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The relationship between *Otiorhynchus ukrainicus* (Korotyaev, 1984), *Otiorhynchus rotundus* (Marseul, 1872) and *Otiorhynchus smreczynskii* (Cmoluch, 1968): a hybrid speciation reconstruction

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Original article

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Otiorhynchus smreczynskii (Cmoluch, 1968) is a ubiquitous weevil species. Despite being common, the species is relatively unknown and most research has focused on its role as a plant pest. In our work, we compared *O. smreczynskii* and the closely related *Otiorhynchus rotundus* (Marseul, 1872) and *Otiorhynchus ukrainicus* (Korotyaev, 1984) based on molecular data. This was the first time that the molecular data of *O. ukrainicus* has been obtained. We used mitochondrial CO1 and the nuclear markers CAS and ArgK. Based on this data, we created phylogenetic trees, calculated genetic distances and conducted species delimitation using the PTP method. We also analysed the allozymes, proving that all the studied specimens of *O. smreczynskii* are hybrid triploids. The lower interspecific divergence (COI: 0.49%) indicates a recent speciation event. These results show that with a high probability, *O. smreczynskii* originated from *O. rotundus* and *O. ukrainicus*.

Key words: Coleoptera, Curculionidae, Otiorhynchini, *Otiorhynchus*, molecular markers, parthenogenesis, taxonomy.

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Otiorhynchus (Germar, 1822) is a genus of the subfamily Entiminae (Schoenherr, 1823) – one of the largest among the weevils (Thompson 1992). Due to its ample size, the classification (division into tribes and subtribes) is still unclear (Oberprieler *et al*. 2007). *Otiorhynchus* contains about 1500 species, mainly from the Palearctic region (Magnano 1998), whereas its monophyly is doubtful (Hlaváč 2011). All the species from this genus are phytophagous, with the larvae feeding on roots and adults on the foliage of their host plants. Their imago is mainly nocturnal (Wanat & Mokrzycki 2018). Many of them are significant plant pests, e.g. the black vine weevil (*O. sulcatus*) and strawberry root weevil (*O. ovatus*) (Keskin 2007).

The weevils studied in this work are: *Otiorhynchus smreczynskii*, *Otiorhynchus ukrainicus* and *Otiorhynchus rotundus*, which are species that belong to the subgenus *Podoropelmus* (Reitter 1912). They are very similar in appearance and it can be difficult to distinguish them without a proper key. Furthermore, *O. smreczynskii* and *O. rotundus* are known to feed

on the same plant species (mainly *Syringa vulgaris* and *Ligustrum vulgare*). This makes them common denizens of anthropogenic habitats such as parks and squares.

O. smreczynskii is quite well distributed throughout Europe (Korotyaev *et al*. 2018), where it inhabits lowland deciduous forests. Due to the use of its host plants for ornamental purposes, it is widespread in urban areas (Yunakov *et al.* 2018) and has been widely introduced.

Only parthenogenetic lineages of *O. smreczynskii* are known, whereas *O. rotundus* is a bisexual species that is found in central and south-east Europe, in lowland broadleaf forests near large rivers (Yunakov *et al.* 2018) and in cities (Korotyaev *et al.* 2018). *O. ukrainicus* occurs only in southern Ukraine (Crimea), and was discovered and described in 1984 by Korotyaev. It is known to be a bisexual species. Unfortunately, in his work, there is not much information regarding the feeding plants of adult *O. ukrainicus*. Korotayev found them in steppes and shrublands where the larvae were feeding on *Fragaria sp.* roots. Our samples of weevils from the territory of Ukraine (even *O. ukrainicus*) were all collected on lilacs (*Syringa vulgaris*); in Poland, however, they were picked from privets (*Ligustrum vulgare*).

It is important to mention that the features included in Korotayev's publication distinguishing *O. smreczynskii* from *O. ukrainicus* are quite subjective – such as the size (4.5-6.0 mm for *O. smreczynskii*; 3.0-4.5 mm for *O. ukrainicus*), as well as the number of dimples on the pronotum (12-14 for *O. smreczynskii*; 12-16 for *O. ukrainicus*) (Korotyaev *et al.* 2018) – and there is no evidence that these features cannot vary between them. This significant morphological similarity raises the question of the genetic relationship between the parthenogenetic O. *smreczynskii* and the other two Mendelian species.

In the Molecular Weevil Identification Project (Stüben *et al.* 2015) *O. smreczynskii* and *O. rotundus* were synonymised and considered to be two forms of one species (bisexual and parthenogenetic). Later, based on detailed morphological data (Gosik *et al.* 2023), this synonymising was deemed to be unsubstantiated. The genetic relationships between *O. smreczynskii* and *O. rotundus* have also been estimated based on the COI sequence variation (Gosik *et al.* 2023). However, there was no available molecular data from *Otiorhynchus ukrainicus*. In our study, we aimed to obtain this data and to conduct a comparative analysis of the mitochondrial and nuclear gene variations in all three species. Based on previous studies that demonstrated the hybrid nature of all the studied parthenogenetic weevils (Morozov-Leonov & Nazarenko 2017; Nazarenko & Morozov-Leonov 2018), we assumed the possible origin of *O. smreczynskii* to be a hybridisation of *O. ukrainicus* and *O. rotundus*.

