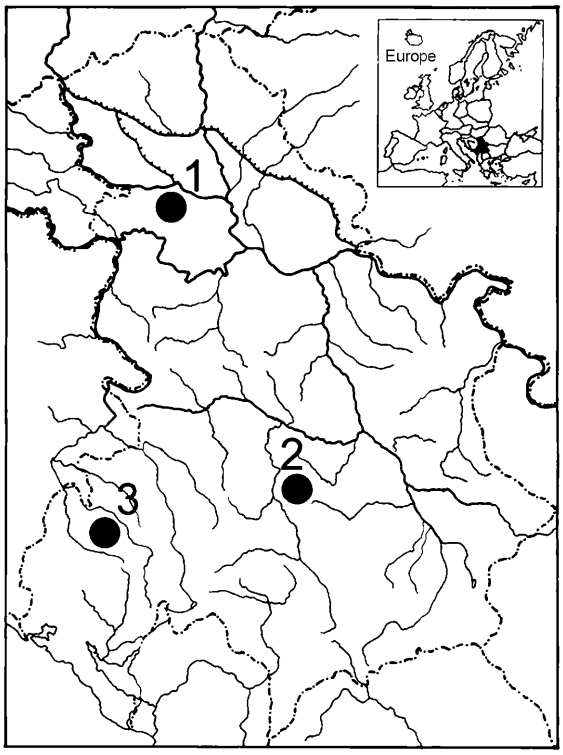

Fig. 1. Origins of populations of *Cheilosia orthotricha*, *C. canicularis* and *C. himantopus* analyzed for allozymes: 1 – Fruška Gora, Serbia (19°50′E, 45°10′N); 2 – Kopaonik, Serbia (20°40′E, 43°15′N); 3 – Durmitor, Montenegro (19°00′E, 43°11′N).

cannot be identified as one or the other taxon (Vujić & Šikoparija, 2001). It has been hypothesized that the taxon is one species with two generations (Vujić & Claussen, 1994) or it is indeed two independent species (Stuke & Claussen, 2000).

Given this taxonomic dilemma and the distribution of the focal taxa in similar habitats, a more detailed analysis of the biology (Vujić & Šikoparija, 2001) and intra- (Stamenković et al., in press) and interpopulation analysis of genetic variability are necessary. Thus, the objective of this paper was three-fold: (1) to analyze using allozymes the population-genetic variability in sympatric and allopatric populations of *C. orthotricha, C. canicularis* and *C. himantopus*, (2) to investigate the pattern of variation in the COI sequences, and (3) to validate or invalidate the status of the controversial species *C. canicularis* and *C. himantopus*.

MATERIAL AND METHODS

Sample collection and identification

The species *C. japonica* and *C. yesonica* were not available for DNA analysis. Samples of the species *Cheilosia orthotricha, C. canicularis* and *C. himantopus* were collected from Fruška Gora (hilly area in the Pannonian plain) and two high Dinaric mountains, Durmitor and Kopaonik, in Serbia and Montenegro (Fig. 1, Table 1). Specimens were stored at –20°C until used in the analysis.

Species identification, prior to preparation of whole body tissue extracts, was based on the morphological taxonomic characters defined for the *Cheilosia canicularis* complex of species (Stuke & Claussen, 2000), such as arista characteristics in males and females, distiphallus in males and tergites III and IV in females. Black hairs on mesonotum in females served as an ancillary trait for delineating *C. canicularis* (black hairs present) and *C. himantopus* (no black hairs in populations from the Balkans).

Allozyme analysis

All populations were included in the allozyme analysis in order to determine molecular markers that could be used to identify the taxa studied. Due to the small number of specimens, populations COKOP and CHFG were not included in the population – genetic analysis, and genetic differentiation was quantifiable based only on COFG, CCDUR, CHDUR and CHKOP (Table 1).

