



Morphological and Molecular (*COI* mtDNA) Diversity of the Polyzonal Species of Grass Flies *Meromyza nigriseta* Fedoseeva, 1960 (Diptera: Chloropidae)

Tatiana A. Triseleva*, Varos G. Petrosyan, Alexandra A. Yatsuk & Andrej F. Safonkin

Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, 33 Leninsky Prospekt, 119071 Moscow, Russia;
E-mail: triselyova@yandex.ru

Abstract: The population diversity of the Euro-Siberian species *Meromyza nigriseta* Fedoseeva, 1960 was analysed using morphometric and molecular genetic data obtained (*COI* mtDNA locus) based on 12 populations from the central part of its geographical range. The colour variations of the mid mesonotal stripes were insufficient for identification of inter-population diversity. The size of the anterior processes of postgonites (AP) and the unique sequence of the *COI* mtDNA gene can serve as markers of the population diversity. Seven haplotypes with genetic distance from 0.1 to 0.3% were identified. We recorded H1 haplotype in samples from all regions, except Tula (T), Brest (B) and Kaliningrad (Kl) populations. The load-graph in the plane of the principal components revealed a strong influence of the total (S_t) and main (S_{map}) part of AP. The Moscow (M) and Ural (Ur) populations of *M. nigriseta* differed most substantially from T and Kl populations by the projecting part of the area of AP (S_{pap}), S_{map} and S_t . In addition, S_t of AP increased from Ur to the western population and S_{map} from U to Kl ones. *Meromyza nigriseta* originated in the grasslands and steppe ecosystems of Eastern Europe and then during the period from 44,000 to 130,000 years invaded new regions.

Key words: morphology, postgonites, grass flies, microevolution, Palaearctic

Introduction

One of the most demonstrative examples of morphological diversity in insects is the size and shape variations of their external reproductive structures (EBERHARD 1985, MASLY et al. 2011). For insects, one of the mechanisms for maintaining biodiversity and microevolution is based on variations of morphological features within populations (VIA 1990). In a number of studies, it is assumed that characteristics of the structure of the sexual apparatus, including non-discrete characteristics of its size and shape, have a significant role in maintaining diversity through the restriction of panmixia.

Modern analyses of population diversity are based on an integrated approach combining morphological features and molecular genetic characteristics, including mitochondrial DNA (SCHMIDT & SPERLING 2008). Mitochondrial DNA (mtDNA) is considered an important tool in evolutionary and population genetics due to its small size and lack of introns and recombination (MITROFANOV et al. 2002), which facilitates the assessment of mitochondrial DNA variability. *COI* and *COII* genes are widely used as the markers of choice in the studies of intra- and inter-population groupings (SCHROEDER & SCHOLZ 2005, SCHEFFER & WINKLER 2008,

*Corresponding author

KHRABROVA et al. 2013). Such markers allow analysing the geographical structure of populations using females in contrast to morphological features, which characterise the population based on both sexes (SPERLING et al. 1999).

The grass flies of the genus *Meromyza* Meigen, 1830 (Diptera: Chloropidae), which include more than 90 species, are widespread throughout the Northern Hemisphere above 40° north latitude (NARCHUK & FEDOSEEVA 2011). The identification key is based on morphological features and the structure of postgonites (AP), which is an element of male reproductive apparatus (NARCHUK & FEDOSEEVA 2010). In females, there were no significant differences in the sexual apparatus, including the copulative sac; however, we have developed the species diagnostics based on the COI gene (SAFONKIN et al. 2016, TRISELYOVA et al. 2014). Due to the structure of postgonites and unique nucleotide sequences of mtDNA COI locus, we used the genus as a model for the analysis of population variability and the process of microevolution in reproductive behaviour (SAFONKIN et al. 2016, 2018). The only data have been obtained for the Holarctic species *M. saltatrix* L., 1761, which demonstrates the existence of two phenotypes (described by the setae on the lower surface of genae) and two haplogroups

