Identification of the species in the *Cheilosia melanura* group (Diptera, Syrphidae) occurring on the Balkan Peninsula. I: Allozyme markers

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Allozyme variability, at eight isozyme loci of the *Cheilosia melanura* group, was determined. Zymograms identified species-specific alleles and their combinations at *Aat*, *Fum*, *Gpi*, *Hk-2*, *Hk-3*, *Pgm* loci. They also indicated the independent evolution of *Cheilosia bergenstammi* Becker, 1894; *C. bracusi* Vujić & Claussen, 1994; *C. carbonaria* Egger, 1860; *C. lenis* Becker, 1894; *C. lenta* Becker, 1894; *C. melanura* Becker, 1894; *C. rhynchops* Egger, 1860 and *C. vernalis* (Fallén, 1817). A genetic-biochemical key, based on molecular markers of adults, which allows identification and separation among species of the *melanura* group on the Balkan Peninsula, is presented.

Zusammenfassung

Die Allozym-Variabilität an acht Isoyzmloci der *Cheilosia melanura*-Gruppe wurde bestimmt. Zygogramme zeigen artspezifische Allele und ihre Kombinationen an den Loci *Aat*, *Fum*, *Gpi*, *Hk-2*, *Hk-3*, *Pgm*. Außerdem zeigen sie die unabhängige Evolution von *Cheilosia bergenstammi* Becker, 1894, *C. bracusi* Vujić & Claussen, 1994, *C. carbonaria* Egger, 1860, *C. lenis* Becker, 1894, *C. lenta* Becker, 1 894; *C. melanura* Becker, 1894; *C. rhynchops* Egger, 1860 und *C. vernalis* (Fallén, 1817). Ein auf molekularen Markern der Imagines basierender genetisch-biochemischer Schlüssel zur Bestimmung und Unterscheidung der auf der Balkanhalbinsel vorkommenden Arten der *melanura*-Gruppe wird vorgelegt.

Introduction

The genus *Cheilosia,* with more than 80 species on the Balkan Peninsula, has been the subject of many recent taxonomic, genetic and phylogenetic investigations. The *melanura*-group comprises 11 closely related taxa in this area (Vujić 1996): *Cheilosia bergenstammi* Becker, 1894, *C. bracusi* Vujić & Claussen, 1994, *C. carbonaria* Egger, 1860, *C. chloris* (Meigen, 1822), *C. cynocephala* Loew, 1840, *C. lenis* Becker, 1894, *C. fraterna* (Meigen, 1830), *C. lenta* Becker, 1894, *C. melanura* Becker, 1894, *C.*

rhynchops Egger, 1860 and *C. vernalis* (Fallén, 1817). The *melanura* group is characterised by the following morphological features in the adult fly: antennal pits separated; eyes covered with hairs (at least in upper half) in both sexes; mouth-edge more or less expressed; scutellar margin usually with long black hairs or bristles; legs completely dark (*C. lenta* and *C. rhynchops*) or with tibiae pale at both ends; sternites shining or slightly dusted; male genitalia very similar in shape. The close relationships existing between these species have recently been confirmed by morphological characters of the larvae (Stuke 2000). Although the separation of *C. bergenstammi*, *C. chloris*, *C. cynocephala*, *C. fraterna*, *C. melanura* and *C. vernalis* larvae is still uncertain, it has been proven that all analysed species from the *melanura* group belong to one clade (two sister groups) (Stuke 2000).

There are some unsolved taxonomic problems (Vujić et al. 1994) and much uncertainty and confusion in the determination of adults in this group, especially the females (Speight 1999). Two pairs of species, *melanura-lenis* and *rhynchops-lenta*, have been considered semispecies, based on the occurrence of intermediate morphological forms (Vujić et al. 1994). Problems in identification of other species groups have also been discussed: *bracusi-fraterna*, *carbonaria-cynocephala*, *chlorus-fraterna-bracusi*, *cynocephala-melanura-vernalis*, *lenis-bergenstammi* (Speight 1999). It has been suggested that *C. vernalis* comprises more than one species (Speight 1999). Taxonomic and evolutionary problems were the main reasons for initiation of genetic analysis of the *melanura* group species available on the Balkan Peninsula. The results presented in this paper enable identification of the *melanura* group species using allozymes. The results of morphological analyses, and an identification key, will be published separately (Vujić & Claussen, in preparation).