Genetic variability was studied by standard 5% polyacrylamide gel electrophoresis, following Munstermann (1979) (ACO, EST, FUM, GPD, GPI, HAD, HK, IDH, LDH, MDH, ME, PGM, SOD) and Pasteur et al. (1988) (AAT, SDH), with slight modifications (Milankov, 2001). The Tris-Boric-EDTA buffer system (pH 8.9) was used to assay esterase (E.C. 3.1.1.? EST; locus *Est*-?), fumarate hydratase (4.2.1.2. FUM; *Fum*), glucosephosphate isomerase (5.3.1.9. GPI; *Gpi*), hexokinase (2.7.1.1. HK; two loci: *Hk-2, Hk3*), malic enzyme (1.1.1.40. ME; *Me*), phosphoglucomutase (2.7.5.1. PGM; *Pgm*), sorbitol dehydrogenase (1.1.1.14. SDH, *Sdh*) and superoxide dismutase (1.15.1.1. SOD; *Sod*). A Tris-Citric buffer system (pH 7.1) was used to assay aconitase (4.2.1.3. ACO; *Aco*), aspartate amino transferase (2.6.1.1. AAT; *Aat*), glycerol 3-phosphate dehydrogenase (1.1.1.8. GPD; *Gpd-2*), β-hydroxy acid dehydrogenase (3.1.1.31. HAD, *Had*), isocitrate dehydrogenase (1.1.1.42. IDH; *Idh-1, Idh-2*), lactate dehydrogenase (1.1.1.27. LDH, *Ldh*) and malate dehydrogenase (1.1.1.37. MDH; *Mdh-1, Mdh-2*).

Depending on the metabolic function and regional distribution of enzymes, different body parts were used for the analysis of isozyme variability (head in 0.2 ml homogenate: AAT, FUM, HAD, LDH, MDH, ME; thorax in 0.3 ml: ACO, EST, GPD, GPI, HK, IDH, PGM, SDH, SOD). Duration of electrophoretic run at 90 mA (135–220 V) was 3–4 h.

TABLE 1. Population codes, based on the species and place of origin (number of specimens used for allozyme analysis in parenthesis) and collection dates.

	Fruška Gora	Kopaonik	Durmitor
C. orthotricha	COFG (16) 05/1996; 03/2000; 03/2001	COKOP (4)* 04/2001; 05/2001	–
C. canicularis	–	–	CCDUR (21) 07/1998; 08/1999; 06/2000
C. himantopus	CHFG (3)* 04/1998; 04/2001	CHKOP (13) 08/1997; 06/1998; 04/2001; 06/2001	CHDUR (15) 06/1998; 05/2001; 06/2001; 06/2002

* omitted from population-genetic analysis due to the small number of specimens

TABLE 2. Allelic frequencies at 10 loci in populations of *Cheilosia orthotricha*, *C. canicularis* and *C. himantopus* (see Table 1 for population codes).

Locus	Allele	COFG	COKOP	CCDUR	CHDUR	CHKOP	CHFG
Aat	*a*	1.000	1.000	–	–	–	–
	b	–	–	1.000	1.000	1.000	1.000
	n	16	4	14	9	9	2
Aco	*a*	1.000	1.000	–	–	–	–
	b	–	–	1.000	1.000	1.000	1.000
	n	6	2	4	8	6	2
Est-?	*a*	1.000	1.000	–	–	–	–
	b	–	–	1.000	–	–	–
	c	–	–	–	1.000	1.000	1.000
	n	16	4	17	10	9	2
Fum	*a*	0.889	1.000	–	–	–	–
	b	0.111	–	1.000	1.000	1.000	1.000
	n	9	2	11	11	12	2
Idh-1	*a*	1.000	1.000	–	–	–	–
	b	–	–	1.000	1.000	1.000	1.000
	n	11	4	17	9	13	3
Idh-2	*a*	1.000	1.000	–	–	–	–
	b	–	–	1.000	1.000	1.000	1.000
	n	11	4	17	9	13	3
Mdh-2	*a*	1.000	1.000	–	–	–	–
	b	–	–	1.000	1.000	1.000	1.000
	n	14	2	13	15	10	2
Me	*a*	–	–	1.000	1.000	–	1.000
	b	0.833	–	–	–	1.000	–
	c	0.167	1.000	–	–	–	–
	n	6	2	4	8	6	2
Pgm	*a*	–	–	0.095	0.500	0.143	–
	b	1.000	1.000	0.905	0.500	0.857	1.000
	n	15	2	21	10	7	2
Sdh	*a*	1.000	1.000	–	–	–	–
	b	–	–	1.000	1.000	1.000	1.000
	n	5	2	4	6	2	2

The electrophoresis of individuals from different populations was conducted in the same gel for direct interspecific comparison. Loci were numbered and alleles marked alphabetically with respect to order of increasing anodal migration.