(by mtDNA COI locus) correlating with the size of the anterior processes of postgonites (SAFONKIN et al. 2018). The analysis of the complex of the studied traits has allowed evaluating micro-evolutionary trends in this species, which are associated with the clinal variability of the size of postgonites and the restriction of panmixia between populations across its geographical range. No similar studies have been carried out for grass flies with narrower ranges, including *Meromyza nigriseta* Fedoseeva, 1960. At the same time, analyses of the role of the latitude of the species range in the intra- and interpopulation diversity may allow evaluating general patterns of micro-evolutionary processes. The hypothesis tested in this study is that the size of male genital apparatus regularly varies across the geographical range of a species, which leads to intraspecific diversification in COI mtDNA gene and is associated with partial reproductive isolation of populations.

This study aims to analyse morphological features such as colour variability in mesonotum stripes and morphometry of the anterior processes of postgonites in the Eurasian polyzonal species *M. nigriseta* in the central part of its geographical range. The results of this analysis are compared with the data of the mtDNA COI. We also propose a hypothesis of the relative time of divergence of the studied populations.

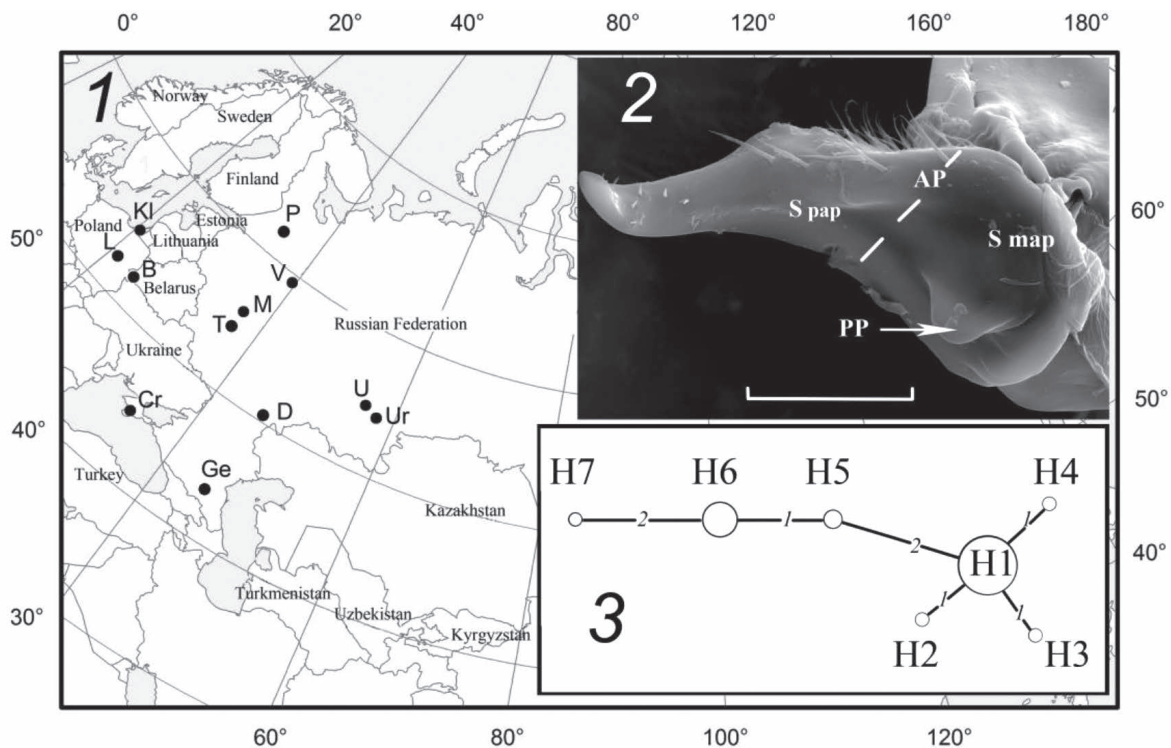


Fig. 1. Collection sites (1.1). The image of the anterior (AP) and posterior (PP) processes of postgonite (1.2): dotted line – the line of separation of the projecting and main part of AP, scale-bar 50 nm. Median network of haplotypes (H1–H7) of *M. nigriseta* (1.3): figures on branches are number of nucleotide substitutions. H1 (n = 26): V, Ge, D, Cr, P, Ur; H2 (n = 1): Ur; H3 (n = 1): Ur; H4 (n = 1): P; H5 (n = 3): B, Kl; H6 (n = 12): B, Kl, T; H7 (n = 1): Kl.

Materials and Methods

Sampling

The adults were collected by mowing in June 2012–2018. Both dry material and material fixed in 96% ethanol were used. Samples were collected from the following sites (Fig. 1.1): Russian Federation: floodplain of the Moskva River, Biological Station of the Moscow State University, Zvenigorod, Moscow Region (altitude $H \approx 141$ m a.s.l.) (M); surroundings of Tula ($H \approx 161$ m a.s.l.) (T); Vologda ($H \approx 114$ m a.s.l.) (V); Kaliningrad ($H \approx 7$ m a.s.l.) (Kl); Petrozavodsk ($H \approx 121$ m a.s.l.) (P); South Ural Natural Reserve ($H \approx 311$ m a.s.l.) (Ur); the floodplain of