ZOOSYSTEMATICA ROSSICA

ISSN 2410-0226 [online] 0320-9180 [print]

Zoological Institute, Russian Academy of Sciences, St Petersburg ▪ https://www.zin.ru/journals/zsr/

Vol. 28(2): 305–316 ▪ Published online 27 December 2019 ▪ DOI 10.31610/zsr/2019.28.2.305

RESEARCH ARTICLE

Analysis of the differences between *Syntormon pallipes* and *S. pseudospicatus* (Diptera: Dolichopodidae): morphological and molecular data

Анализ различий *Syntormon pallipes* и *S. pseudospicatus* (Diptera: Dolichopodidae): морфологические и молекулярные данные

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Abstract. The recent catalogues of the family Dolichopodidae considered *Syntormon pallipes* (Fabricius, 1794) and *S. pseudospicatus* Strobl, 1899 as separate species. In this study, we used three approaches to estimate the significance of differences between the two species: molecular analysis (COI and 12S rRNA sequences), analysis of leg colour characters and geometric morphometric analysis of wing shape. The morphological data confirmed the absence of significant differences between *S. pallipes* and *S. pseudospicatus* found in the DNA analysis. Significant differences in the wing shape of two species have not been revealed. Hence, according to our data, there is no reason to consider *S. pseudospicatus* as a distinct species.

Резюме. В последних каталогах семейства Dolichopodidae *Syntormon pallipes* (Fabricius, 1794) и *S. pseudospicatus* Strobl, 1899 рассматриваются как отдельные виды. Для оценки значимости различий между ними были использованы три подхода: анализ молекулярных последовательностей (COI и 12S рРНК), анализ цветовых признаков ног и сравнение формы крыльев методами геометрической морфометрии. Отсутствие существенных различий между *Syntormon pallipes* и *S. pseudospicatus*, выявленное по результатам анализа ДНК, подтверждено морфологическими данными. Анализ формы крыла не выявил достоверных различий между двумя видами. Таким образом, по результатам нашего анализа нет оснований считать *S. pseudospicatus* самостоятельным видом.

Key words: morphology, mitochondrial DNA, ribosomal RNA, wing shape, Diptera, Dolichopodidae, *Syntormon*

Ключевые слова: морфология, митрохондриальная ДНК, рибосомальная РНК, форма крыла, Diptera, Dolichopodidae, *Syntormon*

ZooBank Article LSID: urn:lsid:zoobank.org:pub:1C9AF107-CE95-4C78-BE39-0C6DA789B38B

Introduction

The cosmopolitan genus *Syntormon* Loew, 1857 (Dolichopodidae, Diptera) contains more than 110 species including over 50 species described from the Palaearctic Region (Grichanov, 2017). Some of recent catalogues (e.g., Negrobov, 1991; Yang et al., 2006) considered *Syntormon pallipes* (Fabricius, 1794) and *S. pseudospicatus* Strobl, 1899 as different species. According to the key by Negrobov (1975), the two species of *Syntormon* are distinguished by the colour and struc-

ture of male hind leg. The diagnostic characters of *S. pallipes* are as follows: the hind basitarsus has two hooks fused almost to apex, thehind tibia has a dense row of bristles, the apical part of hind tibia and the base of hind tarsus are dark (Figs 1–2). *Syntormon pseudospicatus* is characterised by the following characters: the hind basitarsus has two hooks divided almost to the base, the hind tibia is covered with sparse bristles, the apical part of hind tibia and the base of hind tarsus are yellow (Fig. 3). However, these characters vary between individuals. At the same time, morphological differences including colour have not been found in the females of *S. pallipes* and *S. pseudospicatus*.

Grichanov (2001, 2013) considered *Syntormon pallipes* and *S. pseudospicatus* as the same species, with *S. pseudospicatus* as a junior synonym, because the two forms have no morphological differences in the male genitalia, while the variations in the hind leg coloration can be treated as colour forms. *Syntormon pallipes* and *S. pseudospicatus* are often collected together; therefore, they can hardly be regarded as subspecies. Below, we consider them as "forms".

The subspecies *S. pallipes longistylus* Grichanov, 2001 known from Madagascar, has the hypopygium morphology similar to that of both the forms but possesses the antenna very distinct in the length ratio of antennomeres; otherwise it is closer to *S. pseudospicatus* (Grichanov, 2001). It is worth noting that two more variations of *S. pallipes* were described, *S. pallipes uncitarsis* Becker, 1902 from Egypt and *S. pallipes immaculatus* Santos Abreu, 1929 from the Canary Islands, differing from *S. pallipes* only in the morphology of the male antenna and in the colour of the abdomen (i.e. in the characters that are not species-diagnostic); *S. pallipes immaculatus* and *S. pallipes uncitarsis* have been considered the synonyms of *S. pallipes* and *S. pseudospicatus*, respectively (material not examined in this study; see Yang et al., 2006; Grichanov, 2013). Both *S. pallipes* and *S. pseudospicatus* are very common in many countries of the Palaearctic, occurring also in the Afrotropical and Oriental regions.

In order to solve the long-lasting taxonomic problem in *Syntormon*, we have analysed molecular and morphological differences between the two forms. To estimate the significance of the described differences, we used three approaches: molecular analysis, shape analysis using geometric morphometry approach, and the comparison of the relative length of dark and yellow parts of the hind tibia.