Cases of the emergence of a new biological species capable of autonomous reproduction are widely known in nature, and have been studied by researchers for many years. As a rule, this speciation type is characteristic of plants (Levy & Feldman 2022). However, hybrid speciation is also known in animals, including vertebrates (Sanches *et al.* 2021). In particular, many parthenogenetic forms of animals are of a hybrid origin. Parthenogenesis, to avoid the problems associated with meiotic disorders in interspecific hybrids, has been proven to be very common among animals (Jaron *et al.* 2021; Sperling & Glover 2023).

Moreover, at least for some parthenogenetic hybrid forms, a repeated origin has been confirmed (Pongratz *et al.* 2003). Another argument in favour of this possibility is that different lineages within the same parthenogenetic species may have different mtDNA haplotypes (Cywinska & Hebert 2002). For this reason, parthenogenetic forms can be considered to be the most promising model species for studying hybridisation processes, especially in cases of successful speciation due to such hybridisation.

Weevils are especially rich in parthenogenetic species (Insecta, Coleoptera, Curculionidae) (Rożek *et al.* 2009). Therefore, it is very promising to study the morphological and genetic features of parthenogenetic forms of weevils, in comparison with their parental Mendelian species.

Materials and Methods

Materials collection

Forty-one specimens were collected across Poland and Ukraine from 2018-2020 (Table 1, Fig.1) using sweep nets or by direct collecting from plants. All the specimens were a priori identified to the species level, using the keys of Smreczyński (1966) or Dieckmann (1980) for weevil identification, and the scientific names were in accordance with Alonso-Zarazaga *et al.* (2017), Wanat & Mokrzycki (2018) and The Polish Biodiversity Information Network (PolBIN) database. All the individuals were preserved in 99.8% ethanol and stored at -20°C.

Table 1

Species collection and GenBank accession numbers

Sequence_ID	Organism	Country	Genbank accession numbers
O.smreczynskii_OSPL32	*Otiorhynchus smreczynskii*	Poland	OR819430
O.smreczynskii_OSPL42	*Otiorhynchus smreczynskii*	Poland	OR819431
O.smreczynskii_OS11	*Otiorhynchus smreczynskii*	Ukraine	OR819432
O.smreczynskii_OS12	*Otiorhynchus smreczynskii*	Ukraine	OR819433
O.smreczynskii_OS22	*Otiorhynchus smreczynskii*	Ukraine	OR819434
O.rotundus_OR31	*Otiorhynchus rotundus*	Ukraine	OR819435
O.rotundus_OR32	*Otiorhynchus rotundus*	Ukraine	OR819436
O.smreczynskii_OS41	*Otiorhynchus smreczynskii*	Ukraine	OR819437
O.smreczynskii_OS42	*Otiorhynchus smreczynskii*	Ukraine	OR819438
O.smreczynskii_OS43	*Otiorhynchus smreczynskii*	Ukraine	OR819439
O.ukrainicus_OU51	*Otiorhynchus ukrainicus*	Ukraine	OR819440
O.ukrainicus_OU52	*Otiorhynchus ukrainicus*	Ukraine	OR819441
O.ukrainicus_OU53	*Otiorhynchus ukrainicus*	Ukraine	OR819442
O.rotundus_OR61	*Otiorhynchus rotundus*	Ukraine	OR819443
O.rotundus_OR62	*Otiorhynchus rotundus*	Ukraine	OR819444
O.ukrainicus_OU71	*Otiorhynchus ukrainicus*	Ukraine	OR819445
O.ukrainicus_OU72	*Otiorhynchus ukrainicus*	Ukraine	OR819446
O.ukrainicus_OU73	*Otiorhynchus ukrainicus*	Ukraine	OR819447
O.ukrainicus_OU74	*Otiorhynchus ukrainicus*	Ukraine	OR819448
O.smreczynskii_OS81	*Otiorhynchus smreczynskii*	Ukraine	OR819449
O.smreczynskii_OS82	*Otiorhynchus smreczynskii*	Ukraine	OR819450
O.smreczynskii_OS84	*Otiorhynchus smreczynskii*	Ukraine	OR819451
O.smreczynskii_OS85	*Otiorhynchus smreczynskii*	Ukraine	OR819452
O.smreczynskii_OS86	*Otiorhynchus smreczynskii*	Ukraine	OR819453
O.smreczynskii_OS87	*Otiorhynchus smreczynskii*	Ukraine	OR819454
O.ukrainicus_OU91	*Otiorhynchus ukrainicus*	Ukraine	OR819455
O.smreczynskii_OSUM	*Otiorhynchus smreczynskii*	Poland	OR819456
O.smreczynskii_OSJG	*Otiorhynchus smreczynskii*	Poland	OR819457
O.smreczynskii_OSKR	*Otiorhynchus smreczynskii*	Ukraine	OR819458
O.smreczynskii_OSB	*Otiorhynchus smreczynskii*	Ukraine	OR819459
O.smreczynskii_OSBR	*Otiorhynchus smreczynskii*	Ukraine	OR819460
O.rotundus_ORP	*Otiorhynchus rotundus*	Ukraine	OR819461
O.rotundus_ORK	*Otiorhynchus rotundus*	Ukraine	OR819462
O.rotundus_ORB	*Otiorhynchus rotundus*	Ukraine	OR819463
O.ukrainicus_OU02	*Otiorhynchus ukrainicus*	Ukraine	OR819464
O.ukrainicus_OUOD	*Otiorhynchus ukrainicus*	Ukraine	OR819465