Material and methods

Sample Collection: Samples of *Cheilosia bergenstammi* Becker, 1894; *C. bracusi* Vujić & Claussen, 1994; *C. carbonaria* Egger, 1860; *C. lenis* Becker, 1894; *C. lenta* Becker, 1894; *C. melanura* Becker, 1894; *C. rhynchops* Egger, 1860 and *C. vernalis* (Fallén, 1817) were collected from three geographical regions: Dubasnica Mountain E 21°59', N 44°01' (DUB, Serbia); Kopaonik E 20°40', N 43°15' (KOP, Serbia); Durmitor Mountain E 19°00', N 43°11' (DUR, Montenegro). Specimens of *C. bergenstammi* (KOP: 5, DUR: 20), *C. bracusi* (DUB: 1, KOP: 1, DUR: 9), *C. carbonaria* (DUR: 11), *C. lenis* (KOP: 7, DUR: 7), *C. lenta* (KOP: 12, DUR: 7), *C. melanura* (DUB: 2, DUR: 24), *C. rhynchops* (KOP: 8, DUR: 12) and *C. vernalis* (DUR: 1) were assayed. The genetic structure of the Durmitor population of *C. vernalis* (*Fum, Gpd-2* and *Idh-1* loci were monomorphic) and genotypes at the studied loci, except *Aat*, were known from previous gene-enzyme analyses (34 specimens) (Milankov 2001).

Allozyme Analysis: Electrophoresis is a biochemical method that can detect differences among proteins coded by alleles of different genes. It allows the study of

isoenzyme/isozyme (molecular forms of enzymes coded by alleles of different loci) (Hunter & Market 1957) and alloenzyme/allozyme (coded by alleles of the same locus) (Prakash et al. 1969) variability. Mobilised by electrophoresis (power of electric field, buffers, support medium), polypeptides move at different rates according to characteristics of their molecules, such as electric charge, size and shape. The resulting electrophoretic pattern is called a zymogram (Hunter & Market 1957). Since primary protein structure makes up net electric charge and determines secondary, tertiary and quaternary structure, it can be inferred that detected differences in mobility are due to genetic structure and are in correlation with evolutionary divergence, thus pointing to the degree of difference between studied taxa (Richardson et al. 1986; Pasteour et al. 1988). However, not all genetic differences (allele variability of introns, silent allele variability of exons, amino acid substitutions that do not change electrophoretic mobility) can be detected by electrophoresis (Richardson et al. 1986). Therefore, only point mutations, in gene coding regions that could modify the net charge of an enzyme, can be detected by electrophoresis (Nei 1987). After an electrophoretic run, incubation, visualisation of enzyme activity and gel fixation, zymograms are analysed. The final and the most important stages are genetic interpretation of zymograms and explaining isozyme/allozyme bands on gels.

Isozyme variability was studied by standard 5% polyacrylamide gel electrophoresis, following Munstermann (1979) (FUM, GPI, HK, PGM, GPD, IDH) and Pasteour et al. (1988) (AAT), with slight modifications (Milankov 2001). Different enzyme groups involved in fundamental cell processes such as glycolysis (HK, GPD, GPI), gluconeogenesis (PGM), Krebs cycle (IDH, FUM) and cytoplasmatic transport (AAT) were included in this study. The Tris-Boric-EDTA buffer system (pH 8.9) was used to assay fumarate hydratase (E.C.4.2.1.2. FUM; locus *Fum*), glucosephosphate isomerase (5.3.1.9. GPI; *Gpi*), hexokinase (2.7.1.1. HK; two loci: *Hk-2, Hk-3*) and phosphoglucomutase (2.7.5.1. PGM; *Pgm*). A Tris-Citric buffer system (pH 7.1) was used to assay aspartate amino transferases (2.6.1.1. AAT; *Aat*), glycerol 3-phosphate dehydrogenase (1.1.1.8. GPD; *Gpd-2*) and isocitrate dehydrogenase (1.1.1.42. IDH; *Idh-2*).