the Belaya River, Ufa ($H \approx 90$ m a.s.l.) (U); Simferopol, Crimea ($H \approx 247$ m a.s.l.) (Cr); Dyakovka Village, Krasnokutsky District, Saratov Region ($H \approx 36$ m a.s.l.) (D); Georgievsk, Stavropol Region ($H \approx 302$ m) (Ge); vicinity of Brest, Republic of Belarus ($H \approx 142$ m a.s.l.) (B); Republic of Poland: Lomianki, Masovian Province ($H \approx 77$ m a.s.l.) (L).

Morphological analysis

The identification of grass flies by a complex of external features was performed according to the original description of the species and the identification key to grass flies (FEDOSEEVA 2003). From each collection site, the characters “colour of stripes of mesonotum and scutellum” and “presence/absence of black setae on the lower surface of genae” were analysed. Grass flies have a marked mid stripe and two lateral stripes on the mesonotum. The mid stripe passes through the scutellum. The lateral stripes of the mesonotum are always black in colour whereas the mid one is from black to dark brown, less often light brown. We divided males and females separately of each sampling into four colour groups: C1 – completely black mid stripe of the mesonotum; C2 – one third of the mid stripe of the mesonotum brown; C3 – one third of the mid stripe of the mesonotum and the scutellum brown; C4 – other colour variations of the mesonotum. Collection sites and number of males/females were as follows: M: 25/48, T: 36/45, V: 25/45, Kl: 5/4, P: 4/3, U: 23/14, Ur: 51/34, Cr: 22/42, D: 32/15, Ge: 1/1, B: 48/25, L: 22/30.

Morphometric analysis

Postgonites of grass flies consist of anterior (AP) and posterior (PP) processes (Fig. 1.2). The structure of the postgonite surface was studied using the image acquired with CamScan MV 2300 scanning electron microscope (Czech Republic) (Fig. 1.2). PP of *M. nigriseta* is small, tightly adjoins to AP, therefore,

for comparative analysis, AP of male postgonites (with division into the main (MAP) and projecting (PAP) parts) was selected (dotted line in Fig. 1.2). Measurements of the selected postgonites were carried out using images acquired with Keyence VHV-1000 light microscope (Japan) with an integrated data analysis program and with a standard setting throughout the study. We calculated the total area of AP (S_t), the area of MAP (S_{map}) and the area of PAP (S_{pap}) (Fig. 1.2) and recorded values in micrometres. The number of specimens for analysis was as follows: M – 41, T – 5, V – 12, Kl – 7, P – 3, Ur – 16, U – 8, Cr – 23, D – 12, Ge – 6, B – 10 and L – 5.