For the molecular analysis, we used sequences of the mitochondrial gene encoding the protein cytochrome c oxidase subunit I (COI). In addition, sequences of the 12S rRNA gene from previous studies were also used (Caterino et al., 2000; Bernasconi et al., 2007).

Colour characters of legs are widely used in the systematics and identification of Dolichopodidae. However, the ranges of variability of these characters are still not studied in detail.

Geometric morphometry is a perspective method for quantifying shape variation based on the Cartesian coordinates of landmarks. Recent studies have demonstrated that the comparison of insect wing shape can be used for the allocation of phenetic units within species (Imasheva et al., 1995; Hoffman & Shirriffs, 2002; Vujić et al., 2013), for separating morphologically similar species (Schutze et al., 2012; Torres & Miranda-Esquivel, 2015) and for studying evolutionary trends in the development of shape characters (Pepinelli et al., 2013).

Material and methods

The molecular analysis was carried out using the material collected from Iran in 2017 and preserved in ethanol. To analyse morphological features, the materials representing seven populations were selected from the collection of the Department of Ecology and Systematics of Invertebrate Animals of the Voronezh State University. These materials have been collected in different years (from 1926 to 1994; see below).

Analysis of DNA sequences

The mtDNA COI sequences were analysed using the polymerase chain reaction (PCR), which was carried out by the Sintol Enterprise (Russia). The PCR was made using one microliter of extracted DNA, 0.5 microliter of each primer and one unit of Taq-polymerase. The PCR was carried out as follows: (1) initial DNA denaturation at 95°C for three min.; (2) 36 cycles, each consisting

Figs 1–3. Morphological variability of the hind leg in *Syntormon pallipes* and *S. pseudospicatus*. **1**, *S. pallipes* from Pskov Province of Russia; **2**, *S. pallipes* from Abkhazia; **3**, *S. pseudospicatus* from Iran.

of three steps: denaturation at 95 °C for one min., annealing at 48–54 °C for one min., and elongation at 72 °C for 1.5 min.; (3) final elongation at 72 °C for three min. The primers used for amplification and sequencing were taken as described in previous studies (Simon et al., 1994; Simmons & Weller, 2001; Bernasconi et al., 2007). The sequences obtained were aligned manually using MUSCLE multiple alignment program.

The analysed molecular matrix included sequences of the mtDNA COI (578 characters) gene and the 12S rRNA genes (354 characters) (see Electronic supplementary material 1). Using the key provided by Negrobov (1975), the population from the Iranian province Markazi was identified as *Syntormon pallipes*, while the population from the adjacent Iranian province Lorestan was identified as *S. pseudospicatus*.

Species of the genus *Campsicnemus* Haliday, 1851 belonging together with *Syntormon* to the subfamily Sympycninae (Negrobov, 1991; Yang et al., 2006; Grichanov, 2017), were selected from GenBank (Table 1) and used as an outgroup for the trees. The trees were built using the maximum parsimony model in TNT program (Goloboff et al., 2008). To find the most probable relationships of species, a consensus tree was constructed. In addition, we carried out phylogenetic reconstruction using the neighbour-joining (NJ) analysis on the COI dataset using PAUP software (Swofford, 2001). Reliability of inner branches was estimated by the bootstrap method based on 1,000 pseudo-replicates.

Morphological analysis

By means of the methods of traditional and geometric morphometry, we studied 142 males from the following seven populations: Bulgaria (28 specimens), Finland (8), Estonia (5), Tajikistan, the Kondara Gorge (38), Tajikistan, Dushanbe (32), Russia, the White Sea (14), and the Crimea (17 specimens). We identified all specimens as *Syntormon pallipes* or *S. pseudospicatus* using the key by Negrobov (1975).

Table 1. A list of dolichopodid species used in the molecular analysis.

Species	Locality	GenBank accession number	
		COI	12S rRNA
	Syntormon Loew, 1857		
S. pallipes (Fabricius, 1794)	Belgium, Denderhoutem	DQ456944[A]	DQ464801[A]
S. pumilus (Meigen, 1824)	Belgium, Froidfontaine	DQ456913[A]	DQ464869[A]
S. zelleri (Loew, 1850)	Austria, Kaunerleeuw	DQ456917[A]	DQ464761[A]
S. flexibilis Becker, 1922	Hawaiian Islands	KM282746[B]	KM283083[B]
S. denticulatus (Zetterstedt, 1843)	Belgium, Froidfontaine	DQ456910.1[A]	DQ464866.1[A]
S. pallipes (Fabricius, 1794)	Iran, Markazi	MK158951.1*	MK110582.1*
S. pseudospicatus Strobl, 1899	Iran, Lorestan	MK128993.1*	MK246923.1*
	Campsicnemus Haliday, 1851		
C. scambus (Fallén, 1823)	Belgium, Zonhoven	DQ456904[A]	DQ464849[A]
C. loripes (Haliday, 1832)	Belgium, Zonhoven	DQ456897[A]	DQ464836[A]
C. picticornis (Zetterstedt, 1843)	Belgium, Zonhoven	DQ456908[A]	DQ464856[A]

[A] Bernasconi et al. (2007). [B] Goodman et al. (2014). * The material of these two species was provided for the analysis by Azam Ahmadi (see Electronic supplementary material 1). The material of other species was taken from the GenBank.