Insect specimen electrophoresis was conducted in the same gel for direct inter-population and inter-specific comparison. Loci were numbered and alleles marked alphabetically, with respect to increasing anodal migration. Since all analysed enzymes had anodal migration and isozymes did not overlap, the multilocus HK isoenzyme system was marked numerically: 2 for the less mobile and 3 for more mobile isozyme. Based on literature data (Pasteour et al. 1988) it can be assumed that the mitochondrial *Hk-1* locus also exists.

Different body regions were used for the analysis of isozyme variability, depending on metabolic function and regional distribution of enzymes (head+0.1ml homogenate: AAT, FUM, PGM; thorax+0.15ml: GPD, GPI, HK, IDH). Duration of electrophoretic run at 90mA (130-220 V) was 3.00-3.5 hrs.

Analysis: Genetic interpretation of zymograms was based on Mendel's laws for codominant genes of autosomal loci, depending on enzyme structure (monomers and oligomers) and homo- and heterozygous specimens. Since allozyme data provide information about genetic variation in natural populations and genetic differentiation between taxa of different hierarchical levels at single loci, the results can point to eiher complete identity (the same genotypes occur with identical frequencies in the two populations), complete differentiation (the two populations do not share any genotypes) or different proportions of overlap of the distribution of genotype frequencies (Ayala & Powell 1972). When two populations have complete differentiation (Ayala & Powell 1972) or when any alleles common for two taxa occur at frequencies less than 0.05 in one of the two taxa (fixed difference) (Richardson 1986), given loci are diagnostic. In this study, a locus was considered diagnostic if the probability for correct identification of individuals belong to species of the *Cheilosia melanura* group was 100% (complete differentiation) or higher than 95% (frequency common allele in one of two species is less than 0.05). Loci with overlapping allelic frequencies of 0.15 and 0.20 (correct diagnosis with at least 85% and 80% probability, respectively), or a combination of discriminatory loci, were also used for distinguishing species.

Results

Genetic interpretation of zymograms for *Cheilosia bergenstammi, C. bracusi, C. carbonaria, C. lenis, C. lenta, C. melanura, C. rhynchops* and *C. vernalis* revealed the presence of species-specific alleles at *Aat, Fum, Gpi, Hk-2, Hk-3,* and *Pgm* loci (Tab. 1).

The AAT zymogram enabled the species *C. lenta, C. rhynchops, C. bergenstammi* ($Aat^{b/b}$), *C. melanura* ($Aat^{b/b}$, $Aat^{c/c}$) and *C. bracusi* ($Aat^{b/b}$, $Aat^{c/c}$) to be differentiated from *C. lenis* ($Aat^{a/a}$), as well as from *C. carbonaria* ($Aat^{c/c}$). *C. rhynchops* and *C. lenta* ($Gpi^{c/c}$) were separable from *C. bergenstammi* and *C. lenis* ($Gpi^{a/a}$) as well as *C. carbonaria, C. vernalis* ($Gpi^{b/b}$), *C. melanura* ($Gpi^{b/b}$, $Gpi^{c/c}$) and *C. bracusi* ($Gpi^{b/b}$, $Gpi^{a/a}$) using the alleles and their combinations at the *Gpi* locus. The analysis of FUM zymograms revealed the presence of Fum^a allele in the populations of *C. melanura, C. bracusi, C. lenis* and *C. carbonaria*. In sympatric populations of *C. rhynchops* and *C. lenta* from Durmitor Mountain different alleles were registered (Fum^b and Fum^a, respectively), while in those from Kopaonik Mountain identical alleles were registered but with different frequencies. A major allele (>0.5) in the populations of *C. rhynchops* was Fum^b, while in *C. lenta* it was Fum^a. In the population of *C. bergenstammi* from Durmitor Mountain both Fum^a and Fum^b alleles were found. *C. lenis* and *C. carbonaria*

