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Research Article

Dysdera parthenogenetica sp. nov. (Araneae, Dysderidae): A Unique Case of Parthenogenesis in Spiders

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We studied the parthenogenetic lineages of the spider *Dysdera hungarica* (Araneae: Dysderidae). Based on our data, we consider them to constitute a separate taxon, *Dysdera parthenogenetica* sp. nov. Morphologically, the new species differs mainly by slightly reduced female copulatory organs. The ovaries contain meiotic cells, suggesting that automictic thelytoky occurs in this species. *D. parthenogenetica* sp. nov. colonised areas west of the ancestral sexual species *D. hungarica*, especially the Pannonian region; the distribution areas of these species show minimal overlap. The distribution pattern of *D. parthenogenetica* sp. nov. suggests that the obligate thelytoky in this species originated through geographic thelytoky. *D. parthenogenetica* sp. nov. has been found in a significantly larger variety of habitats than *D. hungarica*, including agroecosystems. Therefore, the parthenogenesis of *D. parthenogenetica* sp. nov. is associated with the ability to populate even habitats without tree or bush cover, often disturbed, which is unfavourable for other *Dysdera* species. According to the analysis of selected nuclear (ITS2) and mitochondrial markers (COI), *D. parthenogenetica* sp. nov. showed low genetic diversity (single COI haplotype and two closely related ITS2 haplotypes) in contrast to the ancestral *D. hungarica*. By separation of *D. parthenogenetica* sp. nov., *D. hungarica* becomes a paraphyletic species. *D. hungarica* is thus one of the first documented cases of paraspecies among spiders. Although *D. parthenogenetica* sp. nov. exhibits minimum genetic variation at the analysed molecular markers, it displays considerable karyotype diversity. The transition to parthenogenesis was accompanied by a decrease in diploid number through chromosome fusions. Karyotypes of *D. parthenogenetica* sp. nov. diverged considerably from those of *D. hungarica*. Potential hybrids between these species would likely produce gametes with defective genomes. There is also a behavioural barrier between these two taxa. Females of *D. parthenogenetica* sp. nov. refuse to mate.

Keywords: automictic thelytoky; chromosome; *Dysdera hungarica*; geographic parthenogenesis; karyotype; new species; nucleolus organiser region; paraspecies; phylogeography

1. Introduction

In thelytoky, one of the types of parthenogenetic reproduction, generations are formed by females arising from unfertilised eggs. This type of reproduction occurs in many animal groups. In some clades, it is more frequent within taxa with limited vagility than in highly mobile animals [1]. There are two hypotheses explaining this impact of vagility. First, to be fixed, the newly arisen parthenogen must be isolated from its sexual ancestor (destabilising hybridisation hypothesis [2]). Second, the better dispersal capacity provided by the parthenogenetic mode of reproduction does not represent any advantage in species where sexually reproducing counterparts have efficient dispersal ability. In such species, all environmentally suitable regions are already colonised by sexual populations that are more long-term stable than parthenogenetic lineages [1]. This could explain why parthenogenesis is so rare in the order Araneae (spiders): most spiders are excellent dispersers,

employing silk fibres as balloons for aerial dispersal [3, 4]. Among over 52,000 described species [5], parthenogenesis has been confirmed in only a few species. All parthenogenetic spiders are thelytokous. The cases of *Theotima minutissima* Petrunkevitch, 1929 (Ochyroceratidae) [6], *Triaeris stenaspis* Simon, 1891 (Oonopidae) [7], and *Dysdera hungarica* Kulczyński, 1897 (Dysderidae) [8] are best documented. Furthermore, Platnick and Duperre [9] reported some parthenogenetic lineages in *Heteroonops spinimanus* Simon, 1892 (Oonopidae). Finally, Lake [10] observed the emergence of spiderlings from unfertilised eggs in *Holconia insignis* Thorell, 1870 (Sparassidae). Several other spider species with unknown males have also been suspected to be parthenogenetic (e.g., *Coelotes troglocaecus* [11]).

In harvestmen, another arachnid order that, in contrast to spiders, lacks the capacity for aerial dispersal, 0.31% of species are known to be thelytokous (in spiders, the proportion of thelytokous species is 0.003% [12]). The best-documented cases of thelytoky in harvestmen are in the genus *Leiobunum*. Some Japanese *Leiobunum* species are known for automictic and facultative thelytoky [12]. In the automictic type of thelytoky, meiosis is maintained [1, 12].

Thelytoky has not been studied in detail in any spider to date. Therefore, we would like to contribute to understanding parthenogenesis in spiders. In this study, we focus on the haplogyne spider *D. hungarica* (Dysderidae). Haplogyne spiders are early-diverging groups of the infraorder Araneomorphae, in which females still have dead-end spermathecae, not yet flow-through as in the more derived groups referred to as entelegyne spiders. *D. hungarica* is the only native European spider proven to be parthenogenetic. Only thelytokous lineages occur in the western part of its distribution area [8, 13]. Dysderidae is the only spider family endemic to the Palearctic (a second such family might be the Fonteferreidae, but the separation of the Portuguese species *Fonteferrea minutissima* Wunderlich, 2023 into a separate family will have to be verified by further studies). In particular, dysderids occur throughout the Mediterranean, Near East and Central Asia, with few representatives in adjacent areas [5, 14, 15]. The vast majority of dysderids are endemic to small areas [16]. *Dysdera* is one of the most diversified spider genera in the Mediterranean. To date, 331 species have been described [5]. Moreover, the number of species is probably much higher, as suggested by the increasing number of new species described in the past few decades (see [5]). This genus often includes complexes of sibling species only distinguished by subtle morphological differences. Also, the copulatory organs, which are otherwise highly divergent among spider species [17], are very similar among the species of these complexes [18, 19]. Most *Dysdera* species are dietary specialists that feed on terrestrial isopods [20–22]. Dysderids are exceptional among araneomorph spiders due to their weak dispersal ability; they have never been observed to balloon [16]. To understand the evolution, current functioning, and taxonomic status of the parthenogenetic lineages, we analysed several aspects of their biology, including distribution, habitats and morphology. Through a study of molecular markers, we sought to answer whether these parthenogenetic forms are monophyletic and what their phylogenetic relationships are with related sexual taxa. We also investigated whether they have indeed completely lost the ability to reproduce sexually with males from sexual populations, specifically whether they have developed a behavioural precopulatory barrier in the form of a reluctance to mate, whether their copulatory organs have been reduced and whether their karyotypes are different from those of sexual individuals, which would indicate incompatibility of karyotypes of parthenogenetic lineages and sexual populations.

In the framework of our study, we also analysed the karyotype evolution of parthenogenetic lineages. Karyotype changes could accompany the transition to thelytoky and be involved in forming reproductive barriers between sexuals and parthenogens. Thelytokous species are frequently polyploid [23]. Moreover, they often exhibit highly dynamic karyotypes. Particular lineages of thelytokous species can differ by chromosome rearrangements (e.g., [24–29]) or even ploidy [29, 30]. Such karyotype diversification is promoted by their reproductive isolation. In addition to basic karyotype data, we also detected nucleolus organiser regions (NORs). Changes in the NOR position can reflect the operation of some chromosome rearrangements. These rearrangements can be involved in the formation of reproductive barriers.

The comprehensive data obtained allowed us to conclude that the parthenogenetic lineages studied are a distinct taxon, which we formally described as *Dysdera parthenogenetica* sp. nov.

2. Methods

2.1. Phylogenetic Analyses. Based on molecular data, we determined the phylogenetic relationships of parthenogenetic lineages with related sexual taxa. For phylogenetic analyses, the right leg IV was obtained from each analysed individual. The tissue was fixed and stored in 96% pure ethanol until ready for use. DNA was extracted using a NucleoSpin Tissue XS kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Two aliquots of the obtained DNA were stored at −20 °C. The extracted DNA was amplified as described by Literák et al. [31] using primers targeting two loci, the nuclear ITS2 locus and the mitochondrial COI locus. To amplify ITS2, we used the primers ApicITS2FW1 (5′-AACTCTGAGCAGTGGATCACT-3′ [32]) and RITS (5′-TCCTCCGCTTATTGATATGC-3′ [33]). To amplify COI, we used nested PCR with the outer primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′ [34]) and C1-N-2194 (5′-CTTCTGGATGACCAAAAAATC-3′ [35]) and the inner primers DysCO1fwIN (5′-TRGTRGGAACGGCTATAAGAG-3′) and DysCO1rvIN (5′-AAATAGGGTCTCCTCCACCCGC-3′) (both [18]). The DNA fragments were purified using USB Exo-SAP-IT (Affymetrix, Santa Clara, CA) and subjected to bidirectional Sanger sequencing using an ABI 3130 DNA Analyser (Applied Biosystems, Foster City, CA, USA). The resulting consensus DNA sequences were submitted to the GenBank database under the accession numbers KJ941210–KJ941224, KJ941226–KJ941234, KJ941236–KJ941244, KJ941246–KJ941248, KJ941261–KJ941270, KJ941274–KJ941275, KJ941277–KJ941279, KJ941281–KJ941298, KJ941300–KJ941302, KJ941309–KJ941310, KJ941312–KJ941315, and KJ941319–KJ941322 (see

Supporting Information 1). In several samples, we successfully amplified only one of the two tested DNA loci.