Population -genetic analysis

Statistical analysis of allozyme data was performed using the computer program BIOSYS-2 (Swofford & Selander, 1981, modified by Black, 1997). The analysis included determining genotypic and allelic frequencies, the frequency of polymorphic loci (*P*), and the mean observed and expected heterozygosity (H_o, H_e), corrected using Levene's (1949) formula for small samples. The deviation between H_o and H_e of separate variable loci was evaluated using Wright's inbreeding coefficient (F_i; Wright, 1951) and mean *F*-statistics (Weir, 1996). Nei's genetic identity (1978) was used to compare gene frequencies among species or populations, and to construct a dendrogram using clustering with the Unweighted Pair Group Method with Arithmetic Average (UPGMA) (Sneath & Sokal, 1973) .

TABLE 3. Deviation of genotype frequencies from Hardy-Weinberg equilibrium at polymorphic loci in populations of *Cheilosia orthotricha*, *C. canicularis* and *C. himantopus*.

Locus	Population	H_o	H_e	F_{is}	*P*
Fum	COFG	0	1.882	1.000	0.0015
Me	COFG	0	1.818	1.000	0.0075
Pgm	CCDUR	0	3.707	1.000	0.0000
	CHDUR	0	5.263	1.000	0.0009
	CHKOP	0	1.846	1.000	0.0044

H_o = Observed number of heterozygosity; H_e = Expected number of heterozygosity; F_{is} = Fixation index (Wright, 1951); *P* = Level of significance.

DNA sequencing

A total of ten specimens from all six populations (Table 1) and one specimen of *C. canicularis* from Sweden (Skåne, Fyledalen) were used for DNA sequencing. DNA was extracted from legs or other parts of single frozen individuals (–20°C) using the Nucleospin Tissue DNA extraction kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocols and then re-suspended in 50 µl of ultra-pure water. Amplification was attempted for COI and internal transcribed spacer region (ITS2) rRNA genes, but the latter was unsuccessful (probably due to the age of the specimens). Remains of specimens, including male genitalia, used for the morphological studies and for DNA extraction are deposited at the Finnish Museum of Natural History (Helsinki, Finland).

PCR reactions were carried out in 25 µl reaction aliquots containing 2 µl DNA extract, 1 µl of each primer (at 10 pmol/µl), 0.25 µl of DNA polymerase (5U/µl), 2 µl 2.5 mM $MgCl_2$, 2.5 µl 10 × Buffer II (MBI Fermentas, St. Leon-Rot, Germany) and 4 µl 200 mM dNTP (GeneAmp, Applied Biosystems, Foster City, CA, USA) and ultra-pure water. Thermocycler conditions were initial denaturing at 95°C 2 min, 29 cycles of 30 s denaturing at 94°C, 30 s annealing at 49°C, 2 min extension at 72°C, followed by a final extension of 8 min at 72°C. The universally conserved primers used for amplifying and sequencing the COI fragment (738-bp) were the forward primer C1-J-2183 (5'-CAA CAT TTA TTT TGA TTT TTT GG-3') (alias JERRY) and the reverse primer TL2-N-3014 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') (alias PAT) (Simon et al., 1994). PCR products were purified using the GFX PCR Purification Kit (GE Healthcare Biosciences, Little Chalfont, UK) and then sequenced (with the PCR primers) in both directions using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) at one-fourth of the recommended volumes on an ABI PRISM 377 (Applied Biosystems, Foster City, CA, USA) semi-automated DNA sequencer. The sequences were edited for base-calling errors and assembled using Sequence Navigator™ (version 1.01) (Applied Biosystems, Foster City, CA, USA).

RESULTS

Allozyme variability

Diagnostic allozyme loci

Delineation of all three species was possible based only on allozymes coded by the alleles at the *Est*-? locus that were unique for the populations of *C. orthotricha*, *C. canicularis* and *C. himantopus* (Table 2). Alleles at

TABLE 4. Estimates of genetic variability parameters in populations of *Cheilosia orthotricha*, *C. canicularis* and *C. himantopus* (standard error in parenthesis).