→ **Tab. 1. Allelic frequency at diagnostic loci of the *Cheilosia melanura* group** (DUB: Dubasnica Mountain; KOP: Kopaonik Mountain; DUR: Durmitor Mountain).

Taxon		*melanura*		*bracusi*			*rhynchops*		*lenta*	
Locus	Allele	DUR	DUB	DUR	KOP	DUB	DUR	KOP	DUR	KOP
Aat	*a*	-	-	-	-	-	-	-	-	-
	b	0.667	-	0.833	-	-	1.000	1.000	1.000	1.000
	c	0.333	-	0.167	-	-	-	-	-	-
Fum	*a*	1.000	-	1.000	-	-	-	0.333	1.000	0.875
	b	-	-	-	-	-	1.000	0.667	-	0.125
Gpi	*a*	-	-	0.222	-	-	-	-	-	-
	b	0.958	1.000	0.778	-	-	-	-	-	-
	c	0.042	-	-	-	-	1.000	1.000	-	1.000
Hk-2	*a*	-	-	-	-	-	-	-	-	-
	b	-	-	-	-	-	1.000	1.000	1.000	1.000
	c	-	-	-	-	-	-	-	-	-
	d	-	-	-	-	-	-	-	-	-
	e	1.000	1.000	1.000	1.000	1.000	-	-	-	-
Hk-3	*a*	-	-	-	-	-	-	-	-	-
	b	-	-	-	-	-	1.000	1.000	1.000	1.000
	c	-	-	-	-	-	-	-	-	-
	d	-	-	-	-	-	-	-	-	-
	e	1.000	1.000	1.000	1.000	1.000	-	-	-	-
Pgm	*a*	-	-	-	-	-	0.083	-	-	0.046
	b	0.190	0.500	0.375	1.000	1.000	-	-	-	0.046
	c	0.810	0.500	0.625	-	-	0.083	0.125	-	-
	d	-	-	-	-	-	0.833	0.875	0.200	-
	e	-	-	-	-	-	-	-	0.600	0.455
	f	-	-	-	-	-	-	-	-	0.228
	g	-	-	-	-	-	-	-	0.200	0.182
	h	-	-	-	-	-	-	-	-	0.046

Taxon		*bergenstammi*		*lenis*		*carbonaria*	*vernalis*
Locus	Allele	DUR	KOP	DUR	KOP	DUR	DUR
Aat	*a*	-	-	1.000	1.000	-	-
	b	1.000	1.000	-	-	-	-
	c	-	-	-	-	1.000	-
Fum	*a*	0.250	-	1.000	1.000	1.000	-
	b	0.750	1.000	-	-	-	-
Gpi	*a*	1.000	1.000	1.000	1.000	-	-
	b	-	-	-	-	1.000	1.000
	c	-	-	-	-	-	-
Hk-2	*a*	-	-	1.000	1.000	-	-
	b	-	-	-	-	-	-
	c	1.000	1.000	-	-	-	1.000
	d	-	-	-	-	1.000	-
	e	-	-	-	-	-	-
Hk-3	*a*	-	-	1.000	1.000	-	-
	b	-	-	-	-	-	-
	c	1.000	1.000	-	-	-	1.000
	d	-	-	-	-	1.000	-
	e	-	-	-	-	-	-
Pgm	*a*	-	-	-	-	-	-
	b	1.000	1.000	-	-	0.545	1.000
	c	-	-	-	-	0.364	-
	d	-	-	-	-	0.091	-
	e	-	-	0.500	-	-	-
	f	-	-	0.500	0.572	-	-
	g	-	-	-	0.286	-	-
	h	-	-	-	0.143	-	-