Molecular genetic analysis

Total DNA was extracted from whole flies (in males, the abdomen tip was removed to take the measurement of postgonites), using the reagents “Diatom-200” (“Izogen”, Moscow) and according to the manufacturer’s protocol. Polymerase chain reaction (PCR) was performed with forward primer CI-J-2183 CAACATTTATTTTGATTTTTTGG and reverse primer TL2-N-3014 TCCAATGCAC-TAATCTGCCATATTA developed by Simon et al. (1994). Extraction of DNA, PCR and sequencing were performed in the Laboratory of the molecular diagnostic methods of the Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences as previously described (TRISELYOVA et al. 2014). The fragment analysed included 770 base pairs of the second part of the COI gene. A total of 45 sequences were analysed. The GenBank accession numbers of the haplotypes represented by samples from different regions were: KT 781132, MH 428646, MH 428648, MH 428651, MH 428652, MH 428647, KT 781133, KT 781131, MH 428644, MK 125079, MH 428645, MH 428653, MK 125078, MK 125080, MH 428650. Data processing was carried out using MEGA 5 software packages (TAMURA et al. 2011), Network ver. 4.6.1.1 (BANDELT et al. 1999) and ARLEQUIN ver. 3.5 (EXCOFFIER & LISCHER 2011).

Statistical analysis

We used two-way analysis of variance (ANOVA) to determine the colour variability of mid stripe of the mesonotum between sexes and populations. We used multivariate analysis of variance (MANOVA) to determine differences between means of the morphometric (S_t , S_{map} , S_{pap} , S_t / S_{pap}) parameters using the general linear model (GLM) (ZAR 2010, PETROSYAN 2014). If MANOVA based on criteria Wilks’s lambda (Λ), Pillai’s trace (V), Hotelling-Lawley trace (U) and Roy’s largest root (Θ) rejected

Table 1. Sample size, colour variation of mid stripe of mesonotum in the populations of *M. nigriseta* from 12 localities of the species range. Legend: C1 – black, C2 – one third of mid stripe is brown, C3 – one third of mid stripe and scutellum are brown, C4 – other colour variations; M – male; F – female.

Collection sites	Number of individuals								Total number of individuals		Colour variations, %	
	C1		C2		C3		C4					
	M	F	M	F	M	F	M	F	M	F	M	F
Moscow Region	16	20	9	25	0	2	0	1	25	48	2 (8.0)	4 (8.3)
Tula	4	2	18	25	13	15	1	3	36	45	4 (11.1)	4 (8.9)
Vologda	9	20	14	25	0	0	2	0	25	45	3 (12.0)	2 (4.4)
Kaliningrad	0	0	5	2	0	2	0	0	5	4	1 (20.0)	2 (50.0)
Petrozavodsk	3	3	0	0	1	0	0	0	4	3	2 (50.0)	1 (33.3)
Ufa	3	2	16	11	4	1	0	0	23	14	3 (13.0)	3 (21.4)
South Ural Natural Reserve	7	4	24	13	18	12	2	5	51	34	4 (7.8)	4 (11.8)
Crimea	1	2	19	22	2	16	0	2	22	42	3 (13.6)	4 (9.5)
Dyakovka Village	1	0	10	7	20	8	1	0	32	15	4 (12.5)	2 (13.3)
Georgievsk	0	0	0	1	1	0	0	0	1	1	1 (0.0)	1 (0.0)
Brest	0	0	20	6	26	19	2	0	48	25	3 (6.3)	2 (8.0)
Lomianki	3	7	9	12	10	10	0	1	22	30	3 (13.6)	4 (13.3)
Number of individuals with definite colour (colour frequencies)	47 (0.16)	61 (0.2)	144 (0.49)	149 (0.49)	95 (0.31)	85 (0.28)	8 (0.03)	12 (0.04)	294	306		