We imported the newly generated sequences of mitochondrial and nuclear DNA and representative publicly available sequences into the program MEGA5 (Pennsylvania State University, University Park, PA, USA), aligned them by ClustalW (University College Dublin, Ireland), and trimmed the aligned sequences. The trimmed ITS2 locus corresponded to nts 11–408 (411 bp) of KJ941232 of *D. hungarica*. The trimmed COI locus corresponded to nts 45–557 (513 bp) of KJ941282 of *D. parthenogenetica* sp. nov. The maximum likelihood fits of 24 nucleotide substitution models, which were performed as described by Řezáč et al. [18] with all sites used for the analyses, including the gaps. For each model, we calculated the Bayesian information criterion, Akaike information criterion (corrected), and maximum likelihood values. Following the determination of the best-fit model, we used the respective model to construct a phylogenetic tree. For the COI data, we used the Tamura–Nei model [36]. The non-uniformity of evolutionary rates among the sites was modelled using a discrete gamma distribution (+G) with 5 rate categories. For the ITS2 data, we used the Kimura 2-parameter model [37]. We employed the bootstrap procedure with 1000 replicates. For tree inference, we used the nearest-neighbour-interchange as the maximum likelihood heuristic method of choice, and the initial tree was generated using the neighbour-joining algorithm. We further calculated evolutionary divergence over sequence pairs within and between groups. The genetic distances were calculated as the number of base substitutions per site from averaging over all sequence pairs between groups. The analyses were conducted using the same models as the corresponding maximum likelihood trees. All positions containing gaps and missing data were excluded from the final analysis.

To validate the data obtained from the maximum likelihood analyses, we employed Bayesian inference. We converted the ClustalW alignments generated in MEGA5 to the Nexus format in Mesquite 3.04 to infer the tree topologies using a Bayesian approach. We then performed the Bayesian analysis in MrBayes 3.2.5 using the mixed model of nucleotide substitution. The Bayesian analysis included four Monte Carlo Markov chains for 10,000,000 generations and trees sampled every 1000th generation, with the average standard deviation of split frequencies not exceeding 0.015. We discarded the first 25% of samples as burn-in. After discarding the burn-in data, we used the remaining dataset to generate a 50% majority consensus tree with indicated posterior probabilities of branches. We visualised the resulting trees in FigTree 1.4.2. We obtained the following summary statistics for analyses performed: average standard deviation of split frequencies 0.0018 (ITS2) and 0.0017 (COI), the maximum standard deviation of split frequencies 0.007 (ITS2) and 0.011 (COI), average potential scale reduction factor 1.000 (ITS2) and 1.012 (COI), and maximum potential scale reduction factor 1.000 (ITS2) and 1.017 (COI).

2.2. Karyology. We compared karyotypes of parthenogenetic lineages and sexual populations to determine potential differences in their karyotypes. Furthermore, we analysed the karyotype evolution of parthenogenetic lineages. Chromosomes were prepared from one specimen belonging to the sexual population (Hungary, Vác) and 10 specimens belonging to five parthenogenetic lineages (Austria, Innsbruck; Czechia, Prague; Czechia, Brno-Hády; Hungary, Balatonfüred; Hungary, Pécs). In the specimen from the sexual population, the chromosome plates were obtained from the testes of an adult male. Preparations contained both mitotic and meiotic plates. In the parthenogenetic lineages, the chromosome preparations were prepared from ovaries, intestines, or the entire content of the abdomens of female nymphs or adult females. These preparations contained only mitotic plates except for sporadic meiotic cells from ovaries.

The chromosome preparation technique was based on Dolejš et al. [38]. Tissues were hypotonized in 75 mM KCl for 25 min and fixed two times (10 and 20 min) in ethanol: acetic acid (3:1). To make a cell suspension, a piece of fixed tissue was dissociated in a drop of 60% acetic acid on a slide using a pair of tungsten needles. The preparation was placed on a histological plate (40°C). The drop was moved by a tungsten needle until it almost evaporated. Slides were stained for 28 min with 5% Giemsa solution in Sörensen buffer (pH 6.8). Selected chromosome plates were observed under an immersion oil objective using a BX50 microscope (Olympus, Tokyo, Japan) equipped with a DP71 CCD camera (Olympus, Tokyo, Japan). The chromosomes from selected mitotic metaphases were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Relative chromosome length was estimated as a percentage of the total chromosome length of the haploid set.

Subsequently, preparations were incubated in xylene and benzine baths (1 min each) to remove the immersion oil, destained by incubation in methanol:acetic acid (3:1) for 3 min, and used for the detection of NORs via fluorescence in situ hybridisation (FISH). The FISH probe for the detection of NORs (18S rDNA region from *Dysdera erythrina* [Walckenaer, 1802]) was generated and labelled with biotin following Forman et al. [39]. Due the conservative nature of 18S rDNA sequence, the probe gives good results for other spiders and arachnids as well. After hybridisation, the probe was detected by streptavidin-Cy3 (Jackson ImmunoResearch, West Groove, PA, USA) with amplification of signals (biotinylated anti-streptavidin [Baria, Prague, Czechia], streptavidin-Cy3). Chromosomes were counterstained with 4′,6-diamidino-2-phenolindole (DAPI) and photographed using an Olympus IX81 microscope (Olympus, Tokyo, Japan) equipped with an ORCA-AG CCD camera (Hamamatsu, Hamamatsu, Japan). Images were pseudocoloured (red for Cy3, blue for DAPI) and superimposed using Cell^R software (Olympus Soft Imaging Solutions, Münster, Germany).

2.3. Behavioural Incompatibility. Based on mating experiments, we wanted to determine whether parthenogenetic females were willing to mate with males from sexual populations. Ten males and five females belonging to a sexual population (Slovakia: Hrušov) and five females belonging to a parthenogenetic lineage (Czechia: Prague-Ruzyně) were collected in August 2003. Five males were coupled with females belonging to the parthenogenetic lineage. As a control, five

males and females belonging to the sexual population were coupled. Each pair was placed in a Petri dish (diameter 33 mm) for an hour, and the behaviour (mating or avoidance) was recorded.

2.4. Measurements and Views, Scanning Electron Microscopy. To determine potential morphological differences between sexual and parthenogenetic individuals, we compared the dimensions of selected structures. The precision of morphometric measurements was 0.02 mm. The width of the carapace was taken at its widest point. The length of the gnathocoxa was measured from the tip to the base of the pedipalpal coxa. Measurements of the chelicerae were taken as specified in Řezáč et al. [19]. The lateral measurement of the length of the basal cheliceral segment did not include the proximal part, which is hidden within the carapace. The width of this segment was measured in the middle of the lateral side.

To compare selected microstructures of sexual and parthenogenetic individuals by scanning electron microscopy (SEM), specimens preserved in ethanol were used. The prosomas were isolated. The female genitalia were dissected, macerated in 5% KOH until the soft tissues were dissolved and washed in distilled water. Samples were then dried at room temperature, mounted on a stub, coated with gold and examined using SEM (JEOL 6380 LV).

2.5. Morphology of Female Copulatory Organs. Based on data on the morphology of female copulatory organs, we wanted to determine whether copulatory organ atrophy occurred in parthenogenetic females. *Dysdera* spiders possess a specific morphology of copulatory organs. We adopted the nomenclature used by Arnedo et al. [40, 41] for particular structures. Like other haplogyne spiders, *Dysdera* females lack external sclerotised genitalic structures (epigyne). The internal genitalic structure (vulva) is located in the anterior ventral side of the opisthosoma and opens to the exterior through the epigastric furrow. To investigate its morphology under a light microscope, the vulva was dissected and brightened using concentrated glycerol. It is composed of two main diverticles: the anterior diverticle, which includes the spermatheca and the copulatory bursa, and the posterior diverticle, a membranous pocket. The copulatory bursa is bound dorsally by a sclerotised crescent termed the dorsal arch and ventrally by a membrane called the ventral wall. The dorsal posterior margin of the dorsal arch is folded towards the uterus (dorsal fold), whereas the ventral wall is directly connected to the genital opening. In a relaxed state, the lateral walls of the copulatory bursa are flexuous like an accordion, where sclerotised and membranous parts alternate. Ventrally from the dorsal arch, there is another sclerotised arch (ventral arch) separated from the dorsal arch by a fold (ventral fold). The inner side of the ventral wall is longitudinally divided by a groove (medial groove), which anteriorly continues as a channel leading to the spermatheca. The spermatheca is a heavily sclerotised transversal hollow rod with muscles attached anteriorly and glands on its dorsal side. The posterior diverticle hangs on the sclerotised arched transversal bar with muscle anchors. In its middle part, the bar has a wide lobe that locks to the dorsal fold of the anterior part of the vulva, functioning through the action of muscles as a bursal valve.