DNA extraction and analysis

DNA was extracted from the available body parts or whole insects, using a NucleoSpin tissue kit (Macherey-Nagel) with the procedure included in the manufacturer's instructions. Three DNA fragments – mitochondrial cytochrome oxidase 1 (COI) and two nuclear: 28S+ITS2 (CAS) and arginine kinase (ArgK) – were amplified with the PCR technique in a reaction mix consisting of 3 µl of DNA, 2 µl of buffer x10 with $MgCl_2$, 0.6 µl of dNTPs, 0.6 µl of primer F, 0.6 µl of primer R, 0.2 µl of Taq polymerase and 13 µl of molecular water. Amplification was conducted with the primers listed in Table 2.

The amplification was performed in a Mastercycler EpigradientS (Eppendorf) with the following

Fig.1. Geographical distribution of samples used in the studies.

Table 2

Primers used in the studies

Fragment	Primer name	Primer sequence (5'-3')	Reference
COI	LCO1490-JJ HCO2198-JJ	CHACWAAYCATAAAGATATYGG AWACTTCVGGRTGVCCAAARAATCA	Astrin & Stüben 2008
CAS	CAS5p8sFc CAS28sB1d	TGAACATCGACATTTYGAACGCACAT TTCTTTTCCTCCSCTTAYTRATATGCTTAA	Ji *et al.* 2003
ArgK	ArgKG1f ArgKG1r	ATYGGWATCTAYGCTCCYGAQYGC GCCCATWCGTCTCTTRTTRGAAAT	Hernandez-Vera *et al.* 2013

profile: 95°C for 4 min, 95°C for 30 s, 50°C for 1 min and 72°C for 2 min, followed by 35 cycles at 95°C each for 30 s, 50°C for 1 min, 72°C for 2 minutes, and a final extension step at 72°C for 10 min and 1 min at 10°C.

The effectiveness of the amplification was checked using electrophoresis (for 30 min at 100 V) in 1% agarose gel tinted with Midori Green Advance DNA Stain (NIPPON Genetics) (100 mg/ml). The purification of the PCR products was done with the EPPIC Fast kit (A&A Biotechnology). The purified PCR products were sequenced using the BigDye Terminator v3.1. Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, with the same primers that were used in the PCR reaction. The obtained sequencing products were cleaned up using the ExTerminator kit (A&A Biotechnology). Reading of the sequences was conducted by the Genomed company in Warsaw. Later, the sequences were blasted in BLAST NCBI to verify the species identification, exclude a potential contamination in the samples and to find similar homologous sequences (Altschul *et al.* 1990). The obtained sequences were deposited in the GenBank database (for the exact accession numbers, please see Table 1).

Alignment and phylogenetic analysis

The sequences were edited using BioEdit v.7.2.6 (Hall 1999) and then aligned homologically using ClustalX (Thompson *et al.* 1997). The most suitable model of nucleotide substitution was determined by using MrModeltest 2.3 (Nylander 2004) in conjunction with PAUP*4.0b (Swofford 2002). The HKY+GAMMA model was chosen for COI, while K80+GAMMA was chosen for CAS and ArgK. In the case of the phylogeny constructed from concatenated sequences (ArgK+CAS+CO1), the HKY+GAMMA model was chosen.