the null hypothesis, we used one-way analysis of variance for each dependent variable to determine which mean values differed significantly. For multiple comparisons of means, we used Fisher’s LSD *post hoc* test at the 95% confidence level. The visualisation of the results of a comparative analysis of populations selected from 12 localities of the central part of the range based on the dimensional features of the AP (S_t , S_{map} , S_{pap} , S_t / S_{pap}) was carried out using the principal component analysis (PCA). To simplify the interpretation of the results, PCA was applied to standardised data. Since the four variables used were measured in different units and linear combinations were difficult to interpret, in this case standardisation (i.e., replacing the X_i variable with $Z_i = (X_i - \mu) / \sigma_i$, $i = 1, 2, 3, 4$) of each variable simplified the interpretation. Statistical data analysis was performed using Statistica 10 and Biosystem office statistical packages (PETROSYAN 2014).

Results

Morphological analysis

To identify colour variations of the mid stripe of mesonotum in 600 individuals from 12 localities of *M. nigriseta*, we marked four variants, which were found in different populations (Table 1). The analysis showed that 49% of the individuals (293) from

all 12 localities were characterised by the dominant colour (C2). C3-colour type was recorded for 30% of the individuals (180) from 11 localities. The colour types C1 and C2 were characteristic of 18% and 3% of the individuals, respectively. C1 colour type was detected in nine populations and C4 – in eight populations. Two-way analysis of variance (ANOVA) did not detect significant colour differences in males and females ($P > 0.99$) in these populations, but revealed a significant difference in populations by the number of colour types ($P = 0.04$). According to the Fisher’s LSD *post-hoc* test, it was found that in three populations (Ge, P, Kl) the number of colour types was much lower than in others. The colour type C2 dominated in Ge and C2, C3 – in P, Kl. In males and females, black setae were not found on the lower surface of genae.

Molecular genetic analysis

The alignment of the sequences of the mtDNA *COI* site in grass fly samples revealed eight variable sites (5 transitions and 3 transversions), three of which were parsimony-informative. The nucleotide composition of the analysed fragment included 13.4% of cytosine, 40.8% of thymine, 32.8% of adenine and 13.0% of guanine. A total of seven haplotypes were described for the mtDNA *COI* site; four of them were unique, three were recorded in several