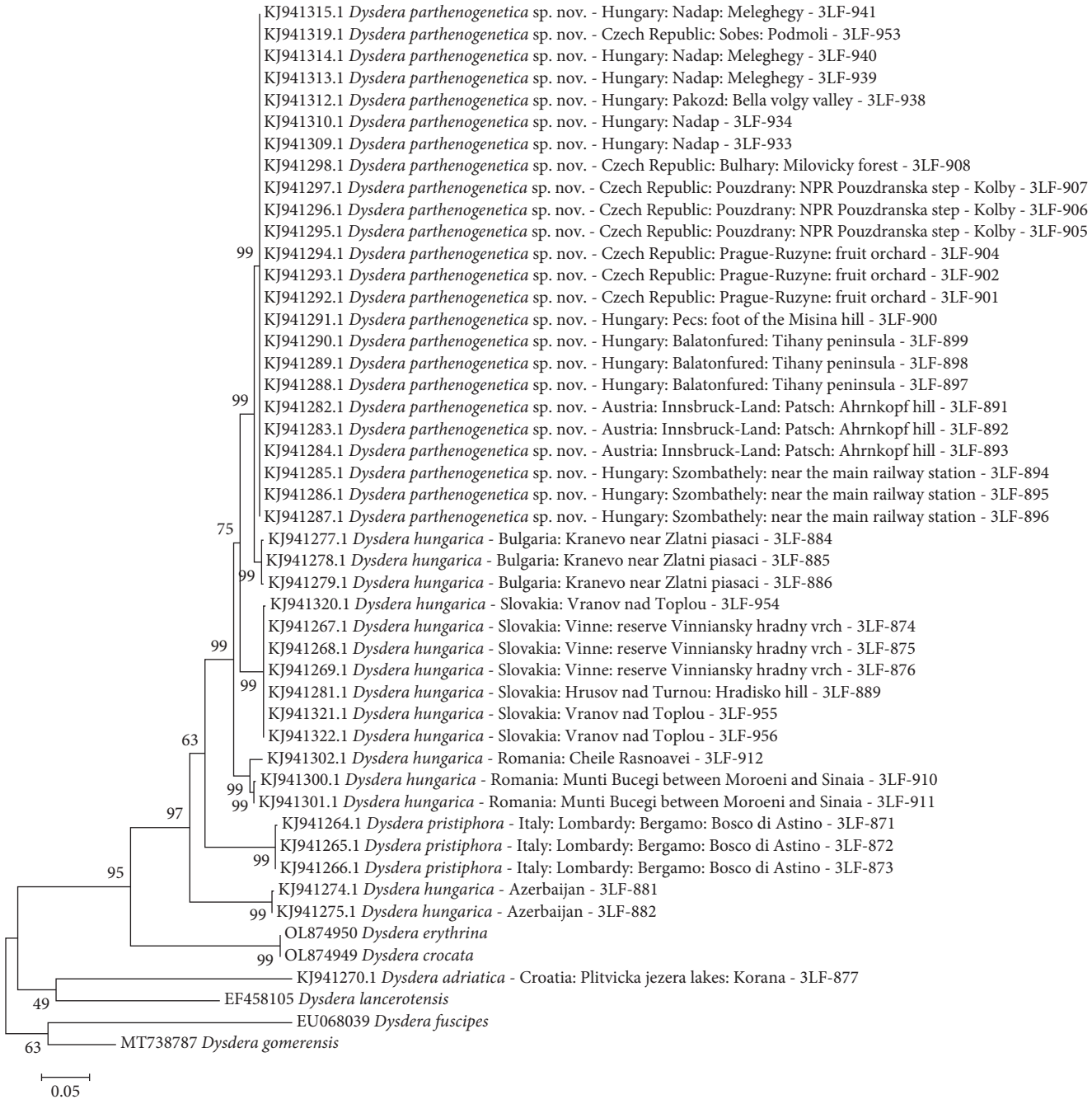
2.6. Distribution, Habitats and Natural History. We compared ranges of parthenogenetic lineages and sexual populations. Furthermore, we determined what habitats parthenogenetic lineages and sexual populations inhabit and whether parthenogenetic lineages inhabit a wider range of habitats. Based on information on distribution, we determined ranges of parthenogenetic lineages and sexual populations, characteristics of these ranges, and their overlap. Data on distribution, habitat, sex ratio (the presence of males was of particular interest), and phenology were obtained from faunistic databases and the literature, samples available in the revised collection, and observations at the visited sites (see Supporting Information 2).

The habitats were classified into the following categories: *Arable:* field, fallow, orchard, vineyard. *Bush:* dry bush, wet bush. *Forest:* dry forest, riparian forest, scree forest, park, forest-steppe, clearing. *Grassland:* dry meadow, damp meadow, steppe grassland, sandy steppe, forest fringe, pasture. *Wetland:* wetland, reed. *Rocky:* quarry.

3. Results

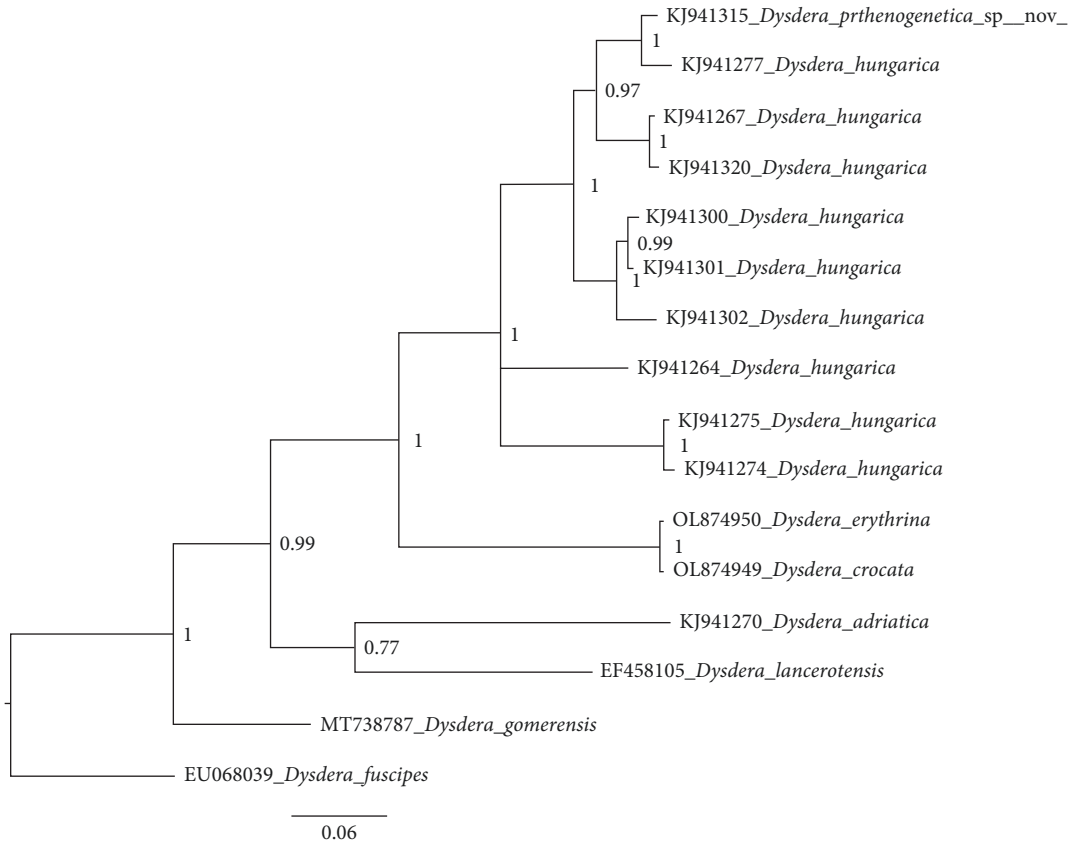
3.1. Phylogenetic Analysis. The analysis of selected DNA loci (COI and ITS2) jointly suggested a phylogeographic pattern: Pannonian populations of *D. hungarica* were variable in both analysed loci and differed from closely related *D. adriatica* Kulczyński, 1897. Note that samples of *D. hungarica* from Azerbaijan did not align with European specimens and formed a separate clade, with *D. pristiphora* Pesarini, 2001 interspersed between European and Azerbaijanian *D. hungarica*. *D. parthenogenetica* sp. nov. from multiple sampling sites was represented by individuals who were identical at their mitochondrial COI locus and formed two closely related haplotypes at their ITS2 locus, one of which was unique for *D. parthenogenetica* sp. nov. and another that was shared with *D. hungarica* (Figures 1 and 2). As mentioned above, the material from Azerbaijan formed a monophyletic clade. Concerning ITS2, this clade represented a sister group of central European *D. hungarica* and *D. parthenogenetica* sp. nov. Concerning COI, it was even less related to central European *D. hungarica* and *D. parthenogenetica* sp. nov. than *D. pristiphora*. This suggests it may represent an independent species. All analysed *D. parthenogenetica* sp. nov. individuals from the Pannonian lowland and surrounding sites of occurrence likely shared an ancestor. All the conclusions from maximum likelihood analyses were confirmed by Bayesian analyses of individuals representing all the analysed haplotypes (Figures 1B and 2B).

Analyses of evolutionary divergence over sequence pairs within and between groups confirmed that the within-group distances were lower in *D. hungarica* and *D. parthenogenetica* sp. nov. than distances between these species and among other closely related species. Estimated average evolutionary divergence in COI within *D. parthenogenetica* sp. nov. was <0.001 and within *D. hungarica* (except isolates from Azerbaijan) was 0.032. The distance between *D. hungarica* and *D. parthenogenetica* sp. nov. was 0.036; the distances of the two species to *D. pristiphora* were 0.103 both, and to *D. gomerensis* Strand,



(A)

Figure 1: Continued.



(B)