1. *Variability in colour characters of the male hind tibia*

We placed the right hind tibia of each specimen on a glass slide and photographed it using a Levenhuk C NG microscope camera. The measurements of the length of tibia and the length of the dark section of tibia were made with the help of Adobe Illustrator CS3 program. The ratio of the tibia length to the length of the dark section of tibia was calculated. The effects of population and form (specimens with different colour characteristics of legs) on the relative length of the dark section of tibia were tested with the analysis of variance (ANOVA).

2. *Geometric morphometric analysis of the wing shape*

We cut off the right wings from the specimens, mounted them in Hoyer's medium on a glass slide and covered with a coverslip. Images of wings were taken using a Levenhuk C NG microscopic camera. We described the wing shape by eight landmarks located at the vein intersections (Fig. 4). The Cartesian coordinates of these landmarks were digitised using tpsDig 2.32 software (Rohlf, 2006). All wings were digitised twice in order to reduce measurement errors.

We computed the centroid size of wing, an isometric estimator of size, and used it as a characteristic of the wing size according to the method described in Zelditch & Swiderski (2004). Then the landmark configurations were scaled to a unit of the centroid size, superimposed so that the centroid of each specimen has coordinates (0, 0), and rotated so that the distance between the landmarks of all specimens became minimal by generalised least squares in the Procrustes superimposition method (Rohlf & Slice, 1990). A new set of variables contained the shape information (Zelditch & Swiderski, 2004). We analysed these variables by the methods of multivariate statistics using MorpholJ (Klingenberg, 2011) and Statistica 10 software.

We used one-way ANOVA to test the differences in the centroid size between the populations and specimens with different colour characteristics of legs. MANOVA (type III sums of squared and cross-products) on Procrustes residuals was performed to estimate the differences between the populations and two forms (*Syntormon pallipes* or *S. pseudospicatus*) in the wing shape. We used

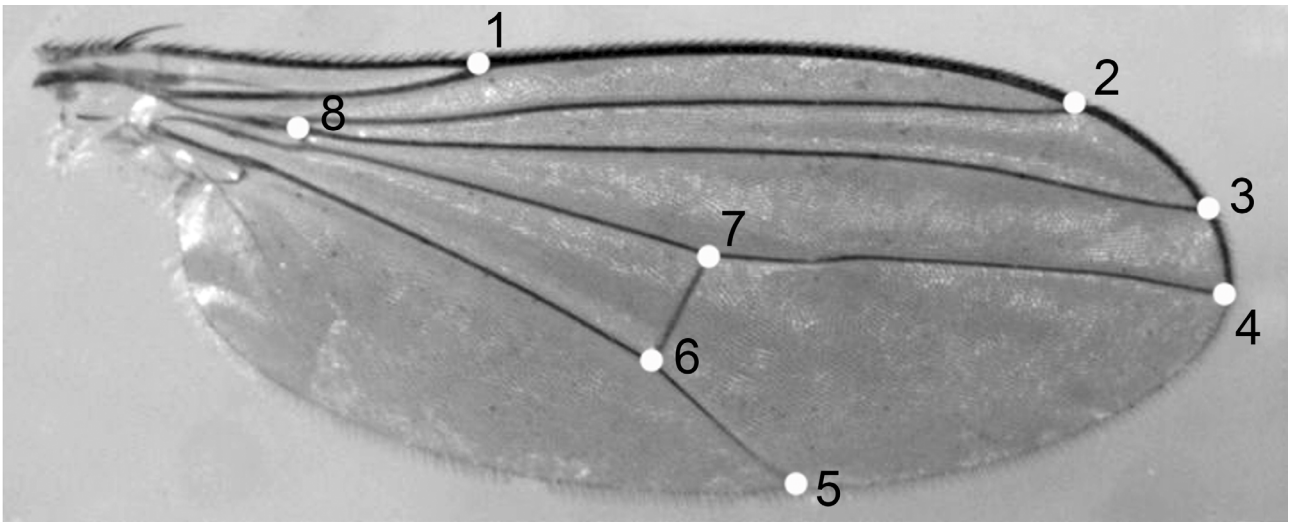

Fig. 4. Wing of *Syntormon pallipes* (male) showing the landmarks used in the study.

canonical variate analysis (CVA) to describe the differences between the groups, and performed principal component analysis (PCA) to investigate the trend of inter-group variation. The statistical significance of pairwise differences was tested using permutation tests with 10,000 replicas on Procrustes distances. Secondly, we used PCA for the detailed investigation and visualisation of the pattern of shape variation. To visualise the shape variation, we used thin-plate spline transformations of landmarks position.

To compare variability in the patterns of landmarks displacements between the forms and between the populations, we used Mantel test of matrix correlation (MC) (Mantel, 1967). The statistical significance of the results was assessed by the permutation test with 1,000 replicates; the null hypothesis stated that the matrices of different factors were completely dissimilar. For a more detailed investigation of the patterns of landmarks displacements, we calculated the angles between the first three principal components (PC1, PC2 and PC3) and tested the null hypothesis stating that the PC vectors for the variations between forms and populations were no more similar than the pairs of random vectors (Klingenberg & McIntyre, 1998).

To investigate the relationships between the populations and forms, the unweighted the pair-group method with arithmetic mean (UPGMA) hierarchical cluster analysis was conducted using Mahalanobis distances (Sneath & Sokal, 1973) obtained by pairwise comparison of analysed specimens from CVA.