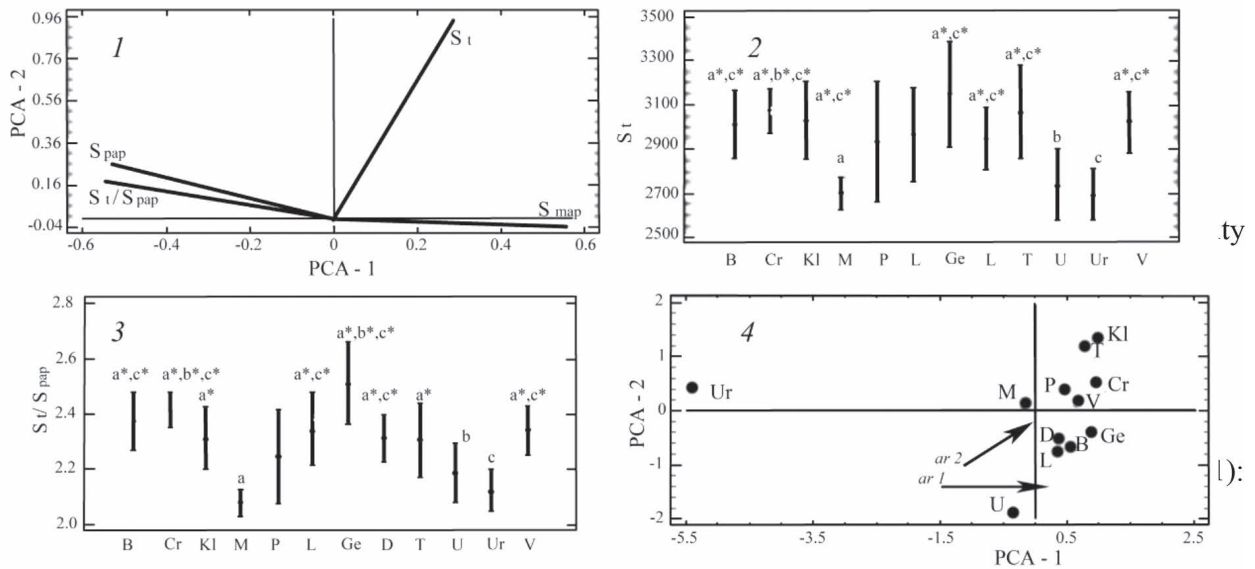


Fig. 2. Analysis of the distribution of populations of the central part of the range of *M. nigriseta* by the sizes of male AP. Load-graph in the plane of the principal components (2.1); average values of S_{map} (2.2) and average values of S_t/S_{pap} (2.3) with Fisher's 95% confidence intervals LSD; scatter chart of the location of populations in the plane of the principal components, determined using the PCA (2.4). Significant differences between populations M (a), U (b), Ur (c) and the rest populations are marked with asterisk (*) ($p=0.05$) in 2-2 and 2-3.

(from 2 to 26) samples. Haplotypic diversity (H) was 0.56 ± 0.07 , while average number of paired differences between haplotypes (P_i) and nucleotide variability (π) were 1.47 ± 0.91 and 0.0019 ± 0.0013 , respectively. The median network of haplotypes of *M. nigriseta* indicated that haplotype 1 (H1) gave rise to other minor haplotypes (Fig. 1.3).

Notably, samples of some populations (Ur, P) were represented by various haplotypes. The population from the South Ural Natural Reserve (Ur) was represented by three haplotypes (H1, H2 and H3), the population from Petrozavodsk (P) – by two haplotypes (H1 and H4). H1 included specimens coming from all regions, except for individuals of Kl, T and B. These populations formed the independent grouping, separated by 2–5 nucleotides from H1 and were represented by three separate haplotypes (H5–H7) (Fig. 1.3). Two out of three detected amino-acid substitutions were in the H7 haplotype ($A \rightarrow G$ (site 476) = $K \rightarrow S$ and $A \rightarrow C$ (site 576) = $D \rightarrow A$), the only sample of which was found in Kl. One amino-acid substitution was in the H3 haplotype from Ur ($A \rightarrow C$ (site 537) = $Q \rightarrow H$).

Morphometric analysis

For standardised data, PCA 1 and 2 explained at least 99% of the total variation. Therefore, populations from different regions could be represented in the system of two principal components. The diagram (Fig. 2.1) showed the connection of a certain compo-

nent with the original morphological features. From the load-graph, we could conclude that the analysed morphological variables form three groups, with the principal components correlating to the greatest extent with the variables of S_{map} and S_t . The correlation coefficients (R) values between PCA-1 and S_{map} and between PCA-2 and S_t were 0.56 and 0.94, respectively. Therefore, S_{map} made a great contribution to PCA-1 and S_t – to PCA-2. The contribution of the variables S_{pap} and S_t/S_{pap} to the principal components was lower: $R = -0.55$ between PCA-1 and both S_{pap} and S_t/S_{pap} ; $R = 0.27$ between PCA-2 and S_{pap} and $R = 0.18$ between PCA-2 and S_{pap} . The analysis of the size of S_t of AP (5247.16 ± 364.98 , with a range from 4373.51 to 6474.56 μm^2) showed no significant differences between populations, except for significantly differing Kl and U populations (GLM-ANOVA: $F = 0.68$; $P = 0.75$). Significantly lower indices of the minimum value of the dimensional characteristic of S_{map} were found in the M, U and Ur populations (Fig. 2.2). Similar differences in populations were reflected by the ratio S_t/S_{pap} (Fig. 2.3). As demonstrated through the scatter chart (Fig. 2.4) of the location of populations based on the average standardised data, several groups could be distinguished. The left side of the diagram included the M, U and Ur populations. The right side groups the rest, amongst which L, B and D; P and V; Kl and T were more closely grouped. The load-graph in the plane of the principal components (Fig. 2.1) indicated a strong influence