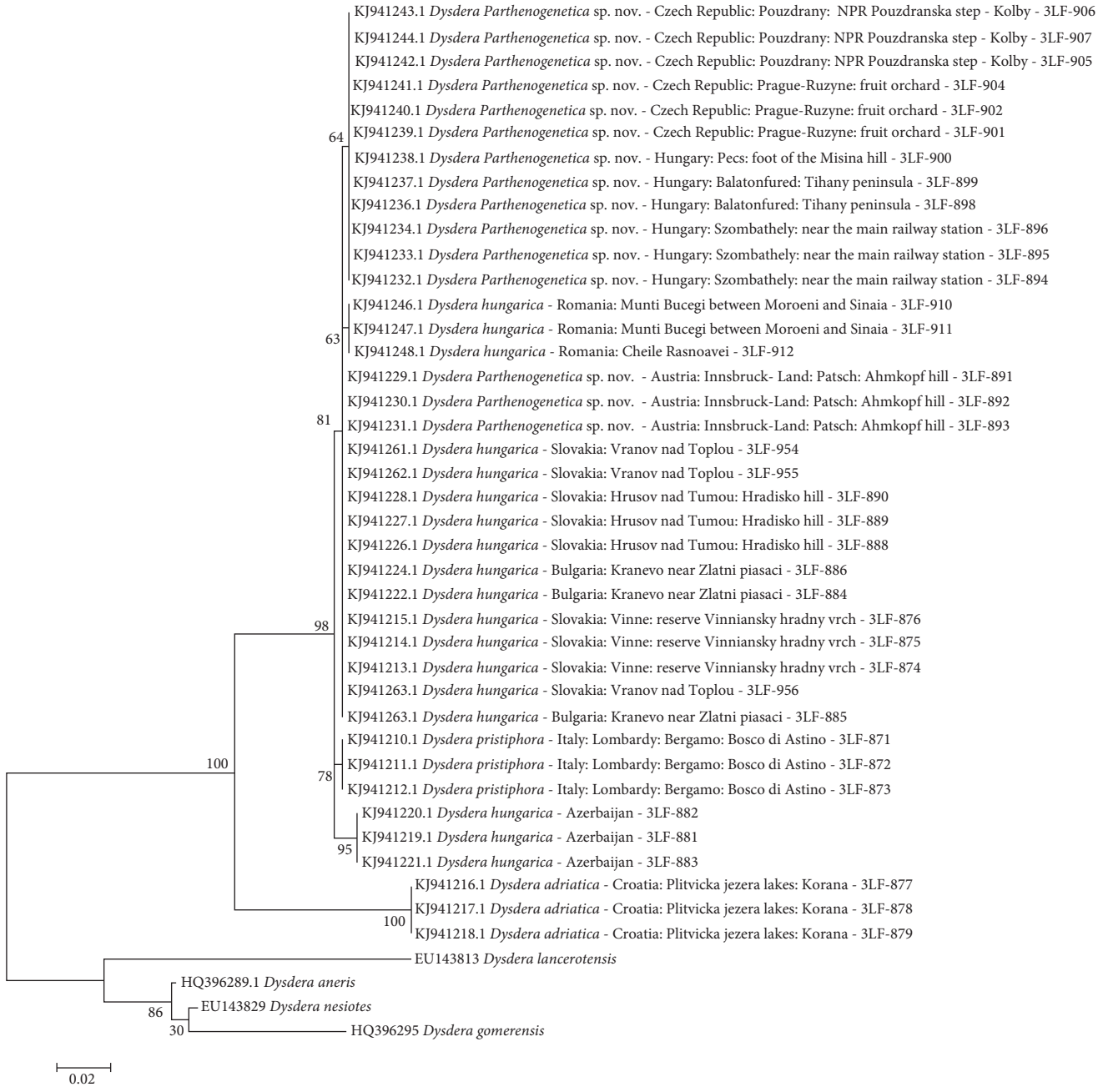
Figure 1: Phylogenetic reconstruction of the *D. hungarica* species complex based on the COI locus. (A) ML approach: Scale bar: 0.05 substitutions per nucleotide. Bootstrap = 1000 replicates. Sample identification codes, sampling sites and species are indicated. The substitution model used was Tamura–Nei, with nonuniformity of evolutionary rates among sites modelled using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites is evolutionarily invariable (+I). (B) Consensus tree resulting from the Bayesian analysis in MrBayes: Scale bar: 0.06 substitutions per nucleotide. The mixed substitution model was used. For the list of the sequences used in the phylogenetic analysis that were not generated in the course of the present study see the Supporting Information 3.

1911 were 0.211 and 0.202, respectively. The isolates of *D. hungarica* from Azerbaijan had a divergence of 0.121 to other *D. hungarica* isolates, 0.115 to *D. parthenogenetica* sp. nov. and 0.132 and 0.245 to *D. pristiphora* and *D. gomerensis*, respectively.

Concerning the ITS2 locus, the estimated average evolutionary divergence within *D. parthenogenetica* sp. nov. was 0.0009 and within *D. hungarica* was also 0.0009 (except isolates from Azerbaijan). The distance between *D. hungarica* and *D. parthenogenetica* sp. nov. was 0.0030; the distances of the two species to *D. adriatica* were 0.099 and 0.101, respectively, and to *D. gomerensis* were 0.210 and 0.212, respectively. The isolates of *D. hungarica* from Azerbaijan had a divergence 0.011 to other *D. hungarica* isolates, 0.013 to *D. parthenogenetica* sp. nov., and 0.102 and 0.204 to *D. adriatica* and *D. gomerensis*, respectively.

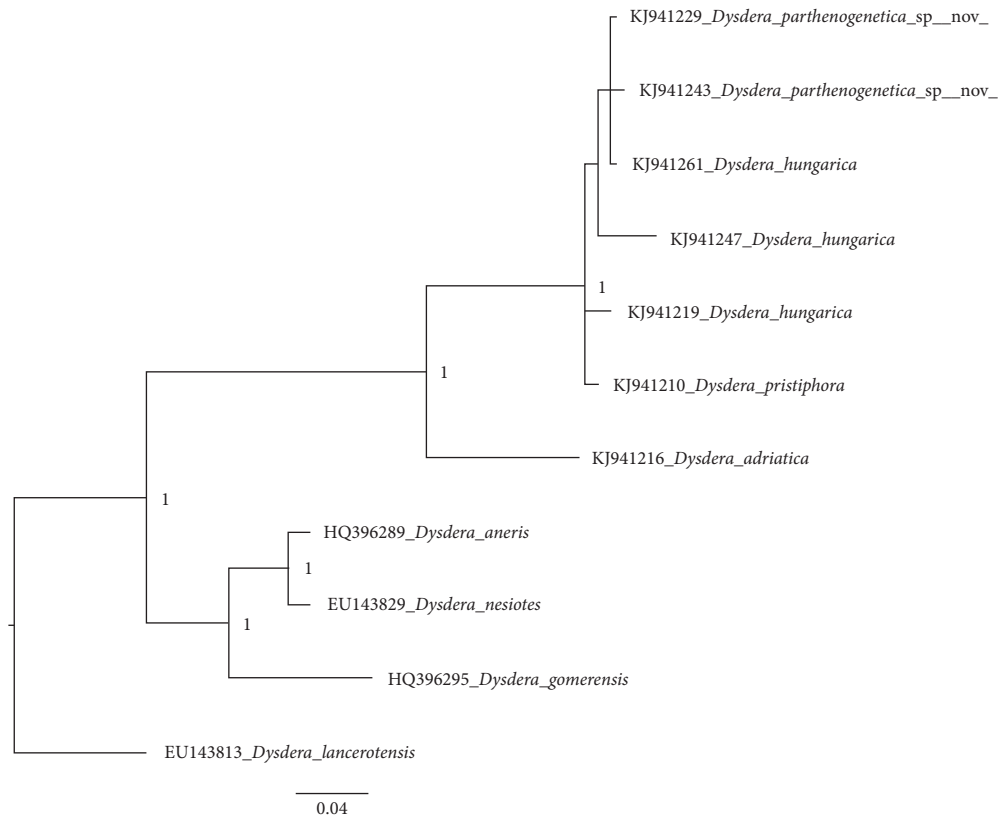
3.2. Karyology. The chromosomes of *D. hungarica* and *D. parthenogenetica* sp. nov. were holokinetic (= holocentric). The male karyotype of *D. hungarica* consisted of eight chromosome pairs decreasing gradually in size and a single X chromosome (Figure 3A), which was more than twice as long as the chromosomes of the longest chromosome pair (Table 1). One short chromosome pair contained an intercalar NOR (Figure 4A). This region manifested as an achromatic constriction in Giemsa-stained chromosomes (Figure 3A).

Concerning *D. parthenogenetica* sp. nov., we karyotyped five lineages. Karyotypes of this species exhibited considerable diversity. Karyotypes of the lineages from Innsbruck (Austria), Brno and Prague (Czechia) were formed by 14 chromosomes. The first three pairs were considerably longer than the remaining pairs, which decreased gradually in size (Figure 3B–D; Table 1). Karyotypes of the Czech lineages and Austrian lineage were very similar. The Austrian lineage differed from the Czech lineages slightly by the lengths of a few chromosome pairs (Table 1). Karyotypes from the Hungarian territory comprised lower numbers of chromosomes. The lineage from Pécs exhibited 12 chromosomes (Figure 3E). As in the Czech and Austrian lineages, the first three pairs were considerably longer than the remaining pairs. However, the difference between the third and fourth pairs was smaller than in these lineages. Moreover, the last three pairs were longer than in the Czech



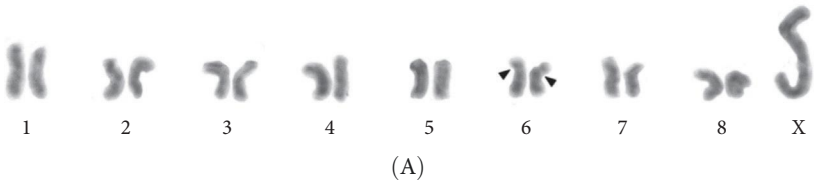
(A)

Figure 2: Continued.



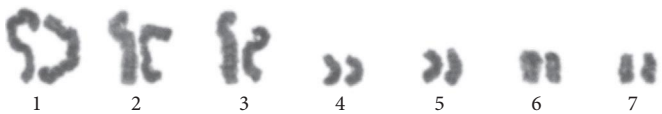
(B)

Figure 2: Phylogenetic reconstruction of the *D. hungarica* species complex based on the ITS2 locus. (A) ML approach: Scale bar: 0.02 substitutions per nucleotide. Bootstrap = 1000 replicates. Sample identification codes, sampling sites and species are indicated. Substitution model used: Tamura 3-parameter, with nonuniformity of evolutionary rates among sites modeled using a discrete Gamma distribution (+G) with 5 rate categories. (B) Consensus tree resulting from the Bayesian analysis in MrBayes: Scale bar: 0.04 substitutions per nucleotide. The mixed substitution model was used. For the list of the sequences used in the phylogenetic analysis that were not generated in the course of the present study see the Supporting Information 3.



(A)

(B)



(C)

(D) (E)

Figure 3: Continued.