Results

Molecular data

The most parsimonious tree describing the phylogenetic hypotheses was constructed after the analysis of molecular characters (tree length = 430 steps; *CI* = 0.8116; *RI* = 0.1884; *RC* = 0.6587) (Fig. 5). Strong similarity between *Syntormon pallipes* and *S. pseudospicatus* has been demonstrated with high statistical support. In addition, *S. pallipes* collected from Belgium is in the same cluster with two samples from Iran. The phylogenetic tree does not give the grounds for separation of *S. pseudospicatus* as a different species.

The result of NJ analysis (Fig. 6) is in agreement with the previous analysis, thus confirming our hypotheses. Of all species, only *Syntormon pallipes* + *S. pseudospicatus* group was reconfirmed in both analyses with strong support (Figs 5–6). The *Campsicnemus* species also formed a stable clade in both trees. Other species (*Syntormon denticulatus* Zetterstedt, 1843, *S. flexibilis* Becker, 1922, *S. pumilus* (Meigen, 1824) and *S. zelleri* (Loew, 1850)) did not demonstrate strong phylogenetic relationships with each other.

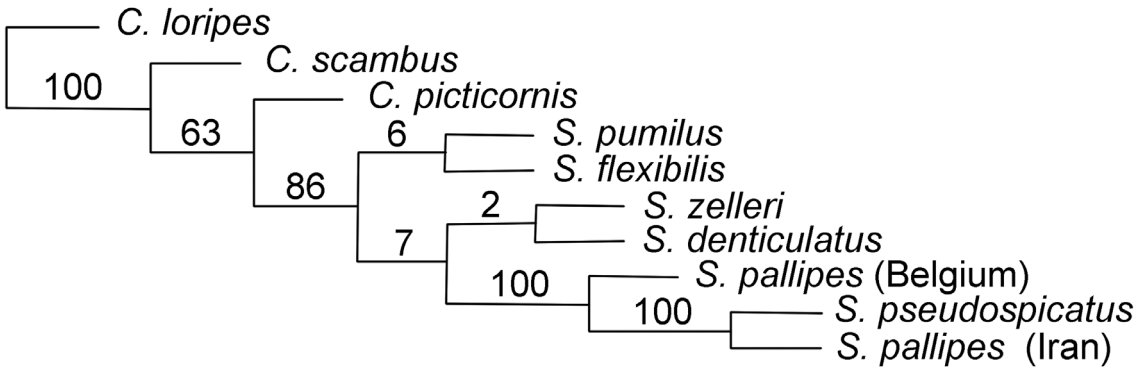

Fig. 5. Phylogenetic relationships between the species of the genus *Syntormon*: maximum parsimony consensus tree of 17 trees of the same length for the combined molecular dataset (COI and 12S). Bootstrap support values from 1,000 pseudo-replicates are indicated above the branches. Three species of the genus *Campsicnemus* were used as an outgroup.

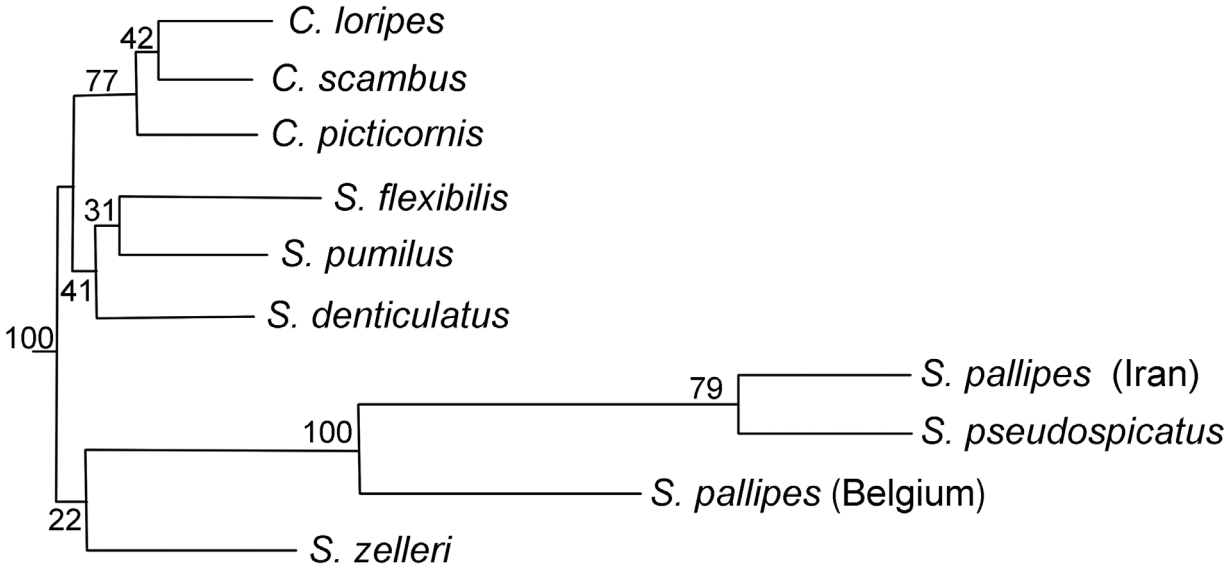

Fig. 6. Phylogenetic relationships between the species of the genus *Syntormon*: neighbour-joining tree from 1,000 bootstrap pseudo-replicates, obtained from the molecular dataset (COI). Bootstrap support values are indicated above the branches. Three species of the genus *Campsicnemus* were used as an outgroup.