We used two methods to determine the phylogeny: Bayesian inference (BI) and maximum likelihood (ML). The BI was run using MrBayes 3.1 (Ronquist & Huelsenbeck 2003; Nylander *et al.* 2004). Each simulation was run twice, with 1 cold and 3 heated Markov chains for 10 million generations, and the trees were sampled every 1000 generations. Convergence of the Bayesian analysis was estimated using Tracer v. 1.7 (Rambaut *et al.* 2018). An appropriate number of sampled trees was discarded as a 'burn-in', and the remainder were used to reconstruct a consensus tree. The ML was run using RAxML v. 8.0.0 (Stamatakis 2014), with a bootstrap resampling of 1000 replicates via the rapid bootstrap procedure of Stamatakis *et al.* (2008) to assign support to branches in the ML tree. Bootstrap support values ≥ 70% were regarded as a significant statistical support. Each tree has two outgroups – *Liophloeus sp.* (own collection) and *Strophosoma faber* (HQ954144.1) for the CO1 marker; *Otiorhynchus desertus* (MT943848.1) and *Polydrusus fulvicornis* (HQ223025.1) for the CAS marker; and *Otiorhynchus auropunctatus* (HQ883883.1) and *Leptopius sp.* (LT799309.1) for ArgK marker. For the concatenated tree there was one outgroup: *Trachodes hispidus* (MK892216.1, KY110307.1, LN888877.1). All outgroup species belong to the same family: Curculionidae. Also, in the CO1 analyses, two sequences from the closely related *Otiorhynchus pauxillus* (KM441226.1, MK892061.1) were added. The trees were later visualised with TreeView 1.6.6 (Page 1996). Genetic distances were computed in MEGA11 (Tamura *et al.* 2021) using an uncorrected 'p' model.

Species delimitation was conducted with the Poisson tree process model (PTP) (Zhang *et al.* 2013). The analysis was conducted using the online service available at https://species.hits.org/ptp/. The rooted phylogenetic tree was uploaded and the analysis was done using the following defaults: 100000 MCMC generations, 100 thinning and 0.1 burn-in.

Allozyme extraction and analysis

We also studied the electrophoretic variability of such proteins as esterase (Es-1-7) and malate dehydrogenase (Mdh). The sample preparation, electrophoretic analysis of enzymes and the data interpretation were performed by standard methods (Mezhzherin & Peskov 1992). The thoracic segments of each weevil were frozen for 12 hours and the proteins were then extracted in a standard way using a Tris-HCl buffer (pH8.0). A vertical electrophoresis in 7.5% polyacrylamide gel (Maurer 1972) and acontinuous system of buffers (Tris-borate-EDTA, pH 8.3) (Peacock & Dingman 1968) were used for the protein separation. Electrophoresis was performed in a Helicon electrophoretic chamber with a current rate of 80 mA and a voltage of 200 V for 2.5 hours.

Results

DNA analysis

Using two phylogenetic methods and three different markers (two nuclear and one mitochondrial), trees of a similar topology were obtained. The phylogeny based on the COI alignment was congruent with the nuclear phylogenies. On each tree, indi-

viduals of *Otiorhynchus ukrainicus* formed a distant clade, but some of the representatives of this species were also grouped with *Otiorhynchus smreczynskii*. *Otiorhynchus rotundus* also formed separate clades, except for 2 individuals that were grouped with *O. smreczynskii* as well. (Fig. 2). It was similarly presented on the tree combining all markers (Fig. 3).

In the COI marker, the genetic distances ranged between 0-28%, and the maximum distance was detected between *Otiorhynchus ukrainicus* from Southern Ukraine and *Otiorhynchus rotundus* from Central Ukraine (28%). In the case of the concatenated markers, they ranged between 0-13%, and the biggest genetic distance was detected between *Otiorhynchus ukrainicus* from Southern Ukraine and *Otiorhynchus smreczynskii* from Eastern Ukraine (13%), as well as between *Otiorhynchus ukrainicus* from Southern Ukraine and *Otiorhynchus smreczynskii* from Southern Ukraine (also 13%) (SM.01, SM.02).

The PTP analysis, which was used to infer putative species boundaries on a given phylogenetic input tree, showed that based on the combined markers (CO1+ArgK+CAS), the estimated number of species ranged from 3 to 26. Maximum Likelihood solutions resulted in 4 species and Bayesian resulted in 21 species. However, they all were not strongly supported (support values of 0.059-0.728) (Fig. 3).

Allozyme analysis

An analysis of the variability of the diagnostic genes encoding enzymes showed that all the studied specimens of *O. smreczynskii* are hybrid triploids. Their genetic composition is either (*RUU*) 1. *O. rotundus* genome and 2. *O. ukrainicus* genomes, or vice versa (*RRU*).

The electrophoresis spectra of the enzymes showed this, and it was especially noticeable for malate dehydrogenase (Fig. 4). Its variability made it possible to uniquely identify individuals of both the parental species and the hybrid form. At the same time, the spectra of all hybrid individuals were asymmetric and demonstrated the effect of the gene dosage. This coincided with our previously obtained data (Nazarenko & Morozov-Leonov 2018).