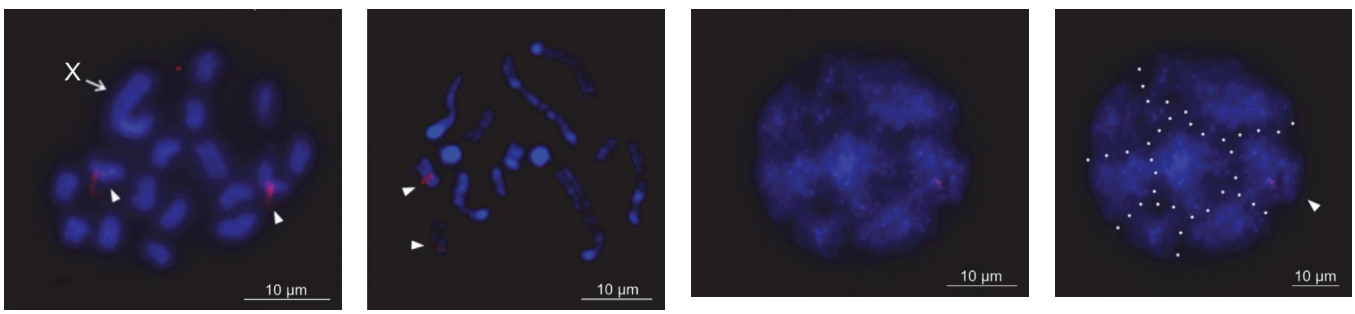
(F)

Figure 3: Karyotypes, based on mitotic metaphases. (A) *D. hungarica*, male, Vác (Hungary) ($2n♂ = 17$, X0). Chromosomes of the sixth pair contain subterminal constriction; (B)–(F) *Dysdera parthenogenetica* sp. nov., females; (B) Lineage from Innsbruck (Austria) ($2n♀ = 14$); (C) Lineage from Brno (Czechia) ($2n♀ = 14$); (D) Lineage from Prague (Czechia) ($2n♀ = 14$). Chromosomes of the fifth pair exhibit a subterminal constriction; (E) Lineage from Pécs (Hungary) ($2n♀ = 12$). One chromosome of the fifth pair exhibits a prominent constriction; (F) Lineage from Balatonfüred (Hungary) ($2n♀ = 10$). Arrowhead: chromosome constriction.

Table 1: Relative chromosome lengths of chromosomes of particular chromosome pairs and the X chromosome of *D. hungarica* and *D. parthenogenetica* sp. nov.

Species, site	1	2	3	4	5	6	7	8	X
D. hungarica♂									
Hungary, Vác (1/4)	11.8 ± 0.5	11.0 ± 0.2	10.4 ± 0.3	9.8 ± 0.3	8.8 ± 0.1	8.2 ± 0.3	7.9 ± 0.2	7.2 ± 0.3	25.3 ± 0.4
D. parthenogenetica sp. nov. ♀									
Austria, Innsbruck (1/4)	25.3 ± 1.2	20.8 ± 0.7	17.2 ± 0.7	11.3 ± 1.0	9.3 ± 0.4	8.5 ± 0.5	7.8 ± 0.2	—	—
Czechia, Brno-Hády (1/5)	26.3 ± 1.4	21.1 ± 0.9	17.6 ± 1.9	10.5 ± 0.7	9.3 ± 0.3	8.5 ± 0.4	6.8 ± 0.4	—	—
Czechia, Prague (6/9)	26.7 ± 0.9	20.5 ± 0.3	17.1 ± 0.9	10.6 ± 0.3	9.4 ± 0.2	8.5 ± 0.3	7.3 ± 0.4	—	—
Hungary, Pécs (1/1)	21.3	19.5	19.3	15.4	13.1	11.5	—	—	—
Hungary, Balatonfüred (1/3)	23.2 ± 0.5	21.2 ± 0.1	19.4 ± 0.4	18.9 ± 0.3	17.4 ± 0.5	—	—	—	—

Note: The numbers in brackets after the locality indicate the number of individuals and plates analysed.



(A) (B) (C) (D)

Figure 4: Detection of NORs (red) using FISH with an 18S rDNA probe. (A) *D. hungarica*, male, mitotic metaphase. One pair of chromosomes contains an intercalar NOR; (B)–(D) *D. parthenogenetica* sp. nov., female, Prague lineage; (B) Mitotic metaphase. One pair of chromosomes includes an intercalar NOR; (C) Diffuse stage. Note the considerable decondensation of chromatin; (D) The same plate, particular bivalents are delimited by a dotted line. One bivalent contains a NOR. Arrowhead: NOR-bearing chromosome (A, B) or bivalent (D). X: X chromosome.

and Austrian lineages (Table 1). The lineage from Balatonfüred displayed only five chromosome pairs, which decreased gradually in size (Figure 3F; Table 1).

Nucleolus organiser regions were detected in the Prague lineage, which exhibited the same pattern as *D. hungarica*. One short chromosome pair contained an intercalar NOR (Figure 4B), which manifested as an achromatic constriction in Giemsa-stained chromosomes (Figure 3D, E).

In addition to mitotic plates, preparations from ovaries of the two *D. parthenogenetica* sp. nov. specimens from Prague contained sporadic plates of prophase of the first meiotic division, namely, the diffuse stage. These nuclei contained seven bivalents, which were considerably decondensed. The sex chromosome bivalent did not differ in its condensation pattern from the other bivalents. One bivalent included a NOR (Figure 4C, D).

3.3. Behavioural Incompatibility. All tested couples of *D. hungarica* copulated. The mode of copulation coincided with those observed in other species of the genus *Dysdera* [20]. After the first encounter, the male jerked its body towards the female, tapping its two frontal pairs of legs to the female's frontal appendages. Then, the male put its first pair of legs on the female's carapace, got under the female's prosoma, embraced the female between the prosoma and opisthosoma by the second pair of legs, put the distal parts of these legs on the dorsal side of the female's opisthosoma and pushed it by its tarsi towards him. Then, the male massaged the epigastric region

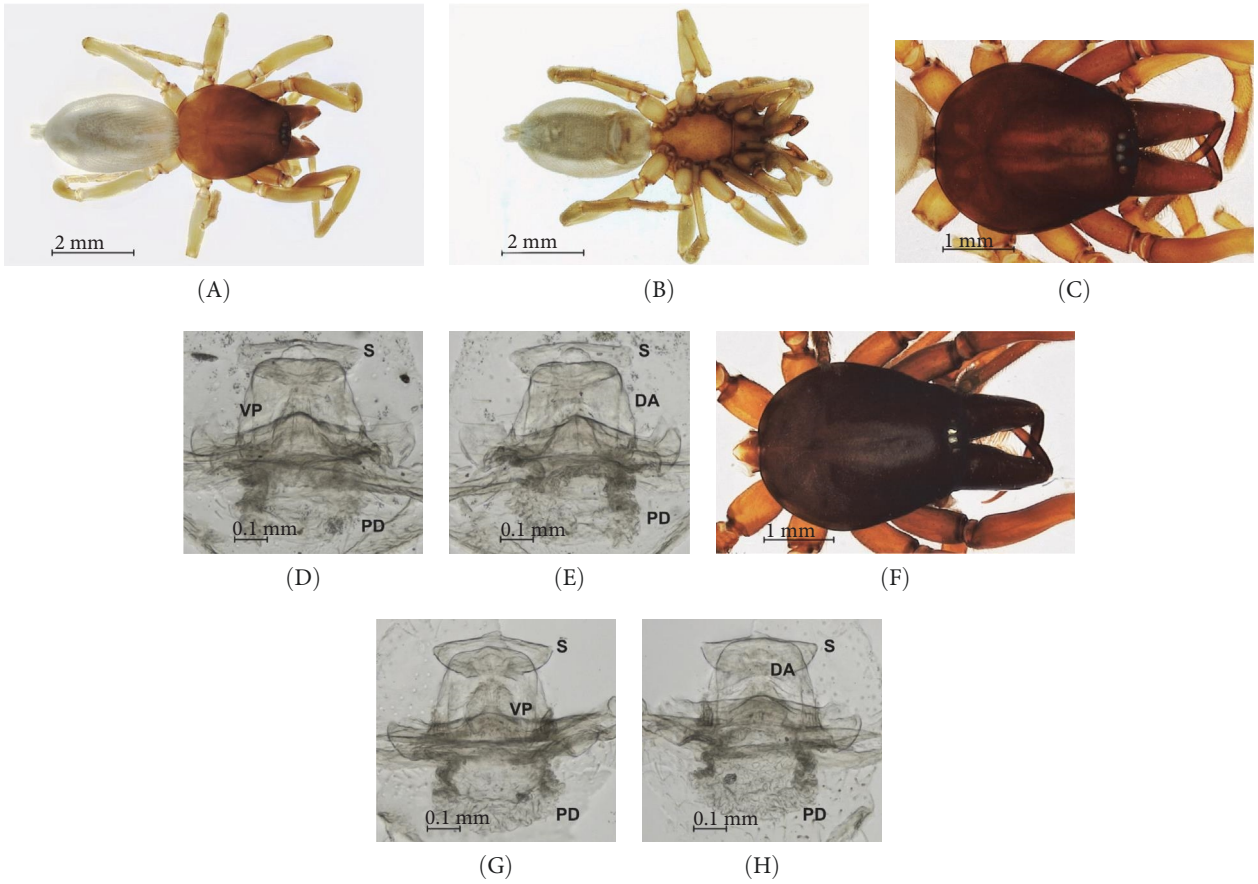


FIGURE 5: Morphology of specimens and vulva. (A–E) *D. parthenogenetica* sp. nov., Czechia, Prague-Ruzyně. (A, B) Holotype, dorsal (A) and ventral (B) views; (C) Prosoma; (D, E) Vulva, ventral (D) and dorsal (E) views; (F–H) *D. hungarica*, Slovakia, Hrušov; (F) Prosoma; (G, H) Vulva, ventral (G) and dorsal (H) views. DA, dorsal arch; PD, posterior diverticle; S, spermatheca; VP, chitinised bands on the ventral plate.

of the female's opisthosoma by chewing movements of the chelicerae and screwing movements of the pedipalps. After that, the male inserted its bulbi simultaneously into the female's bursa copulatrix in front of the epigastric furrow and made screwing movements with their pedipalps.