Diagnostic loci for:						
melanura bracusi	*melanura: rhynchops*	*melanura: lenta*	*melanura: bergenstammi*	*melanura: lenis*	*melanura: carbonaria*	*melanura: vernalis*
-	*Hk-2* *Hk-3* *Gpi**	*Hk-2* *Hk-3* *Pgm**	*Hk-2* *Hk-3* *Gpi*	*Hk-2* *Hk-3* *Pgm* *Gpi* *Aat*	*Hk-2* *Hk-3*	*Hk-2* *Hk-3*

Diagnostic loci for:						
bracusi: rhynchops	*bracusi: lenta*	*bracusi: bergenstanumi*	*bracusi: lenis*	*bracusi: carbonaria*	*bracusi: vernalis*	*rhynchops: lenta*
Hk-2 *Hk-3* *Pgm*** *Gpi*	*Hk-2* *Hk-3* *Pgm* *Gpi*	*Hk-2* *Hk-3*	*Hk-2* *Hk-3* *Pgm* *Aat*	*Hk-2* *Hk-3*	*Hk-2* *Hk-3*	*Pgm**** *Fum****

Diagnostic loci for:						
rhynchops: bergenstammi	*rhynchops: lenis*	*rhynchops: carbonaria*	*rhynchops: vernalis*	*lenta: bergenstammi*	*lenta: carbonaria*	*lenta: lenis*
Hk-2 *Hk-3* *Pgm* *Gpi*	*Hk-2* *Hk-3* *Pgm* *Gpi* *Aat*	*Hk-2* *Hk-3* *Gpi* *Aat*	*Hk-2* *Hk-3* *Pgm* *Gpi*	*Hk-2* *Hk-3* *Pgm* *Gpi*	*Hk-2* *Hk-3* *Pgm* *Gpi* *Aat*	*Hk-2* *Hk-3* *Aat* *Gpi*

Diagnostic loci for:						
lenta: vernalis	*lenis: carbonaria*	*lenis: bergenstammi*	*lenis: vernalis*	*vernalis: bergenstammi*	*vernalis: carbonaria*	*carbonaria: bergenstanumi*
Hk-2 *Hk-3* *Pgm* *Gpi*	*Hk-2* *Hk-3* *Pgm* *Gpi* *Aat*	*Hk-2* *Hk-3* *Pgm* *Aat*	*Hk-2* *Hk-3* *Pgm* *Gpi*	*Gpi*	*Hk-2* *Hk-3*	*Hk-2* *Hk-3* *Gpi* *Aat*

Diagnostic loci permit correct diagnosis of the species with a probability 100%
*Diagnostic loci permit correct diagnosis of the species with a probability ≥95%
**Diagnostic loci permit correct diagnosis of the species with a probability ≥85%
***Diagnostic loci permit correct diagnosis of the species with a probability ≥80%

Tab. 2: A list of diagnostic allozyme loci of the *melanura* group. Diagnostic values were calculated by the method of Ayala & Powell 1972.

1. $Aat^{a/a}$	*lenis*
- $Aat^{b/b}$; $Aat^{c/c}$	2
2. $Hk^{d/d}$	*carbonaria*
- $Hk^{b/b}$; $Hk^{c/c}$; $Hk^{e/e}$	3
3. $Hk^{e/e}$	*melanura, bracusi*
- $Hk^{b/b}$; $Hk^{c/c}$	4
4. $Hk^{b/b}$	5
- $Hk^{c/c}$	6
5. $Pgm^{a/a}$; $Pgm^{c/c}$; $Pgm^{d/d}$	*rhynchops*
- $Pgm^{c/c}$; $Pgm^{f/f}$; $Pgm^{g/g}$; $Pgm^{b/c}$; $Pgm^{a/e}$; $Pgm^{f/h}$	*lenta*
6. $Gpi^{a/a}$	*bergenstammi*
- $Gpi^{b/b}$	*vernalis*