of S_t and S_{map} , while the contribution of S_{pap} and S_t / S_{pap} was significantly lower. Since S_t consists of S_{map} and S_{pap} , the ratio of the main and projecting parts of the AP could be a significant factor in the analysis of population diversity based on selected features.

Discussion

The polyzonal species *M. nigriseta* is widespread from Scandinavia to Bulgaria and from Western Europe to Yakutia. It belongs to the common species of grass flies found in small numbers in collections from all regions of Europe. The identification of *M. nigriseta* using identification keys to species of the genus *Meromyza* (see FEDOSEVA 2003) is possible only based on morphology of male genitalia: bifurcated surstyles with thick spiniform setae and the form of AP. Species identification based on morphological characters of females has not been developed. The main part of samplings from all localities has been composed of the C2 and C3 colour types. Therefore, the variations in the colour of mesonotum stripes could serve as one of the key features for species identification of *M. nigriseta* but are not reliable in identification of the populations from different parts of the range. The feature “the presence of black setae on the lower surface of genae” is not observed in *M. nigriseta*. This feature is used as characteristic of the West-European group species of *Meromyza* (see FEDOSEVA 2003). In the Polish population of the Holarctic *M. saltatrix*, a previously unknown phenotype with black setae (the feature of the West-European species) and the typical AP size of the south population of this species has been revealed (SAFONKIN et al. 2018). This phenotype has been likely formed in a refugium in South-Western Europe during the Quaternary Glaciation (SAFONKIN et al. 2018). The absence of black setae on the lower surface of genae in *M. nigriseta* can be interpreted as an indication for the Eastern-European origin of the species.

Postgonites are the physiologically active structures of the male reproductive system during mating. When studying the mechanism of copulation of Diptera in *Drosophila melanogaster* Meigen, 1830 (Drosophilidae) (KAMIMURA 2010) and *Coproica* sp. Rondani, 1861 (Sphaeroceridae) (LACHMANN 1997), the penetration of postgonites and phallus in the female copulative pouch has been demonstrated. The elements of male genitals should correspond to the shape of the female copulative pouch (YASSIN & ORGOGOZO 2013). The genome protection of a species against the entry of foreign genes at the initial stage of mating may depend on the complementarity of the male and female sex structures (SAFONKIN

2011). Demonstration of significant differences in the shape and size of AP in closely related species of grass flies of *Meromyza* (see SAFONKIN et al. 2016) indicates the importance of this element of the reproductive apparatus, both for interspecific isolation and for maintaining panmixia between individuals of the same population. We have shown in a previous study that in flies of *Meromyza* morphological clustering based on postgonite structure correlated with sequence clustering based on COI locus of mtDNA (TRISELYOVA et al. 2014). These results allow using both males (structure of AP) and females (COI mtDNA gene) for demonstration of inter- and intra-species variability.