In interactions between *D. hungarica* males and *D. parthenogenetica* sp. nov. females, the males tended to court. However, the females generally did not respond to their courtship behavior. Only in one case was the female cooperative. The couple reached the copulatory position, and the male tried for 21 min to insert his bulbi into the female's copulatory bursa. However, he failed.

3.4. Taxonomy. Family Dysderidae C.L. Koch, 1837
Subfamily Dysderinae C.L. Koch, 1837
Genus *Dysdera* Latreille, 1804
Dysdera parthenogenetica Řezáč sp. nov. Figures 5A–E and 6A, B
D. hungarica: [42] : 168, only Figs. 63 and 65 (♀)
D. hungarica: [43] : 44, only Fig. 96.4 (♀)
D. hungarica: [44] : 75, Figs. 1 and 2 (♀)
D. hungarica: [45] : 428, Fig. 8 (♀)
D. hungarica: [14] : 445, Figs. 27–30 (♀)
D. hungarica: [46] : 240, Fig. 297 (♀).

3.4.1. Material. In the lists of type and comparative material below, a three-digit code with the prefix 3LF marks the material used for phylogenetic analyses. Abbreviations used: coll.—collection, leg.—collected by.

3.4.1.1. Type Material. Holotype (Figure 5A, B): CZECHIA—**Bohemia** • 1 ♀, Prague-Ruzyně, an apple orchard in the Czech Agrifood Research Center; 50.0870, 14.2989; 2004; M. Řezáč leg.; pitfall trap; coll. National Museum in Prague P6A 7396.

Paratypes: CZECHIA—**Bohemia** • 4 ♀; Prague-Ruzyně, Czech Agrifood Research Center; 50.0874, 14.3006; 22 Jun 1994; P. Saska et M. Řezáč leg.; orchard; coll. M. Řezáč • 2 ♀; 30 Jun 1994; the other data the same as for preceding • 1 ♀; 15 Sep 1994; the other data the same as for preceding • 1 ♀; 23 May 1996; the other data the same as for preceding • 2 ♀; 10 Jun 1997; the other data the same as for preceding • 1 ♀; 14 Apr 2003 (3LF-901); the other data the same as for preceding • 2 ♀, 2005; the other data the same as for preceding • 1 ♀; 2008 (3LF-902); the other data the same as for preceding • 1 ♀; 24 Apr 2009 (3LF-904); the other data the same as for preceding • 1 ♀; 3 Jul 2014; the other data the same as for preceding • 1 ♀; 24 Apr 2009; the other data the same as for preceding • 2 ♀; Prague 1 - Malá Strana, reserve Petřínské skalky; 50.0855, 14.4009; 9 Jun 2020; J. Straka leg.; orchard; coll. M. Řezáč • 3

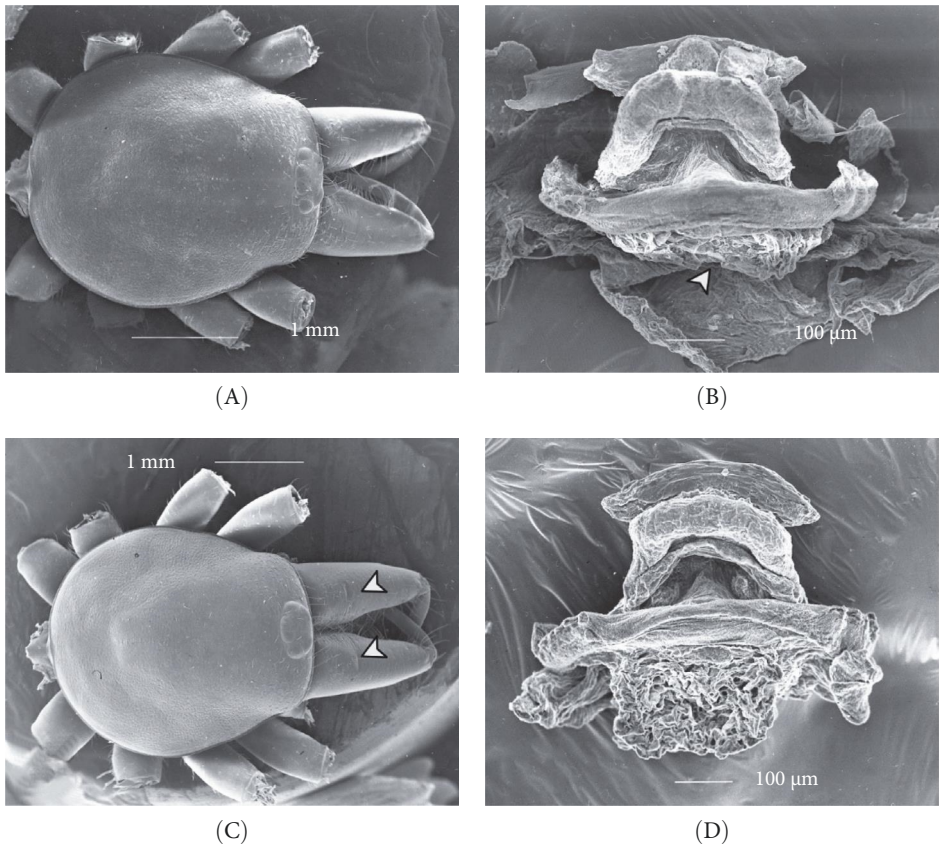


Figure 6: Morphology of prosoma and vulva (SEM images). (A, B) *Dysdera parthenogenetica* sp. nov., Czechia, Prague-Ruzyně. (A) Prosoma, dorsal view. (B) Vulva, dorsal view, arrow—the flaccid shrunken posterior diverticulum. (C, D) *D. hungarica*, Slovakia, Hrušov. (C) Prosoma, dorsal view, arrows—the transverse groove on the anterior base of the basal cheliceral segment. (D) Vulva, dorsal view.

♀, 30 Jun 2020, the other data the same as for preceding • 1 ♀; Prague 9 - Běchovice, registered natural monument Křídový výchoz Na vrchách; 50.0839, 14.8511; 27 May–24 Jun 2017, V. Strnad et M. Řezáč leg.; orchard; coll. M. Řezáč • 2 ♀; the same locality as preceding; 50.0867, 14.6307; 10 May–19 Jun 2017; P. Veselý leg; coll. M. Řezáč • 1 ♀; the same locality and collector as preceding; 19 Jun–17 Jul 2017; coll. M. Řezáč; • 1 ♀, Prague 9—Horní Počernice; 50.1186, 14.6321; 7 Jun 2017; P. Novotná leg.; garden; coll. M. Řezáč • 6 ♀; Velvary; 50.2704, 14.2316; 4 Jun–4 Jul 2016; M. Štrobl leg.; bush; coll. M. Řezáč • 1 juv.; the same locality as preceding; 25 Sep 2020; leg. and coll. M. Řezáč • 1 ♀; Žitenice; 50.5578, 14.1401; 1 Sep 2023; leg. and coll. M. Řezáč; 2 ♀, Žalhostice, reserve Radobýl; 50.5341, 14.0905; 8 Jun–3 Oct 2022, 13 Jun 2023; P. Veselý et P. Moravec leg.; coll. M Řezáč.

3.4.1.2. Other Material. See Supporting Information 2 for the list of studied specimens of *D. parthenogenetica* sp. nov. from Austria, Czechia, Hungary and Slovakia that were not included among the type material.

3.4.1.3. Comparative Material. D. adriatica Kulczyński, 1897. BULGARIA • 4 ♀; Kiustendil area, Bajkal near Izvor; 42.4333, 22.8667; 8 Aug 2005; leg. et coll. M. Řezáč. CROATIA • 1 ♂, 12 ♀; Korana near Plitvička jezera lakes; 44.9000, 15.6000; 22 Jun 2005; leg. et coll. M. Řezáč (3LF-877–879). SLOVENIA • 2 ♀; Planina, Unška Koliševka chasm; 45.8333, 14.2333; 2000; leg. et coll. M. Řezáč.

D. hungarica Kulczyński, 1897. BULGARIA • 4 ♀; Varna area, Kranevo near Zlatni piasaci; 43.333, 28.033; 10 Aug 2005; forest; leg. et coll. M. Řezáč (3LF-884–886). ROMANIA • 2 ♂; Bihor mountains, Hidișelu de Jos; 46.950, 22.050; May–Sep 2004; I. Sas leg.; coll. M. Řezáč (3LF-916) • 3 ♀; the other data the same as previous • 2 ♀; Bucegi mountains, between Moroeni and Sinaia; 45.2860, 25.4992; 27 Sep 2007; V. Opatova leg.; forest; University of Barcelona CRBA001523, CRBA001518 (3LF-910–911) • 1♂; Bucegi Mountains, Cheile Rasnoavei; 45.547, 25.515; 27 Sep 2007; leg. V. Opatova; forest; University of Barcelona CRBA001516 (3LF-912) • 1 ♀; Munti Trascauli, above Fenes; 46.3843, 23.5524; 24 Sep 2007; V. Opatova leg.; forest; University of Barcelona CRBA001520 (3LF-913). SLOVAKIA • 1 ♂, 2 ♀; Hrušov nad Turňou, Hradisko hill; 48.605, 20.641; 19 Aug 2003; bush; leg. et coll. M. Řezáč (3LF-888–890) • 3♂; Vinné, reserve Vinnianský hradný vrch; 48.820, 21.950; 19 Aug 2013; forest; leg. et coll. M. Řezáč (3LF-874–876) • 1 ♂, 2 ♀; Vranov nad Topľou; 48.883, 21.683; 15 Aug 2003; forest; leg. et coll. M. Řezáč (3LF-954–956).