Morphological data

The study of the male hind tibia coloration revealed that the ratio of the length of the dark part of tibia to the tibia length varied from 0 (in specimens from Bulgaria, the Crimea and the White Sea) to 0.49 (Tajikistan, the Kondara Gorge). The ANOVA analysis revealed that the differences in the length of dark part between the populations are statistically significant ($F = 69.10$, $P < 0.001$).

Significant differences in the wing size were found between the populations ($F = 52.95$, $P < 0.0001$), but were not found between the specimens with different hind tibia coloration, i.e. *S. pallipes* and *S. pseudospicatus* ($F = 0.35$, $P = 0.56$) (see Electronic supplementary material 2). The MANOVA analysis has demonstrated that the differences in the wing shape between the populations are highly significant (Wilk's lambda = 0.05, $F = 15.75$, $P < 0.0001$); however, the dif-

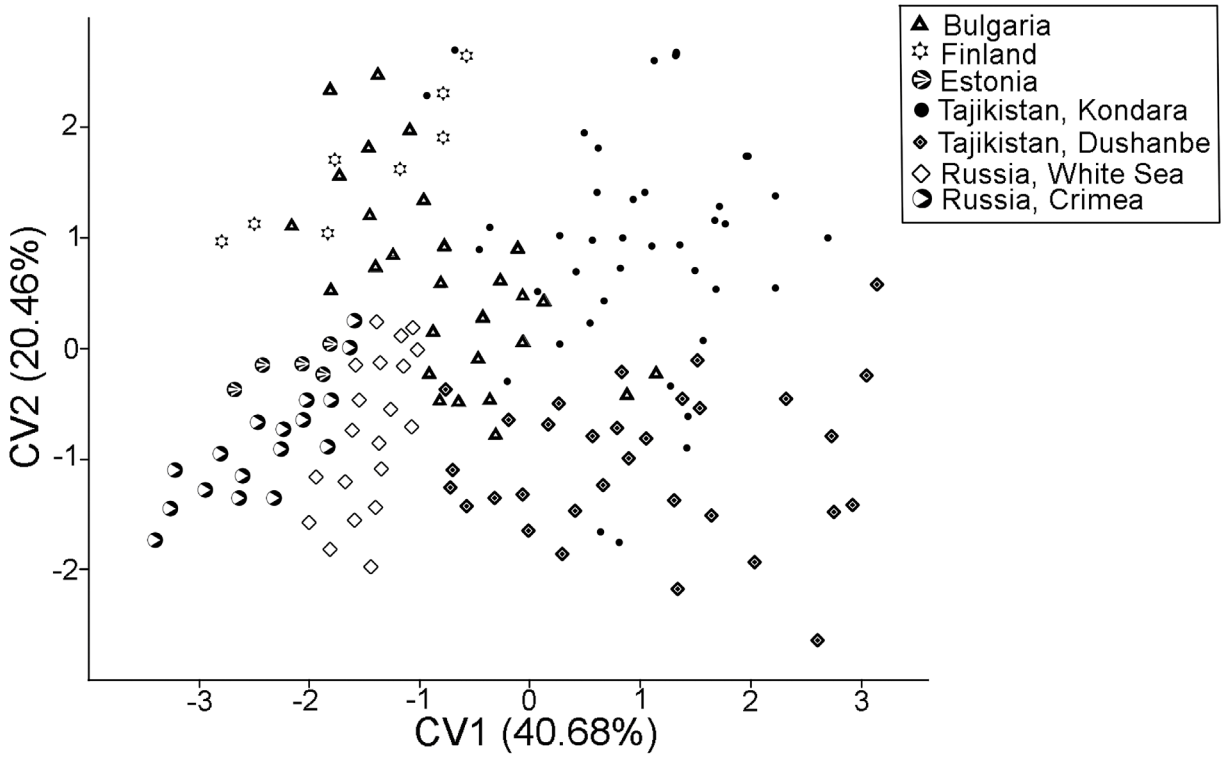

Fig. 7. Scatter plot of individual scores of the two first canonical axes showing variation in the wing shape in seven populations of *Syntormon pallipes*.

ferences between *S. pallipes* and *S. pseudospicatus* were less significant (Wilk's lambda = 0.28, F = 11.81, P < 0.0001). The significant population × form interaction suggests that the difference between the two forms has diverged among populations (see Electronic supplementary material 2).

CVA performed with "population" as a grouping variable resulted in two canonical variate axes accounting for about 61% of the overall wing shape variation (CV1 = 40.68%, CV2 = 20.46%). The scatter plot of the two first canonical axes showed that the specimens clustered into distinct groups belonging to the same population, including the populations that contained both the forms, *S. pallipes* and *S. pseudospicatus* (Fig. 7). Procrustes distances between the populations demonstrated significant differences (P < 0.001) ranging from 0.0348 (White Sea and Tajikistan, Dushanbe) to 0.0111 (Bulgaria and Estonia) (see Electronic supplementary material 3).

Then CVA was used to estimate the difference between *S. pallipes* and *S. pseudospicatus*. In this case, the scatter plot demonstrated broad overlap between the groups (Fig. 8). Procrustes distance between *S. pallipes* and *S. pseudospicatus* was 0.0164 (P < 0.001). The differences between these two forms in the populations were small and statistically insignificant: in Tajikistan, Dushanbe, Procrustes distance between the two forms was 0.0200 (P = 0.3832), in the population from the White Sea, 0.0261 (P = 0.1550) (see Electronic supplementary material 4).