The variability of these esterase-coding genes did not provide for the same reliable diagnosis,

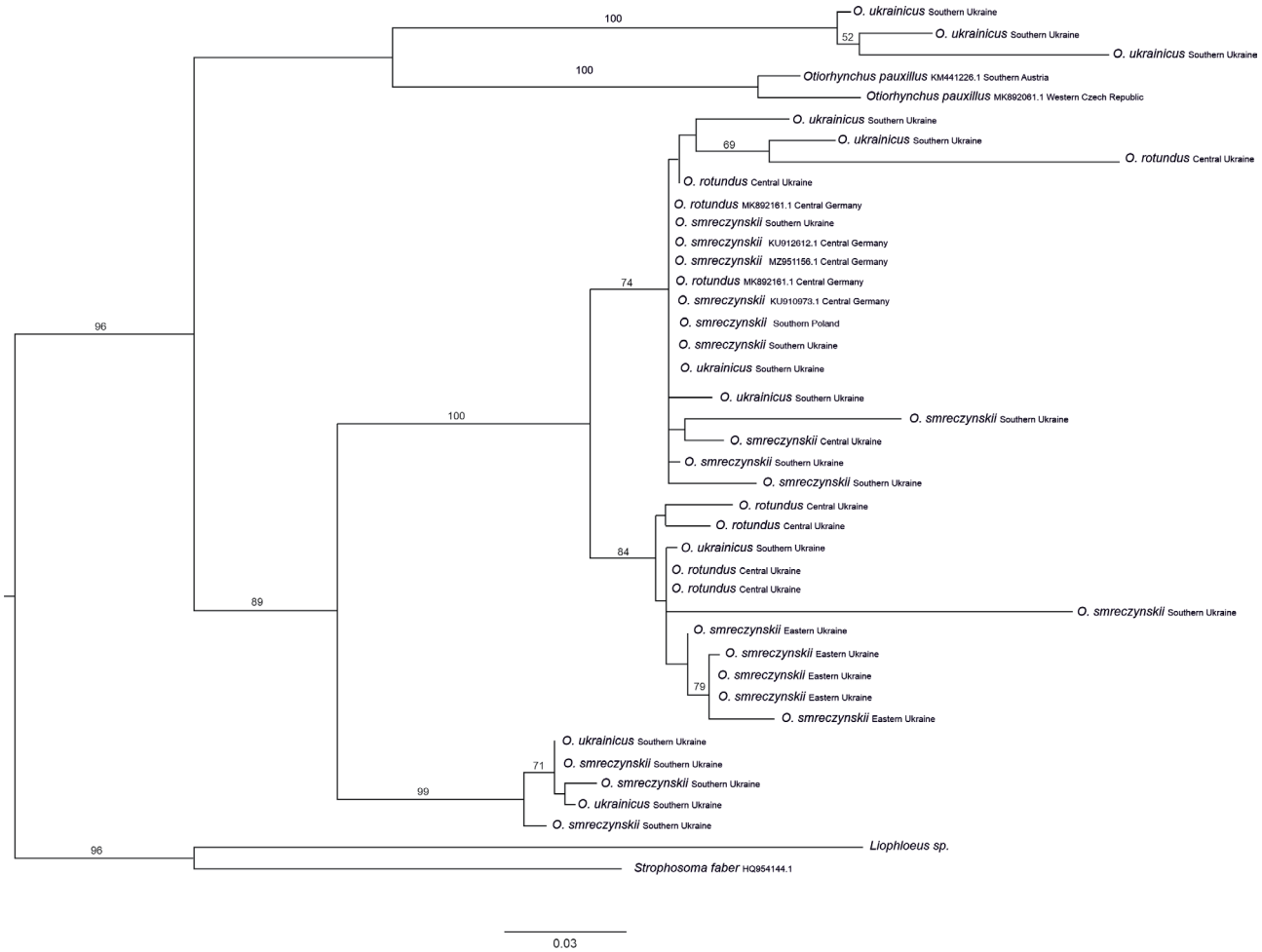

Fig. 2. Maximum-likelihood tree for the COI data. Numbers at nodes indicate the bootstrap value.