Dysdera cf. *hungarica* Kulczyński, 1897. AZERBAIJAN • 1♂, 2 ♀; Baku; 40.4584,49.8387; 2005; E. Huseinov leg.; coll. M. Řezáč (3LF-881–883).

D. pristiphora Pesarini, 2001. SWITZERLAND • 1 ♂; Ticino, Monte Generoso; 26 May 1989; pitfall traps; coll.

Table 2: Lengths of leg and pedipalpal segments of *D. parthenogenetica* sp. nov., holotype.

	Pedipalp	Leg I	Leg II	Leg III	Leg IV
Coxa	0.5	1.1	0.9	0.6	0.7
Femur	1.2	1.9	1.8	1.5	1.9
Patella	0.7	1.2	1.1	0.9	1.1
Tibia	0.6	1.6	1.4	1.1	1.5
Metatarsus	0	1.5	1.4	1.3	1.7
Tarsus	0.8	0.5	0.4	0.4	0.5
Total leg length	3.7	7.7	7.1	5.7	7.4

Note: Measurements are in mm.

Naturhistorisches Museum Basel NMB1560. ITALY • 2 ♂, 1 ♀; Lombardy, Bergamo, Boscodi Astino; 45.71, 9.63; 2005; P. Pantini et O. Lodovici leg.; coll. M. Řezáč (3LF-871–873).

3.4.2. Diagnosis. Dysdera parthenogenetica sp. nov. is morphologically very similar to *D. hungarica*, from which it differs by a shrunken posterior diverticle (Figures 5D, E and 6B; in the latter figure it is marked with an arrow); in *D. hungarica*, it is globular (Figures 5G, H and 6D). It also slightly differs in other characters, but they are not suitable for distinguishing these two species because they are quantitative. First, in *D. parthenogenetica* sp. nov., the transverse groove on the anterior base of the basal cheliceral segment is inconspicuous (Figure 6A); in *D. hungarica*, it is conspicuous (Figure 6C—marked with an arrow). Second, the carapace of *D. parthenogenetica* sp. nov. is lighter (Figure 5C); in *D. hungarica*, it is maroon (Figure 5F), more convex and less shiny. Third, the legs in *D. parthenogenetica* sp. nov. are as dark as the carapace; in *D. hungarica*, they are markedly lighter than the carapace.

D. parthenogenetica sp. nov. differs from *D. hungarica subalpina* Dunin, 1992 (North Caucasus) by its smaller body (in *D. hungarica subalpina*, the carapace is 5 mm long [47]) and sternum, which is less wrinkled, with pits bearing hairs. *Dysdera parthenogenetica* sp. nov. differs from *D. hungarica atra* Mcheidze, 1979, known from Georgia (Lagodekhi) and Azerbaijan [48], by its smaller body size (in *D. hungarica atra*, the carapace is 4 mm long [47]), lighter and smoother carapace (in *D. hungarica atra*, it is dark red-brown, covered with granules [47]), less wrinkled sternum (in *D. hungarica atra*, it is roughly wrinkled with pits bearing hairs [47]), and more numerous leg spination.

Dysdera parthenogenetica sp. nov. differs from *D. pristiphora* (northern Italy) by the morphology of chitinised bands on the ventral plate of the vulva that are longer and parallel (in *D. pristiphora*, they are relatively shorter and slightly convergent anteriorly ([49]: Fig 5).

Dysdera parthenogenetica sp. nov. differs from *D. adriatica* (NW Balkan) by its larger body, darker prosoma, and relatively longer chelicerae. Furthermore, the region behind the eyes is flatter. Concerning the morphology of the vulva, the dorsal arch of *D. parthenogenetica* sp. nov. is almost as long as it is wide, and its posterior edge is more convex (in *D. adriatica*, it is much wider than it is long, and its posterior edge is less convex [42]). The chitinised bands on the ventral plate are wider and parallel (in *D. adriatica*, they are thinner and markedly convergent anteriorly [42]: Figs 71–72).

3.4.3. Description. Female holotype. Carapace. Length 2.6 mm, width 2.1 mm, height 0.7 mm, gently wrinkled, ferruginous. *Sternum*. Almost smooth, glossy, with pits bearing hairs. *Chelicerae*. Paturon length 1.3 mm, width in middle from lateral view 0.6 mm. Frontal side of paturon smooth and slightly convex in lateral view. Fang length 1.3 mm. Groove length 0.8 mm. Distance between distal and proximal teeth 0.5 mm. Basal tooth close to base of groove. Middle tooth is the largest and close to basal tooth (distance between basal and middle teeth 0.15 mm). Distal tooth is the smallest, in middle of groove (distance of distal tooth from distal end of groove 0.4 mm). *Pedipalps*. Gnathocoxa length 1.0 mm. For lengths of other segments, see Table 2. *Legs*. For segment lengths, see Table 2. Spines: tibia (ti) III prodorsally (pd) 2, apically (a) and ventrally (v) 2; metatarsus (mta) III pd 2–3, proventrally (pv) 2, v 2; ti IV pd 1, retrodorsally (rd) 1, v 1, a and v 2; mta IV pd 3, pv 2, a and v 2, retroventrally 1, rd 1. *Opisthosoma*. Length of dorsal hairs 0.1 mm. *Vulva*. Anterior edge of spermatheca only slightly convex (almost straight). Spermatheca straight. Dorsal arch almost as long as wide, middle part of its anterior edge straight and posterior edge strongly convex. Chitinised bands on ventral plate long, wide and parallel.

Males. Nonexistent.

3.4.4. Etymology. Named after parthenogenesis, the obligate mode of reproduction in this species, which is unique among native European spiders.

3.5. Distribution. Dysdera parthenogenetica sp. nov. occurs in the Pannonian lowlands, including SE Czechia, SW Slovakia, NE Austria, Hungary as well as in Wallachia (Romania) (Figure 7, Supporting Information 2). West of this area, some isolated sites exist, particularly in the surroundings of Prague in Czechia and Innsbruck in W Austria (Figure 7). The nominate subspecies of *D. hungarica* occurs in E Slovakia, W Ukraine, NE Hungary (the westernmost known locality of the species in Hungary is Vác), Romania, Serbia, and Bulgaria. It is further recorded from the northern and eastern surroundings of the Black Sea, including Crimea and the northern slopes of the Caucasus. However, the morphological variation of specimens from these regions suggests undescribed taxonomic heterogeneity. *D. parthenogenetica* sp. nov. and *D. hungarica* are almost parapatric based on their distribution areas (Figure 7).

3.6. Ecology. Dysdera parthenogenetica sp. nov. has been found in a variety of habitats. Some of them are unusual for *Dysdera*

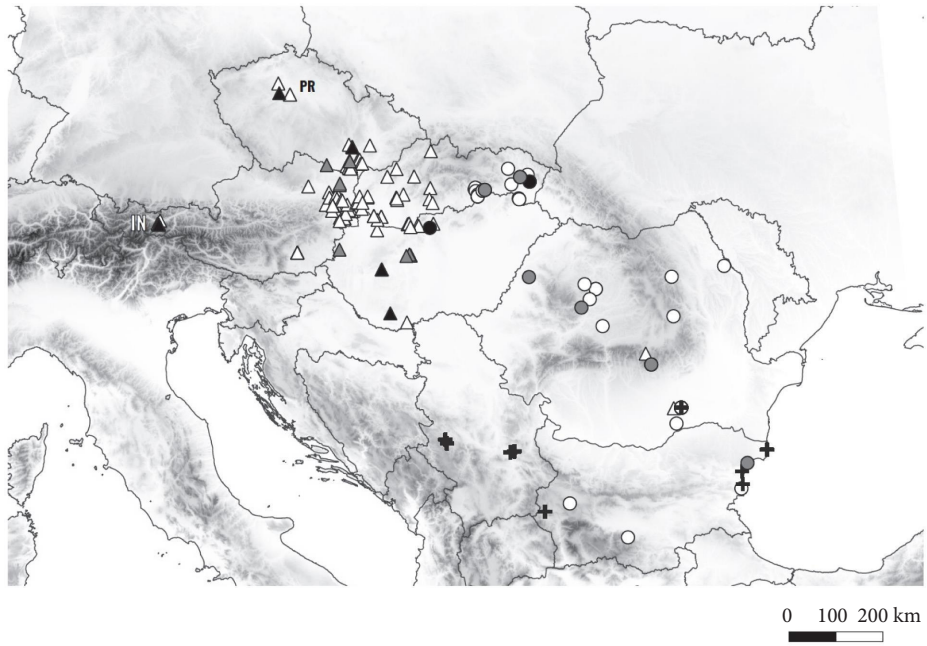


FIGURE 7: Distributions of *D. parthenogenetica* sp. nov. (triangles) and *D. hungarica* (circles and crosses) in Central and Eastern Europe west of the Black Sea. Crosses—sites taken from the literature, white triangles and circles—specimens used for morphological analysis, grey triangles and circles—specimens used for analysis of DNA markers, black triangles and circles—specimens used for analysis of karyological and DNA markers. The isolated sites of *D. parthenogenetica* sp. nov. on the west (PR—Prague, IN—Innsbruck) are presumably a result of introduction by humans.

spiders, such as salt marshes, wetlands with reeds, wet meadows, and agroecosystems (orchards, vineyards, fields) (Figure 8). It also occurs in habitats more typical for *Dysdera* spiders, such as bushes, forest steppes, and forests (mainly with *Quercus* and/or *Carpinus*). In contrast, *D. hungarica* is known only from bushes, forest steppes, and forests. The eggs *D. parthenogenetica* sp. nov. are laid in June–July, and the clutches contain ~20 eggs. They hatch in August (see also Deeleman-Reinhold [13]). After reaching the first nymphal instar, specimens disperse from maternal silk retreats. The spiders mature in autumn of the following year and overwinter as adults. Thus, this species has a biennial life cycle. *D. parthenogenetica* sp. nov. is a dietary specialist on terrestrial isopods (see also [50]).