Population	*A* (SE)	H_e (SE)	H_o (SE)	$P_{(0.95)}$
COFG	1.1 (0.1)	0.026 (0.018)	0.000	11.1
CCDUR	1.1 (0.1)	0.010 (0.010)	0.000	5.6
CHDUR	1.1 (0.1)	0.028 (0.028)	0.000	5.6
CHKOP	1.1 (0.1)	0.014 (0.014)	0.000	5.6

A = Mean number of alleles per locus; H_e = Expected heterozygosity averaged over all loci; H_o = Average frequency of observed heterozygosity; $P_{(0.95)}$ = Frequency of polymorphic loci based on the 0.95 criterion.

another seven loci (*Aat, Aco, Fum, Idh-1, Idh-2, Mdh-2,* and *Sdh*) were species-specific for *C. orthotricha*, and diagnostic for this species. All analyzed populations were identical at eight loci (*Gpd-2, Gpi, Had, Hk-2, Hk-3, Ldh-2, Mdh-1* and *Sod-1*).

Population genetic structure analysis

Of the 18 isozyme loci analyzed, 15 were monomorphic within each of the populations analyzed. The *Fum* and *Me* loci were polymorphic only in COFG, and *Pgm* was variable in CCDUR, CHDUR and CHKOP (Table 2). Additionally, the spatial distribution of alleles at the *Me* locus among populations of *C. himantopus* was observed, with the one allele (Me^a) in CHDUR and CHFG, and a different allele (Me^b) in CHKOP, indicating genetic divergence among conspecific populations.

Only two alternative homozygotes (F_{is} = 1.0), but no heterozygotes, were observed at any of the polymorphic loci within populations. Thus, the observed population structure deviated from the one expected in a randomly mating population (Table 3).

Frequency of polymorphic loci was highest in the COFG population, while the mean number of alleles was identical in all populations analyzed (Table 4).

COI variation

We obtained a 738 bp fragment of the COI gene, corresponding to nucleotide positions 2233 to 2970 in *Drosophila yakuba* sequence (Clary & Wolstenholme, 1985). GenBank accession numbers of the analyzed specimens from six populations are: COFG (VM268-COI: AY800282; VM269-COI: AY800283), COKOP (VM312-COI: AY800284), CCDUR (VM248-COI: AY800280; VM249-COI: AY800281), CHKOP (VM298-COI: AY800285; VM299-COI: AY800286), CHDUR (VM305-COI: AY800287; VM306-COI: AY800288), CHFG (VM309-COI: AY800289). Sequences differed at 38 (5.15%) and 37 (5.01%) nucleotide sites between *C. orthotricha* and *C. himantopus,* and *C. orthotricha* and *C. canicularis*, respectively. Only five nucleotide sites (0.68%) differed between *C. canicularis* and *C. himantopus*. The exception was a single specimen from the CHFG population (VM309), which varied from conspecifics in one nucleotide site and from *C. canicularis* at four sites (Fig. 2). Additionally, data presented herein were identical to the respective sequence data obtained for *C. canicularis* (Serbia: AY533365), *C. himantopus* (Montenegro: AY533352; Sweden, Skåne, 2004 not submitted) and *C. orthotricha* (Montenegro: AY533353) (Ståhls et al., 2004).

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          2222222222222222222222222222222222222222
          2222233344445555566666666777788888888999
          5678902656780333500123357223403557789556
          2762411308174178306877812018447351422173

VM248     CTCTCTTATTTTCTATACAATAGCATGTCTATAAATCTTC
VM249     ........................................
VM298     TC......................G........G.....T
VM299     TC......................G........G.....T
VM306     TC......................G........G.....T
VM305     TC......................G........G.....T
Y140      TC......................G........G.....T
VM309     TC......................G..............T
VM268     T.TATGCTCGCGTATCGTTTCGTT.CACTCTGT.GCACCT
VM269     T.TATGCTCGCGTATCGTTTCGTT.CACTCTGT.GCACCT
VM312     T.TATGCTCGCGTATCGTTTCGTT.CACTCTGT.GCACCT
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Fig. 2. Mitochondrial DNA sites (within the 738 bp fragment spanning COI positions 2232–2970) that varied among populations of *Cheilosia canicularis* (CCDUR: VM248, VM249), *C. himantopus* (CHKOP: VM298, VM299; CHDUR: VM305, VM306; CHFG: VM309; Sweden, Skĺne, Fyledalen: Y140) and *C. orthotricha* (COFG: VM268, VM269; COKOP: VM312).

The majority of the sequence differences between *C. orthotricha* and the *C. canicularis*/*C. himantopus* pair were transitions rather than transversions (62.16%TS, 37.84%TV; 63.16%TS, 36.84%TV, respectively). All DNA substitutions between *C. himantopus* and *C. canicularis* were transitions (Fig. 2). The sequences were AT rich with a mean AT content of 73.62%, while the 3rd codon position exhibited an average AT content of 96%.