Tab. 3: Genetic-Biochemical key for the identification of the species of the *melanura* group

could be identified by *Hk* loci with unique alleles (Hk^a and Hk^d, respectively). Identical alleles at *Hk* loci were registered in the populations of *C. melanura* and *C. bracusi* ($Hk^{e/e}$), *C. rhynchops* and *C. lenta* ($Hk^{b/b}$), *C. bergenstammi* and *C. vernalis* ($Hk^{c/c}$). The largest number of alleles and genotypes was registered at *Pgm* locus. At *Pgm* locus one genotype ($Pgm^{b/b}$) was found in populations of the species *C. bergenstammi, C. vernalis* and populations of *C. bracusi* originating from Kopaonik Mountain and Dubasnica Mountain, two in *C. bracusi* population from Durmitor Mountain and populations of *C. melanura* ($Pgm^{b/b}$, $Pgm^{c/c}$) and three genotypes ($Pgm^{b/b}$, $Pgm^{c/c}$, $Pgm^{d/d}$) in *C. carbonaria* populations from Durmitor Mountain. Specific alleles and genotypes of diagnostic significance were registered in *C. rhynchops* populations ($Pgm^{a/a}$, $Pgm^{c/c}$, frequency 0.083 and $Pgm^{d/d}$), *C. lenta* populations from Durmitor Mountain ($Pgm^{d/d}$, $Pgm^{e/e}$, $Pgm^{g/g}$) and Kopaonik Mountain ($Pgm^{e/e}$, $Pgm^{f/f}$, $Pgm^{g/g}$, $Pgm^{b/e}$, $Pgm^{a/e}$, $Pgm^{f/h}$) and *C. lenis* populations from Durmitor Mountain ($Pgm^{e/e}$, $Pgm^{f/f}$) and Kopaonik Mountain ($Pgm^{f/f}$, $Pgm^{g/g}$, $Pgm^{f/h}$).

The largest number of diagnostic loci (5), or proportion of diagnostic loci (62.5%), that allow distinction of species was registered for the species pairs: *melanura-lenis; rhynchops-lenis; lenta-carbonaria* and *lenis-carbonaria*, followed by: *melanura-lenta; melanura-rhynchops; bracusi-rhynchops; bracusi-lenta; bracusi-lenis; rhynchops-bergenstammi; rhynchops-carbonaria; rhynchops-vernalis, lenta-bergenstammi, lenta-lenis; lenta-vernalis; bergenstammi-lenis; bergenstammi-carbonaria* and *lenis-vernalis* with 4 diagnostic loci. Alleles at *Hk-2, Hk-3* and *Pgm* loci enabled differentiation of *C. melanura* and *C. lenta*. Alleles at *Hk-2, Hk-3* and *Gpi* loci had diagnostic value for species pair *melanura-bergenstammi*, while for *melanura-carbonaria, melanura-vernalis, bracusi-bergenstammi, bracusi-carbonaria, bracusi-vernalis* and *carbonaria-vernalis* the HK isoenzyme system was used. However, separation of *C. rhynchops* and *C. lenta* could be achieved using the alleles at *Pgm* and *Fum* loci with at least 80% probability of correct identification (with 0.20 overlapping

frequency of common alleles). The allozymes at GPI locus allowed exact identification and separation of *C. bergenstammi* and *C. vernalis* (Tab. 2).

Genetic analysis of the investigated taxa revealed the presence of species-specific alleles and their combinations. The allozymes, which they produce, thus allowed identification and separation of the *melanura* group species and enabled construction of a genetic-biochemical key (Tab. 3).