It is known that the clinal morphological variations in insects reflect different patterns of distribution and habitat accessibility (GERLIND et al. 2008, ALVES et al. 2016, YI et al. 2016). For the Holarctic species *M. saltatrix*, the clinal variations of size and ratio of AP parts correlate with phenotype and molecular variability of population (SAFONKIN et al. 2018). The analysis of morphological and molecular genetic differences of the widespread Palearctic polyzonal *M. nigriseta* reveals a variety of population patterns. We assume that on the scatter chart of the location of populations in the plane of the principal components (Fig. 2.4), the abscissa (arrow *a*) characterises the trend of increase of S_{map} . The ordinate indicates the direction of increase of S_t (arrow *b*). As can be seen from the scatter chart (Fig. 2.4), according to the analysis of the cumulative effect of the considered features, the eastern (M, U, Ur and north-western (Kl, T) populations are most distant from the centre. Thus, the size of AP can serve as a phenotypic trait characterising a certain population of *M. nigriseta*. The isolation of these populations, especially Kl, T and adjacent B, is confirmed by the presence of specific haplotypes (Fig. 1.3).

The median network of *M. nigriseta* haplotypes also demonstrates phylogenetic relationships between haplotypes and is characterised by the stellate structure (Fig. 1.3). The predomination of H1 may indicate that this haplotype is ancestral to most populations of the West-European part of the range. Two out of three amino-acid substitutions in the mtDNA locus of *M. nigriseta* have been marked in the north-western population (Kl) and one – in the farthest eastern population, which assumes multidirectional micro-evolutionary transformations in remote populations of the same species by AP sizes. The stellate structure of the median network indicates the conservation of both common and unique region-specific haplotypes. A similar pattern has been found in other insects, *Meromyza saltatrix* (see

SAFONKIN et al. 2018) and *Tortrix viridana* L., 1758 (Lepidoptera, Tortricidae) (SCHROEDER & SCHOLZ 2005). We hypothesise that this pattern is determined by intra-population panmixia, including the stable size of AP in the male reproductive apparatus.

Based on the molecular clock of insect mitochondrial genome, the divergence rate is about 2.3% per 1 million years (BROWER 1994). We recorded a genetic distance between the central and other haplotypes of *M. nigriseta* of 0.1-0.3%. This assumes a possible time of the formation of minor haplotypes and younger populations from 44,000 to 130,000 years. These data on the divergence of populations do not contradict the known facts about the possible divergence of closely related species (using the example of *Drosophila*) for 300,000 years (MASLY et al. 2011).

The larvae of *M. nigriseta* develop in the stems of rootstock grasses *Hierochloa odorata* (L.) P. Beauv., *Leymus ramosus* (Trin.) Tzvelev and sod grasses *Agrostis vulgaris* With., and in the stems of spring wheat in the south part of the range (FEDOSEEVA 2003). It is generally believed that steppes of Eurasia developed in the Oligocene-Miocene. It is a zonal belt of vegetation, stretching from the Danube River plains of Europe to Eastern Mongolia. Rootstock grasses and sod grasses, including the host plants of *M. nigriseta*, dominate in meadow and steppe phytocoenoses, respectively (AVDEEV 2008).

For species of the genus *Meromyza*, up to three centres of species diversity (Western, East Palaearctic and North American) have been recognised (BESHOVSKI 1986). Based on the features “the absence” of black setae on the lower surface of genae” and the prevalence of the common H1 haplotype in Eastern European populations, *M. nigriseta* presumably originated in the grasslands and steppe ecosystems in Eastern Europe and, later, small groups of individuals invaded new regions with different environmental conditions (e.g., the Urals and NW Europe). A similar pattern was noted for populations of *M. saltatrix* in the Moscow Region and the Urals (SAFONKIN et al. 2018). Using the example of revealed morphological and molecular differences of geographically close Moscow and Tula populations of *M. nigriseta*, we can assume the influence of ecological-geographical barriers (such as water barriers, zonality).

Conclusions

The colour variations of mid stripes of mesonotum are insufficient for identification of inter-population diversity. The size of the anterior processes of postgonites and the unique sequence of the COI mtDNA gene can serve as markers of the population diver-

sity in grass flies. The species *M. nigriseta*, while expanding the range and adapting in various eco-geographical conditions, has increased the population diversity, partly resulting from restriction of panmixia due to size differences of the elements of the sexual apparatus.

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