Interspecific relationships

Genetic distance and genetic identity values (Nei, 1978) based on the 18 allozyme loci for the populations analyzed are shown in Table 5. A dendrogram of genetic relationships (Fig. 3) was constructed based on the average genetic identity values, using the UPGMA clustering method. The least genetic differentiation was registered between the conspecific populations CHDUR and CHKOP that formed a cluster. The highest degree of genetic difference was registered between the CCDUR and COFG populations.

The COI sequence of *C. orthotricha* was distinct (5.01% to 5.15% nucleotide divergence) from those of *C.*

TABLE 5. Genetic distance (above diagonal) and genetic identity (below diagonal) calculated after Nei (1978) among populations of *Cheilosia orthotricha*, *C. canicularis* and *C. himantopus.*

Population	COFG	CCDUR	CHDUR	CHKOP
COFG	*****	0.672	0.708	0.586
CCDUR	0.511	*****	0.067	0.119
CHDUR	0.493	0.935	*****	0.065
CHKOP	0.557	0.888	0.937	*****

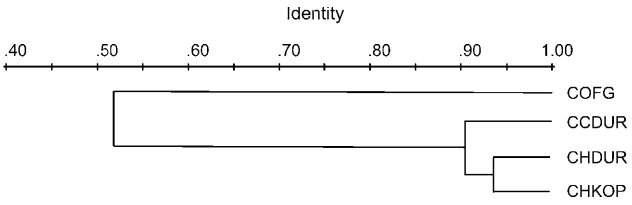

Fig. 3. Dendrogram of genetic relationships among populations of *Cheilosia orthotricha*, *C. canicularis* and *C. himantopus* using unweighted pair group clustering (Sneath & Sokal, 1973) of Nei's, (1978) unbiased genetic identity, based on the allozyme data.

canicularis and *C. himantopus.* Pairwise uncorrected distances between the closely related species *C. canicularis* and *C. himantopus* were 0.54% and 0.68%. The cladogram in Ståhls et al. (2004), and the parsimony analysis of 738 bp of the 10 specimens from 6 populations of *C. orthotricha, C. canicularis* and *C. himantopus* (not shown) produced the same topology as the tree constructed using the allozyme data (Fig. 3).

DISCUSSION

Genetic differences between species

Morphological characters are often insufficient or not exclusive enough for identification of morphologically cryptic taxa in the family Syrphidae. However, allelic and genotypic differentiation of allozyme loci allowed successful delineation of numerous cryptic species of hoverflies (Milankov, 2001; Milankov et al., 2001). Although the diagnostic value of isozyme loci as criteria for defining degree of genetic differentiation and evolutionary independence is not the same for all loci, some patterns have been observed. The loci *Aat, Fum, Hk-2, Hk-3* and *Mdh-2,* monomorphic in the majority of populations analyzed, were valuable diagnostic tools for species of *Merodon* with a high degree of genetic divergence (Milankov, 2001; Milankov et al., 2002c). On the other hand, loci that are frequently polymorphic and exhibit spatial variability, the *Gpi, Had, Me, Pgm* and *Sod-1* loci, are important for identifying of closely related species of the same genus (Milankov, 2001; Milankov et al., 2002b, c).

In addition to unequivocal morphological characters (Vujić & Claussen, 1994; Vujić & Šikoparija, 2001) the eight diagnostic allozyme loci found in this study and mtDNA analysis, confirmed that *C. orthotricha* is indeed distinct from *C. canicularis* and *C. himantopus*. The loci *Aat, Fum, Idh-1, Idh-2* and *Mdh-2,* and for the first time analyzed in hoverflies, the loci *Aco, Sdh* and *Est-?* are diagnostic of *C. orthotricha*. Genetic identity values, similar to those of other species of the family Syrphidae (0.305–0.728; Milankov, 2001) and distinct pairwise distance in the COI mtDNA gene, clearly demonstrate the independent taxonomic status of *C. orthotricha*.

Only a low genetic differentiation was observed between *Cheilosia canicularis* and *C. himantopus*. Complete genetic distinction was recorded only at the *Est* locus, which was monomorphic with a unique allele in all three species analyzed.