Discussion

In contemporary systematics, a large number of molecular techniques are used, for studying molecular markers in taxa at different levels of evolutionary divergence. For example, DNA-DNA hybridization methods are suitable for phylogenetic studies at intermediate taxonomic levels, when evolutionary separation of taxa dates from 2-100 million years ago. Similarly, multi-locus DNA fingerprinting is a suitable method for analysing genetic identity versus non-identity, and parentage. Analysing the evolutionary relationships in conspecific populations and closely related species, as well as studying intra-specific population structure, species limits and species diagnosis, can often be effectively addressed using allozyme electrophoresis, restriction fragment length polymorphism (RFLP), or some other DNA- based technique, like single-stranded conformational polymorphism (SSCP), amplified fragment length polymorphism (AFLP), or random amplified polymorphic DNA (RAPD). Among these various techniques, only DNA sequencing, can usually find application in molecular systematic studies at any taxonomic level (Avise 1994; Caterino et al. 2000; Hillis et al. 1996), even if it is unlikely to be cost-effective . Besides the choice of a suitable method, selecting appropriate molecular markers is also important in systematic studies. Depending on the molecular markers employed, the genetic variability can be used to characterise and identify individuals, populations, species or phylogenetic relationships. Since more mutational variation has been registered for non-coding DNA regions, minisatellite (DNA fingerprinting) and microsatellite loci (microsatellite polymerase chain reaction), the mentioned sequences are used to identify individuals or to analyse paternity within families (DNA fingerprinting, multi-/single locus probes, RAPD, microsatellite PCR). Protein coding regions are DNA sequences with lower levels of mutation and are therefore used as molecular markers in population genetics (isoenzyme electrophoresis, RFLP mtDNA, SSCP and related methods, microsatellite- PCR) and for analysing the evolutionary relationships of closely related species (protein electrophoresis, RAPD, RFLP). For analysing relationships between taxa at supra-generic levels, and reconstruction of phylogeny, methods of molecular biology are recommended: DNA-DNA hybridisation, amino acid sequencing and nucleotide sequencing of mtDNA, for taxa with independent evolution shorter than 20 million years, and rDNA for higher divergency level taxa (Wink 1998). Mitochondral genes are often chosen, since they have different levels of variability and deletions (inversions and insertions are of

minor importance). Being transmitted predominantly through maternal lines, the entire mtDNA molecule represents one non-recombining genealogical unit,with multiple alleles (Hillis et al. 1996). It consists of about 37 genes (22 $mtRNA_S$, 2 rRNA, 13 protein coding genes) and is suitable for phylogenetic analyses at the level of closely related species and above. Nuclear ribosomal DNA repeats in a mosaic form (18S, 5.8S and 28S genes, separated by transcribed and non-transcribed spacers), which consists of highly conservative and variable regions, and is recommended for analysing: phylogeny (conservative sequences: 18S, 5.8S and 28S genes), individual variability (intergenic spacer, IGS), population structure of species (IGS and internal transcribed spacer, ITS regions), evolutionary relationships of closely related species (ITS2 region) and higher level taxa (D2 loop of 28S gene) (Besansky et al. 1992; Caterino et al. 2000).

Relatively low cost, easy resolution of allozyme and isozyme, and the bi-parental nature of allozyme inheritance, are primary reasons why allozyme data are widely used in systematic and evolutionary studies (Hillis et al. 1996; Caterino et al. 2000). Identification of individuals and delineating species boundaries (a taxonomy), quantifying genetic differences and studying genetic relationships among taxa of various classification levels (b taxonomy), plus analysis of population structure of species (g taxonomy), have all been based on allozyme data for a long time. The use of genetic variability of allozyme loci, in phylogenetic analyses, is appropriate for taxa with independent evolution of 50-500 million years, while it is inappropriate for periods longer than 500 million years (Hillis et al. 1996). Isozymes formed by duplication, as a unique event in evolution, can be used for defining direction and position of phylogenetic branches of higher level taxa (Avise 1994). Besides this, due to ontogenetic differentiation and tissue specificity of isozymes, indirect information about the activity of regulatory genes can be obtained (Buth & Murphy 1999).