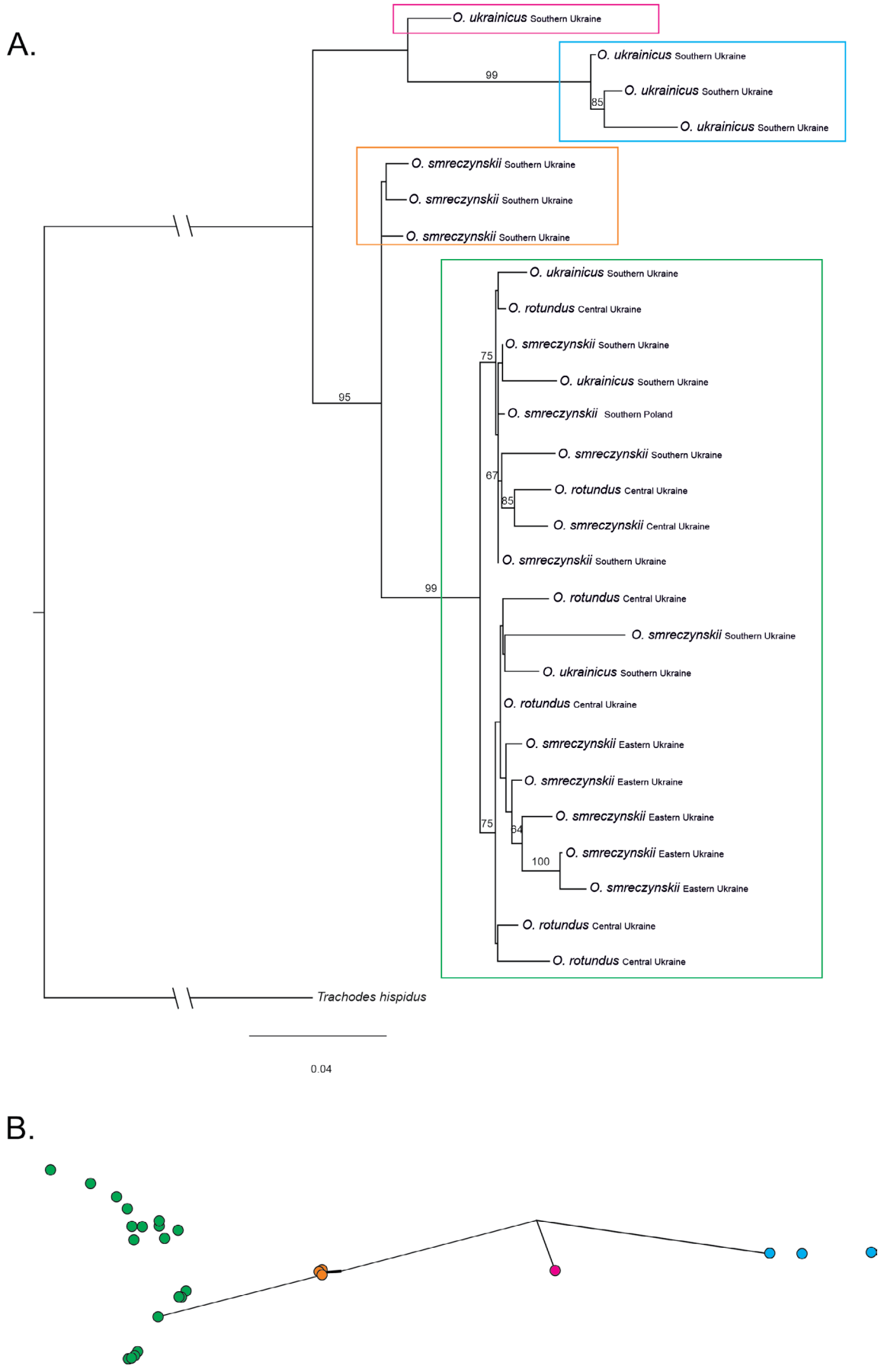

Fig. 3. A – Maximum-likelihood tree for the combined mitochondrial and nuclear data (CO1+ArgK+CAS). Numbers at nodes indicate the bootstrap values. B – Visualisation of PTP the species delimitation results. Different colours represent each clad.

Fig. 4. Electrophoresis spectra of malate dehydrogenase (MDH).

Table 3

Allelic frequencies of diagnostic genes and mtDNA types of the hybrid form *O. smreczynskii* and the parental Mendelian species from different regions of Ukraine

Gene	Allele	Species			
		O. u.	*O. s.*		*O. r.*
		S		E	C
Mdh	109	0.00	0.33	0.67	1.00
	125	1.00	0.67	0.33	0.00
	N	14	6	12	14
Es-5	80	0.07	0.00	0.33	0.00
	91	0.79	0.67	0.00	0.07
	100	0.14	0.33	0.67	0.93
	N	14	6	12	14
mtDNA		*U-type*		*R-type*	

O.u. – Otiorhynchus ukrainicus, O.s.– Otiorhynchus smreczynskii, O.r. – Otiorhynchus rotundus, S – Southern Ukraine, E – Eastern Ukraine, C – Central Ukraine, *U-type – O. ukrainicus type, R-type – O. rotundus type*.

except for the Es5 gene. This gene was almost diagnostic (Table 3). The electrophoretic spectra of the Es5 enzyme showed heterozygosity and asymmetry in all the specimens of *O. smreczynskii* (Fig. 5).