Sequencing revealed a low genetic differentiation between *Cheilosia canicularis* and *C. himantopus* (0.5–0.7% nucleotide difference) and a higher degree of differentiation (5%) between *C. orthotricha* and the species pair *C. canicularis/C. himantopus*. Interpreting the differences in the level of divergence in the COI sequence in the *C. canicularis* group is not straightforward. It is hypothesized that the COI sequence is a slowly evolving gene that may not be a sensitive indicator of speciation (Simon et al., 1994), as illustrated by identical COI sequences in the closely related species *Scaeva dignota* and *S. selenitica* (Syrphidae), while the nuclear ITS2 region showed a 7.5% difference (Ståhls, pers. comm.). The nucleotide variation between *Cheilosia canicularis* and *C. himantopus* is similar to the observed difference in the COII sequence of single specimens of *C. canicularis* and *C. himantopus* (0.7%) (Stuke & Brückner, 2001).

The genetic differentiation observed between *C. canicularis* and *C. himantopus* was stable and suggested genomic independence of these two species. The presence of one diagnostic locus and five consistently different nucleotide sites in sympatric populations of *C. canicularis* and *C. himantopus* (Durmitor, Montenegro) demonstrate that these populations do not exchange genes and are therefore unlikely to be conspecific. The results of morphological analyses support the above. In addition to the morphology of the male genital distiphallus sclerite, and pattern of pilosity on the abdominal tergites in females, only the aristal surface, a more or less clear morphological character, appears to be of diagnostic value in both sexes (Vujić & Šikoparija, 2001).

Genetic variation within species

The level of genetic variation within populations of the *C. canicularis* complex was generally low (Tables 2 and 3). Genetic variability this low is observed only rarely, such as in populations of *Melanogaster nuda* (Ludoški et al., in press) and certain species of the genus *Merodon* (Milankov, 2001). Typically, much higher values are recorded for species from the genus *Cheilosia,* ranging from 0.250 to 0.600 (Milankov et al., 2002a; Ludoški, 2002; Ludoški et al., 2002). Decreased genetic variability in the *C. canicularis* complex could be explained by the genetic system, biology and ecology of the taxon, historic factors such as bottlenecks and founder effect, long-lasting isolation, or natural selection and gene flow. However, not enough information is available to define which of the above factors was the most important.

Genetic and phylogenetic relationships

In spite of the genetic differentiation due to genetic divergence of alleles at the *Pgm* and *Me* loci, a high percentage of loci are genetically identical ($I > 0.95$; 88.89%), which suggests that *C. himantopus* and *C. canicularis* are very closely related. The average value of genetic identity (Nei, 1978) also indicates a low degree of genetic differentiation, with values calculated for conspecific populations of *C. himantopus* ($I = 0.937$) practically identical to those calculated for sympatric populations of *C. himantopus* and *C. canicularis* from Durmitor ($I =$

0.935). Genetic identity observed among populations of *C. himantopus* and *C. canicularis* was higher than values typically observed for closely related species (I = 0.840–0.898; Ludoški, 2002) and similar to those found among conspecific populations of other species of the genus *Cheilosia* (I = 0.928–0.995; Ludoški, 2002; Milankov et al., 2002a).

The time for the first branching in the *Cheilosia* group was estimated, based on the number of allelic substitutions (average genetic distance, D; Nei, 1978), to be ca. 2.9–3.5 Mya for *C. orthotricha* and the *C. canicularis/C. himantopus* pair ($t = 5 \times 10^6 D$; Nei, 1975). Approximate time of divergence within the *C. canicularis/C. himantopus* pair was assessed at ca. 0.3–0.6 Mya. The pairwise sequence divergence in the COI gene, relative to the value of 2.3% per million years estimated for various arthropod taxa (Brower, 1994), suggests that the speciation events between *C. orthotricha* and the *C. canicularis/C. himantopus* pair occurred ca. 2.3 Mya, and the diversification between *C. canicularis* and *C. himantopus* 0.3 Mya, both figures are in (interestingly) good agreement with those obtained from the allozyme data.

The trees constructed based on the allozyme and COI mtDNA data provide evidence of genetic divergence between the evolutionary distant species *C. orthotricha* and the closely related sister species *C. himantopus* and *C. canicularis*. Clear differences were interpreted as indicative of species level differentiation.

We hypothesize that *C. canicularis* and *C. himantopus* have split relatively recently, with little time since speciation for nucleotide substitutions to occur and accumulate. It is possible that the speciation was initiated by biological and ecological separation, which did not involve the gene region analyzed in this study.

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