The use of morphological characters in defining cryptic taxa and evolutionary relationships of closely related species has proven insufficient in many groups of organisms, especially in insects (Narang et al. 1993; Foley et al. 1995; Milankov et al. 2000). The reasons for this are ecological variance, which contributes to the overall phenotype variance, and difficulties in defining the polygenic control of morphological traits and pleiotropic effects. Besides these, the effect of different selection pressures, on various characteristics and difference in the rates of evolutionary changes of genes at the individual loci might be the cause of registered variations. Conversely, allozyme and isozyme data, like many other biochemical-molecular data, are common tools in systematic biology (Wiens 2000), due to their lack of environmental influence, their independence of characters (multiple unlinked nuclear loci), and relatively simple genetic control. It has been established that allozyme data are important in identification of many insect groups (Caterino et al. 2000), including Syrphidae and , especially, in the identification of cryptic species (Milankov 2001; Milankov et al. 2001) and in analysis of evolutionary relationships of taxa up to the genus level (Milankov 2001).

The principal finding of the results presented here is confirmation of the evolutionary independence of the investigated *melanura* group species. Also, information about the alleles at the diagnostic loci was obtained, which can be used in the identification of the species. Identification based on morphological traits is sometimes difficult for the species *melanura* and *lenis*, since there are instances when differential characters are variable (Vujić et al. 1994). However, correct identification could be achieved using allozymes of the diagnostic loci: *Hk-2, Hk-3, Pgm, Gpi* and *Aat*.

Difficulties experienced in differentiating *C. rhynchops* and *C. lenta* using morphological parameters (Vujić et al. 1994) were paralleled in the analysis of zymograms. The descriptive *Pgm* locus enabled correct identification with 80% (sympatric populations from Durmitor Mountain) and 100% probability (sympatric populations from Kopaonik Mountain). It was the other way around in the case of the alleles at the *Fum* locus, which allowed 100% probability for the correct identification of the populations form Durmitor Mountain and 85% for those originating from Kopaonik Mountain. None of the analysed allozymes differentiated the species *C. melanura* and *C. bracusi*. But these two species can be correctly identified using morphological data (Vujić & Claussen 1994). A lack of correlation between morphological and gene-enzyme variability has also been found in other groups of organisms (Sharma et al. 1999).

For the time being, the application of marker loci and keys based on allozyme variability is not possible, due to the lack of reference standards for hoverflies: the species have not yet been bred under laboratory conditions (therefore, no assortative mating is possible) and there is no inbred strain (homozygous for multiple enzyme loci which can use as a standard reference to identify electromorph of respective enzyme genes). The presence of fixed differences at enzyme loci is strong evidence that species are in a state of genetic isolation (the criterion for defining species level status in phylogenetic and biological species concepts) and is used as the basic unit for further study of genetic relationships and phylogenetic analysis. Besides registering additional diagnostic loci, increase in the number of specimens included, in analysed populations and assayed isozyme loci, would allow analyses of population genetic structure, hierarchical population structure (*F*- statistics), genetic relationships among geographically isolated conspecific populations (intraspecific variability) and, based on evolutionary relationships, phylogenetic reconstruction of the taxa in the *melanura* group of the genus *Cheilosia*.

Different numbers of specimens belonging to each of the *melanura* group species were analysed in the study reported here. This is not of a very great significance if two things are taken into account in interpreting allozyme data for taxonomic purposes: firstly, that allele replacement (measured as fixed differences) is a significant biological event, and secondly, that most populations are commonly monomorphic at an average of 85% of isozyme loci, although the range is wide, from 50% to 100% (Nevo 1978). Therefore, a single individual is representative of the whole population for, on average,

85% of electrophoretic characters. Thus, populations of the same species seldom differ by more 10% of loci, and almost never by more than 15% of loci (Baverstock et al. 1977).

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