The results of PCA revealed that the study populations could not be sorted into two clear allocated groups representing the forms that correspond to *S. pallipes* and *S. pseudospicatus* (Fig. 9). The first six principal components are accounted for more than 90% of the total shape variance. The PC1 was accounted for 33.83% of the total shape variation, followed by the variates accounting for 20.62% (PC2), 13.39% (PC3), 9.98% (PC4), 8.66% (PC5) and 4.00% (PC6) of the variance.

As demonstrated by thin-plate splines, PC1 essentially was associated with displacements of landmarks 2, 5, 6 and 7, and the landmarks exhibited a trend towards a more proximal position on the wing in the specimens from the White Sea and in some specimens from Bulgaria and the Crimea,

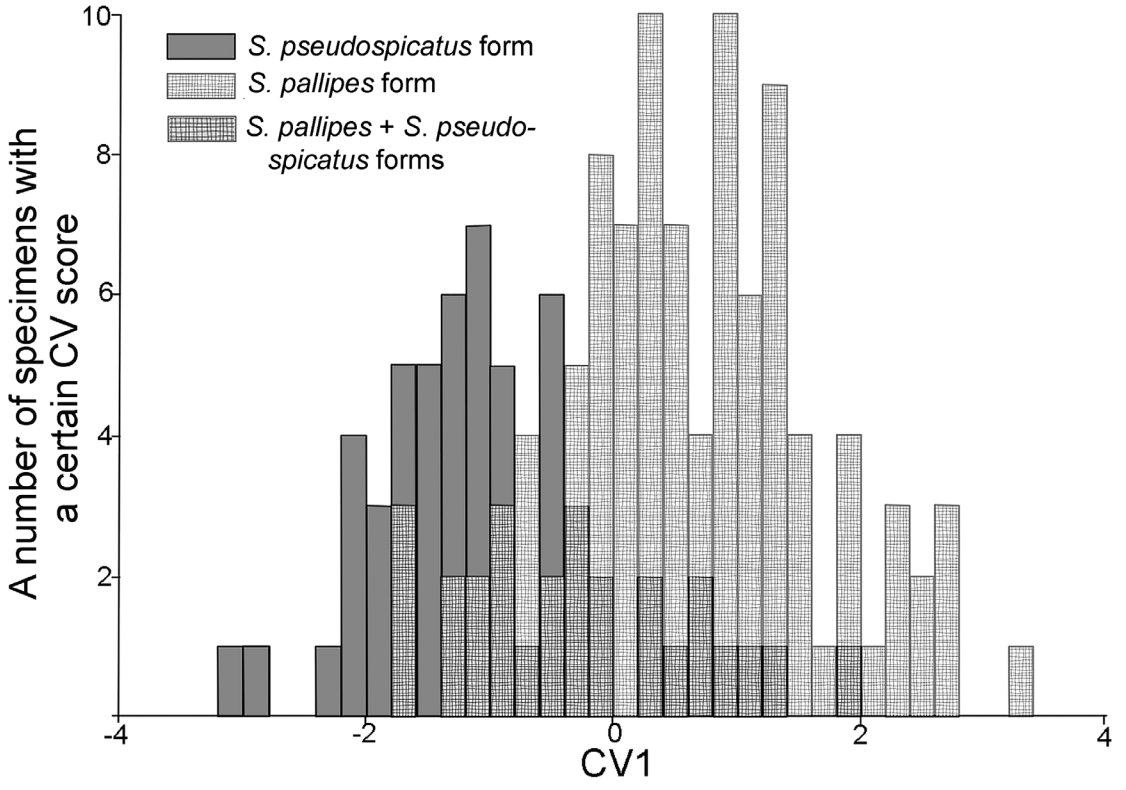

Fig. 8. Scatter plot of individual scores of the first canonical axes showing variation in the wing shape of *Syntormon pallipes* and *S. pseudospicatus*.

while LM1 shifted anteriorly. For PC2, the largest displacements were found in landmarks 1, 2, 5 and 6. These displacements resulted in particular in the increasing length of the posterior cross-vein. In addition, both PC1 and PC2 reflected wing shape variation in the relative width of the wing: the wing width increased from left to right and from bottom to top.

The matrix correlation between the covariance matrices (reflecting the effects between the population and between two forms) were very high and significant: MC = 0.98, $P < 0.0001$. The angles between the PC vectors indicated a high degree of their similarity: 14.61° between PC1 ($P < 0.00001$), 14.48° between PC2 ($P < 0.00001$), and 27.93° between PC3 ($P < 0.0001$).

The UPGMA dendrogram analysis has revealed that there are no clearly identified groups among the specimens examined (Fig. 10). Specimens of both *S. pallipes* and *S. pseudospicatus* were clustered together, as well as specimens from different populations.

Discussion

The genus *Syntormon* was considered in the subfamily Rhaphiinae for a long time (e.g., Becker, 1918; Parent, 1938) due to the elongate antennal postpedicel bearing an apical or subapical arista-like stylus. Later on, the genus was transferred to the subfamily Sympycninae based on the morphological features of the genitalia (Ulrich, 1980); this transfer was later supported by molecular data (Bernasconi et al., 2007). However, the latter subfamily still deserves a more detailed study. At present, it is considered as a polyphyletic assemblage of genera (Bickel, 2009), with *Syntormon* sometimes considered paraphyletic to the other genera of the subfamily (Lim et al., 2010).