Discussion

Combined analysis of mtDNA and enzyme-coding gene variability

The obtained data showed that in the populations from southern Ukraine and Poland, the specimens of *O. smreczynskii* have mtDNA which, based on its nucleotide sequence, shows a close homology with the mtDNA of *O. ukrainicus*. Contrarily, in the case of the populations from Central and Eastern Ukraine, the specimens of *O. smreczynskii* seem to have mtDNA that is highly homologous with that of *O. rotundus*. This means that *RUU* triploid hybrids carry *ukrainicus*-like mtDNA variants (Table 3). Such triploids were found mainly in the populations from southern Ukraine (where they were sympatric with the corresponding parental species *O. ukrainicus*). The association of *RRU* triploids with populations of the second parental species (*O. rotundus*) was not so strict, but still noticeable.

O. smreczynskii origin

The result of the analysis of the three DNA markers showed that *O. smreczynskii* does not form a separate clade. This allows us to assume the origin of this species is from *O. rotundus* and *O. ukrainicus*. The obtained allozyme data showed the hybrid

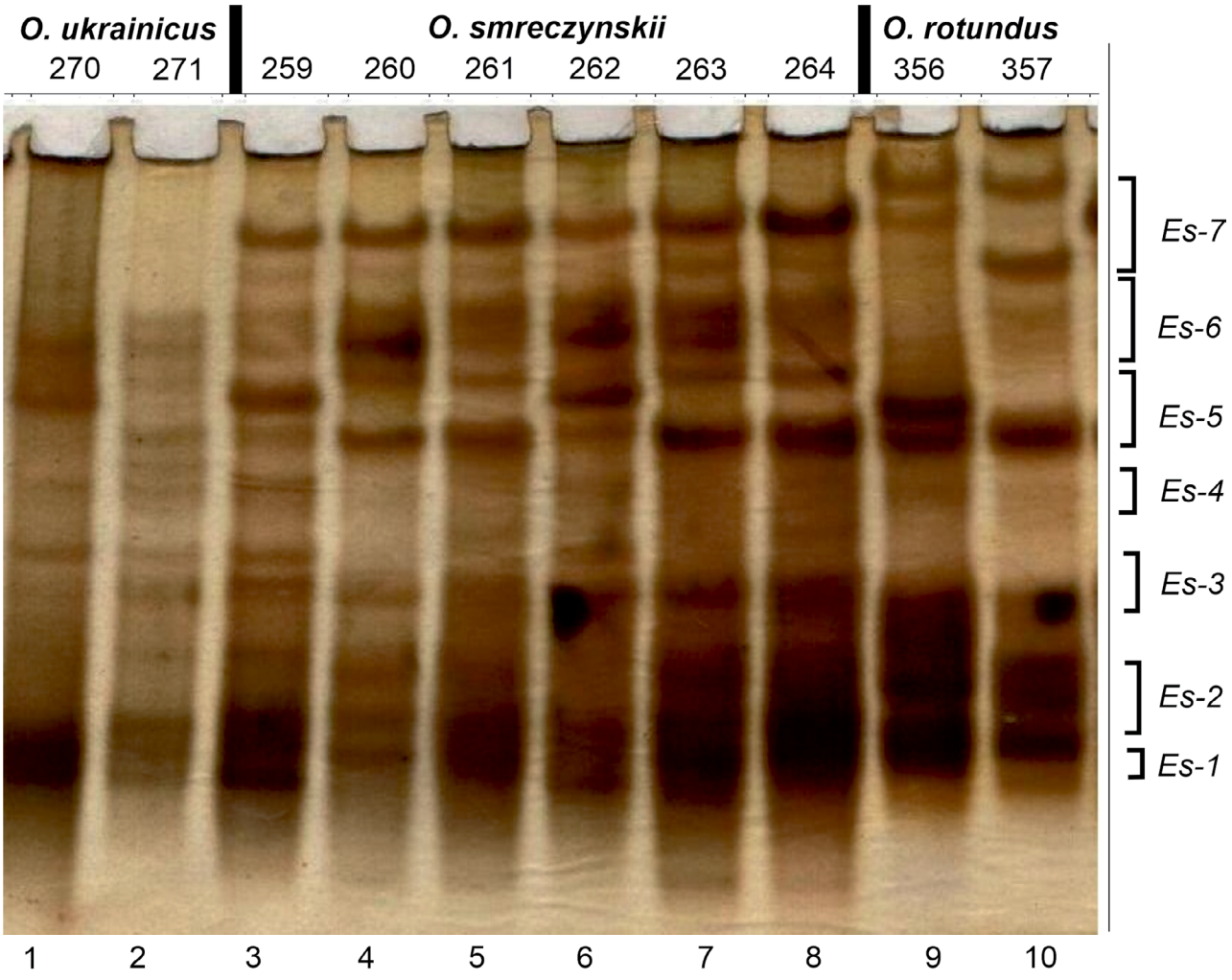

Fig. 5. Electrophoretic spectra of the Es5 enzyme.

nature of *O. smreczynskii*. DNA variability demonstrated that all *O. smreczynskii* weevils have DNA received from *O. ukrainicus* or *O. rotundus,* but do not have their separate DNA. Altogether, the results confirm that *O. smreczynskii* is a hybrid form derived from interspecies crosses between the *O. ukrainicus* and *O. rotundus* species. At the same time, hybridisation of the parental Mendelian species occurred many times, and gave rise to two types of hybrid triploids (*RUU* and *RRU*). Triploids originated mainly from females of the two parent species, which were more frequent in the region where the hybridisation took place. The distinction between *O. smreczynskii* and *O. rotundus* was also confirmed

by Gosik *et al.* (2023), where the morphology and CO1 marker were checked. At the same time, this single marker was not enough to separate them in the Molecular Weevil Identification Project (Stüben *et al.* 2015). The reason for this may be that single locus markers are not always informative enough (Collins & Cruickshank 2013). That is why in our work, we decided to add two nuclear markers to the mitochondrial ones.

The results are comparable with the data obtained in other studies of parthenogenetic species forms and demonstrate the possible ways of hybrid speciation. The literary data shows that the parthenogenetic forms known among animals do not have a single scenario of origin. Despite being widespread even among mammals, spontaneous hybridisation does not always lead to the emergence of a new genotypically distinct species (Adavoudi & Pilot 2022; Taylor & Larson 2019).

Most known parthenogenetic forms arose as a result of hybridisation. This kind of unisexual form occurs, i.e. among lizards or gastropods (Barley *et al.* 2022; Barley *et al.* 2021; Ryskov *et al.* 2017; Spangenberg *et al.* 2017; Moritz & Bi 2011; Johnson & Bragg 1999; Dybdahl & Lively 1995; Moritz 1991, Moritz 1983). However, there is also a known case of non-hybrid parthenogenesis (Johnson 1992). As a rule, parthenogenetic forms have a polyphyletic origin (Barley *et al.* 2022; Barley *et al.* 2021; Spangenberg *et al*. 2017; Moritz & Bi 2011; Johnson & Bragg 1999; Dybdahl & Lively 1995; Moritz 1991; Moritz 1983), although exceptions are also known (Ryskov *et al.* 2017). Finally, these organisms may have different genetic structures: they can be exclusively either diploids (Barley *et al*. 2021; Spangenberg *et al.* 2017), triploids (Dybdahl & Lively 1995; Moritz 1983) or even tetraploids (Moritz & Bi 2011). There are also cases of the detection of a mixture of 2n + 3n in parthenogenetic forms (Johnson & Bragg 1999). An additional factor that increases the likelihood of the rise of a unisexual form can often (though not always) be an infection with parasites. In the case of weevils, such a parasite is a bacteria of the genus *Wolbachia* (Mazur *et al.* 2016); while in the case of gastropods, it is a trematode of the genus *Leucochloridiomorpha* (Johnson 1992). Thus, our data allows us to evaluate how the case of hybridisation we studied compares to others already known. *Otiorhynchus smreczynskii* is a parthenogenetic form of a hybrid origin represented by triploid females in 100% of cases. Moreover, based on the information, we established that the parthenogenetic form *O. smreczynskii* arose in nature as a result of at least two acts of hybridisation of two Mendelian species. The parthenogenetic form from southern Ukraine resulted from hybridisation between *O. ukrainicus* females and *O. rotundus* males; while in the populations of central and southern Ukraine, the opposite situation occurred. It seems that such hybridisation was possible in the case of the migration of the males of one of the species into the range of the second. The hypothetical migration of *O. ukrainicus* males to the north and *O. rotundus* males to the south is the most plausible mechanism for the generation of two genetically distinct variants of *O. smreczynskii*.

However, the correlation between the geographic localisation of the studied specimens and the types of different DNA sequences is not 100 percent. This may indicate that migrations of weevils of the parental species could have also occurred in other directions. A complete reconstruction of the migration routes of both hybridising species will require more extensive research in the future. The issue of the directions and rate of possible migrations of *O. smreczynskii* specimens is also awaiting resolution (as is known, it is wingless, unlike the parental species). Extensive use of molecular methods will allow for a precise verification of the affinity between species (Freeland 2018). In our case, it was helpful to determine the correct relations among *Otiorhynchus*. At this stage, our study confirmed that *O. ukrainicus, O. rotundus* and *O. smreczynskii* are closely related, but firmly separated species.

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Author Contributions

Research concept and design: M.P.; Collection and/or assembly of data: M.P., S.M.-L., V.N.; Data analysis and interpretation: M.P., S.M.-L., V.N.; Writing the article: M.P., S.M.-L., D.L.-C.; Critical revision of the article: S.M., D.L.-C.; Final approval of article: S.M., D.L.-C.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary Materials to this article can be found online at:

http://www.isez.pan.krakow.pl/en/folia-biologica.html

Supplementary files:

SM.01. Genetic distance for CO1 marker.

SM.02. Genetic distance for combined mitochondrial and nuclear markers (CO1+ArgK+CAS).

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