In spite of the fact that Dolichopodidae as a whole is a well-studied family, the ranges of intraspecific variability in colour characters of legs remain poorly studied. *Syntormon pallipes* is a very common species in the tropics and subtropics of the Palaearctic, occurring often on agricultural

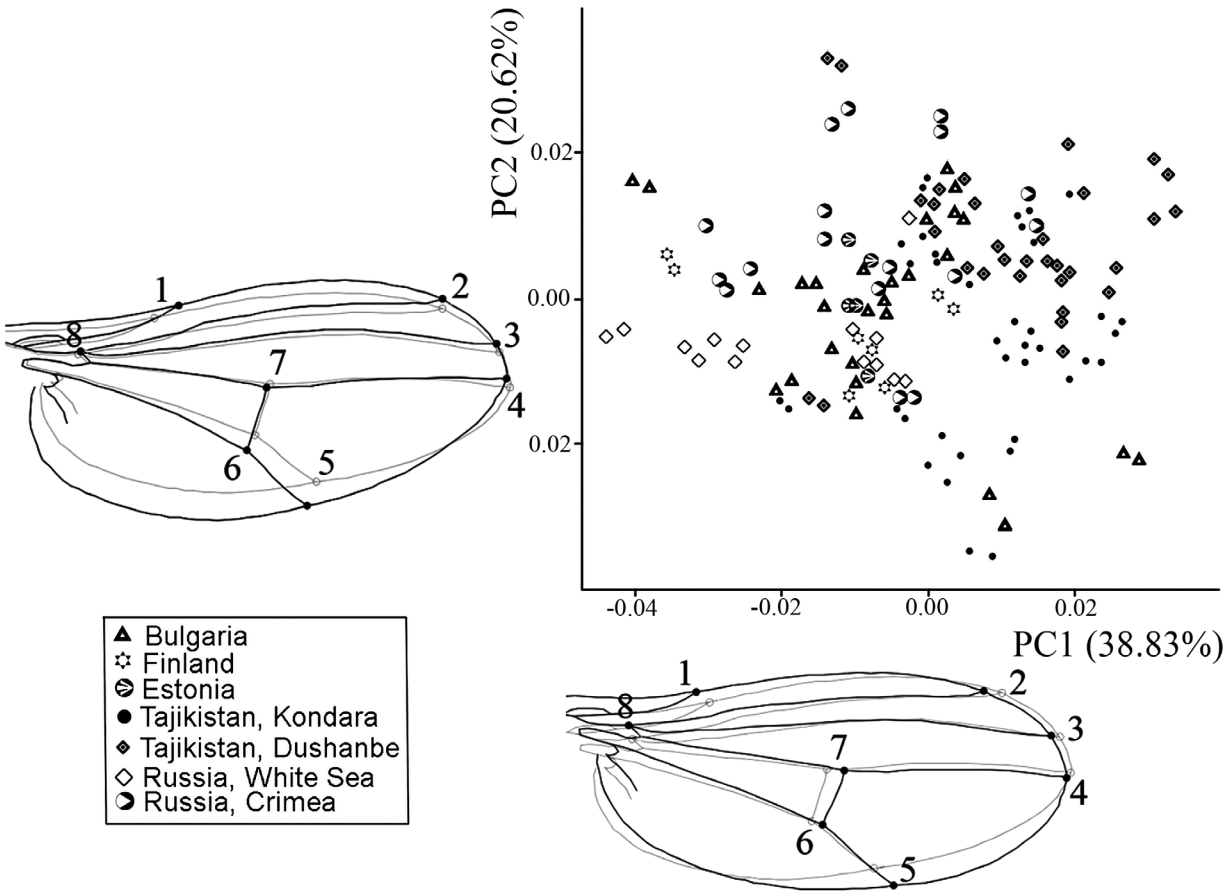

Fig. 9. Scatter plot of principal component analysis of the wing shape in *Syntormon pallipes* and *S. pseudospicatus*. Patterns of shape change along each PC are given at each axis (shown by thin-plate spline interpolation): grey outlines represent a wing with minimum PC-values, and black outlines represent a wing with maximum PC-values.

lands (e.g. Grichanov et al., 2010). Old taxonomic keys distinguish *Syntormon pallipes* from *S. pseudospicatus* by the coloration of the apical part of hind tibia and the base of hind tarsus being dark rather than yellow. Our molecular analysis has demonstrated that the difference between the specimens with different colour characteristics of legs, collected from the same territory, is smaller than the difference between the specimens of *S. pallipes* collected in different countries (Belgium and Iran). This fact itself may not be conclusive, but it is statistically significant so cannot be ignored. Another possible explanation for this fact is that the Iranian specimens (*S. pallipes* and *S. pseudospicatus*) belong to two monophyletic species. However, in such a case, we have to recognise that *S. pallipes* from Belgium is a different species, although being morphologically identical to *S. pallipes* from Iran.

The analysis of population variability in the relative length of dark part of the male hind tibia has revealed a variable degree of the tibia darkening in the studied populations. This result can support the existence of either different species or different forms of the same species. In order to exclude the first hypothesis, we have carried out the comparative analysis of the wing shape in specimens with different colour characteristics of legs. Geometric morphometric analysis of wings is a good approach for discriminating between species (e.g. Schutze et al., 2012), hence we compared the wing shape of specimens from different populations and specimens of both the forms.

The MANOVA and CVA analyses have demonstrated that significant differences in the wing shape are present between the populations of *Syntormon pallipes* + *S. pseudospicatus* from different territories (see results of ANOVA: Electronic sup-

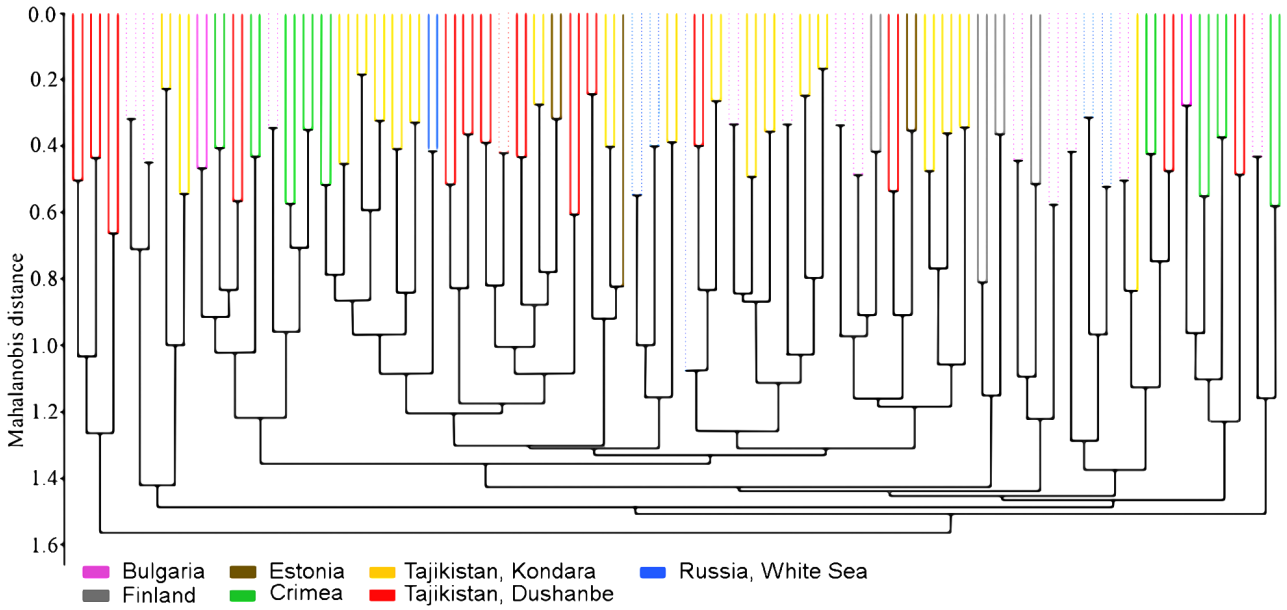

Fig. 10. UPGMA dendrogram of *Syntormon* from the studied populations showing phenetic relationships of wing shape based on the Mahalanobis distances between the specimens (*S. pallipes* – straight line; *S. pseudospicatus* – dash line).

plementary material 2; Figs. 7, 8). Similar interpopulation variability in the wing shape has been revealed in other Diptera species, for example, in *Drosophila serrata* Malloch, 1927 (Hoffmann & Shirriffs, 2002) and *D. melanogaster* Meigen, 1830 (Gilchrist et al., 2000), often showing high correlation with the geographical coordinates of a population habitat. However, the differences in the wing shape between the specimens of *Syntormon pallipes* and *S. pseudospicatus* (neglecting the general interpopulation variability) are not statistically significant. The variance analysis has not revealed reliable differences in the wing shape between the specimens grouped by leg coloration, i.e. the average wing of specimens with yellow hind tibia from various populations does not differ from the average wing of specimens with dark tibia. PCA showed that the variation in the wing shape was distributed in relatively large number of dimensions. We could say that there were two clearly separated species, if we observed at least one distinct trend of variation in shape. According to our study, no certain trends in the wing shape variation between the two forms can be identified. In addition, statistically significant similarity in the patterns of shape variation within one population and between the forms (according to the results of Mantel test and calculation of angles between the first three principal components) suggests that both types of variation are attributed to the same source. Therefore, there is no reason for the separation of *S. pseudospicatus* as a different species.

Further study of intraspecific variation in taxonomically significant characters can reassess their value in the systematics of Dolichopodidae and clarify features of evolutionary and morphological transformations in the family.

Addenda

Electronic supplementary material 1. COI and 12S rDNA genes of *Syntormon pallipes* and *S. pseudospicatus*. Pp. s117–s118.

Electronic supplementary material 2. The effect of population and form on wing size: results of ANOVA and MANOVA. P. s319.

Electronic supplementary material 3. Procrustes distance among wing shape in flies of the populations. P. s320.

Electronic supplementary material 4. Procrustes distance between the two forms in the populations. P. s321.

All electronic supplementary materials are available from: https://doi.org/10.31610/zsr/2019.28.2.305

Acknowledgements

The authors are sincerely grateful to Oleg P. Negrobov (Voronezh, Russia) and Azam Ahmadi (Arak, Iran) for providing the specimens for this study. Igor A. Belousov (Pushkin, Russia), Daniel Miranda-Esquivel (Bucaramanga, Colombia) and Mateus Pepinelli (Toronto, Canada) kindly reviewed an earlier draft of the manuscript. The work is performed within the Program for Basic Scientific Research in the Russian Federation for the Long-Term Period (2013–2020) approved by Order No. 2538-r of the Government of the Russian Federation, dated 27 December 2012.

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Received 17 May 2018 / Accepted 15 December 2019. Editorial responsibility: A.A. Przhiboro