4. Discussion

During the study, we found that parthenogenetic lineages were quite uniform in the molecular markers studied (single COI haplotype and two ITS2 haplotypes differing by a single nucleotide substitution). Concerning ITS2, one haplotype was present throughout all sampling sites except for Innsbruck (western Austria) where another haplotype was found. This haplotype also occured in sexual populations. Shared COI haplotypes among all tested parthenogenetic lineages suggest that they evolved from a common ancestor. The haplotype of ITS2 present in the Innsbruck lineage may have arisen independently of its presence in sexual populations by single nucleotide substitution during the expansion of parthenogenetic lineages upstream of the Danube River outside the Pannonian lowland. Alternatively, this haplotype could persist from sexual ancestors in the Innsbruck lineage due to unfinished lineage sorting. An alternative explanation for the two scenarios of ITS2 heterogeneity is paraphyly of parthenogenetic lineages. However, the identical COI and especially the unique karyotype, shared by parthenogenetic lineages from Austria and Czechia and differing greatly from sexual populations, argue against it. These geographically close lineages have the same diploid number and the same length of most chromosome pairs. It is highly improbable that karyotypes with this degree of similarity arose independently.

The separation of *D. parthenogenetica* is supported by the nature of its distribution range; it is extensive, continuous, and hardly overlaps with the range of sexual populations. Finally, we found that parthenogenetic females refused to mate with males from sexual populations, and their copulatory organs were reduced. Therefore, we considered it justified to separate the parthenogenetic lineages of *D. hungarica* as a separate taxon, *D. parthenogenetica* sp. nov.

4.1. Phylogenetic Analysis. The analysis of selected DNA loci suggests that *D. parthenogenetica* sp. nov. evolved from *D. hungarica* (Figures 1 and 2). Molecular phylogenetic analysis has shown that *D. parthenogenetica* sp. nov. is an ingroup of this species. All analysed *D. parthenogenetica* sp. nov. individuals from the Pannonian lowland and surrounding sites likely evolved from a common ancestor, as they shared a single COI haplotype and two closely related ITS2 haplotypes. The low haplotype divergences are consistent with the recent origin of asexual lineages. The low genetic distances between lineages of the *D. hungarica* complex correspond to intraspecific variation in other taxa (cf., e.g., [51]). This indicates that the

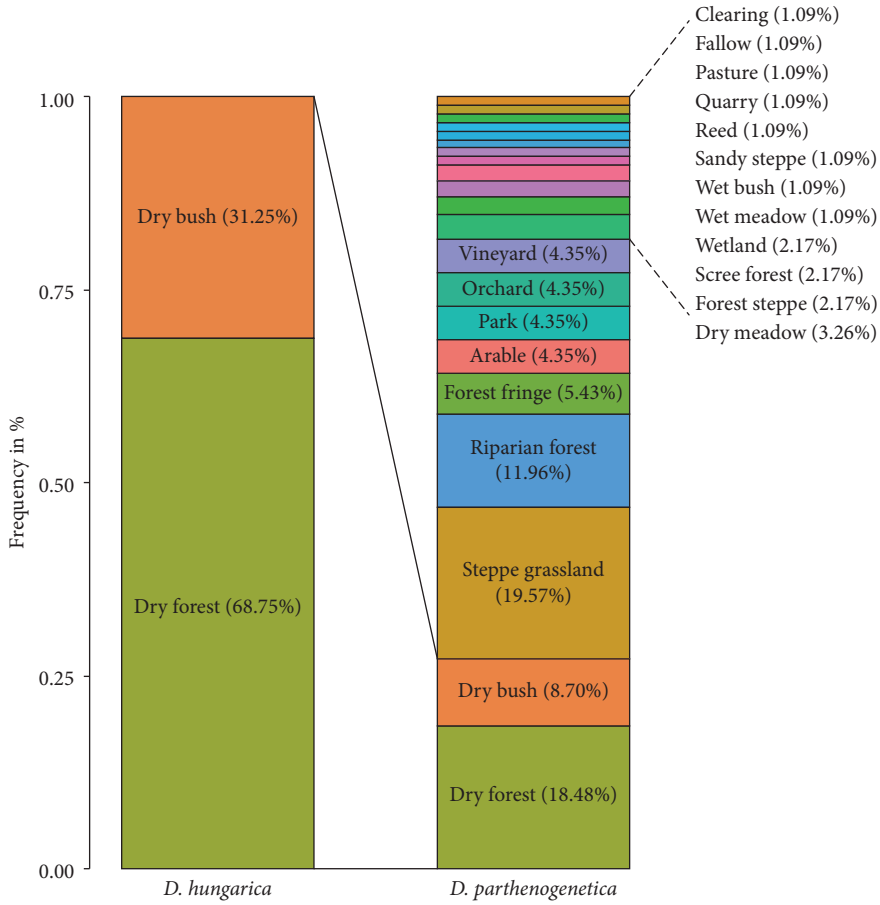


Figure 8: Habitats of *D. hungarica* and *D. parthenogenetica* sp. nov. in Czechia and Slovakia.

speciation event related to the parthenogenesis emergence within this complex occurred only recently. There is also a cytogenetic mechanism that could contribute to the low genetic diversity of *D. parthenogenetica* sp. nov.: the diploid number has decreased during the evolution of this species, which may have led to a reduced number of crossing-overs (see section on karyology below).

4.2. Karyology. Like the other members of the superfamily Dysderoidea [52], the studied species exhibit holokinetic chromosomes, that is, without a localised centromere. The male of *D. hungarica* from Vác (Hungary) shows the same number of chromosomes and sex chromosome system (X0) as the previously karyotyped population from Slovakia [14]. The female karyotype of *D. hungarica* comprises 18 chromosomes, including the X chromosome pair (XX). The female karyotype of *D. parthenogenetica* sp. nov. differs from that of *D. hungarica* in several aspects. First, in *D. parthenogenetica* sp. nov., the number of chromosome pairs is reduced due to chromosome fusions. Therefore, the karyotype of this species is considerably derived in comparison with *D. hungarica*. Second, *D. parthenogenetica* sp. nov. exhibits a considerable diversity of karyotypes. The respective parthenogenetic lineages often differ by diploid number or the length of the chromosome pairs. We suppose that the karyotype diversification of *D. parthenogenetica* sp. nov. has been promoted by the parthenogenetic mode of reproduction and by specific features of holokinetic chromosomes, which facilitate the transmission of some chromosome rearrangements, such as fusions. In holokinetic chromosomes, kinetic activity is spread along the whole chromosome axis [53]. Hence, even fused chromosomes can bind microtubules and segregate properly during cell division. Third, the karyotypes of most parthenogenetic lineages of *D. parthenogenetica* sp. nov. include several prominent chromosome pairs. A comparison of the relative chromosome lengths in *D. hungarica* and the parthenogenetic lineages of *D. parthenogenetica* sp. nov. suggests that the longest chromosome pair of latter species is formed by X chromosomes. The other prominent pairs likely arise from chromosome fusion.

Karyotypes of the parthenogenetic lineages from Czechia and Austria ($2n♀ = 14$) are closest to the ancestral female karyotype, which persists in *D. hungarica* ($2n♀ = 18$). Karyotypes of these lineages can be inferred from the karyotype of *D. hungarica* by two fusions, which gave rise to two prominent chromosome pairs. As already mentioned, the first chromosome pair of *D. parthenogenetica* sp. nov. is composed of X chromosomes. The length of prominent pairs formed by fusion is very similar in all the analysed lineages from these areas. This suggests that each of these pairs was formed in a common ancestor of these lineages by the fusion of certain two pairs (e.g., pair no. 3 arose probably by the fusion of the third and eighth pairs of *D. hungarica*). The slight differences in the length of a few

chromosome pairs between the Innsbruck and Czech lineages could be a result of translocations. Interestingly, the most derived karyotypes of *D. parthenogenetica* sp. nov. occur in Hungary, the region closest to the area inhabited by the ancestral species, *D. hungarica*. It is possible that after its origin, *D. parthenogenetica* sp. nov. was only facultatively parthenogenetic and not fully separated from *D. hungarica*. Its area of occurrence overlapped with that of *D. hungarica*, which could lead to the possible crossing of both species. In the course of selection, the forms of *D. parthenogenetica* sp. nov. with highly derived karyotypes would have the advantage of complete reproductive isolation.

To reveal rearrangements of NOR-bearing chromosomes during the evolution of *D. hungarica* and *D. parthenogenetica* sp. nov., NOR pattern of these species has been compared. NORs were detected by FISH. In Dysderoidea, that is, in spiders with holokinetic chromosomes, this method of NOR detection has not been employed thus far. In contrast to entelegynes (e.g., [54]), synspermiate spiders often bear NORs on sex chromosomes [55, 56]. This is also the case for *Dysdera crocata* C.L. Koch, 1838 [55, 57], which is currently the only species of Dysderoidea whose NORs have been detected. The species analysed in the present study exhibit a different NOR pattern, namely, one intercalar locus on a shorter chromosome pair. This pair is not formed by X chromosomes. Given that *D. hungarica* is an early-diverging lineage of *Dysdera* [58], this pattern could be ancestral within this genus. One or two NOR loci are symplesiomorphy of opisthothele spiders [59]. Both *D. hungarica* and *D. parthenogenetica* sp. nov. exhibit the same NOR pattern. Therefore, the original NOR pattern was not changed by any chromosome rearrangement in *D. parthenogenetica* sp. nov.

In sum, our data suggest that differentiation of *D. parthenogenetica* karyotype from the ancestral state found in *D. hungarica* has included several fusions. In holokinetic organisms, the potential of chromosome fusions to restrict gene flow is lower than in organisms with standard chromosome structure due to inverted meiosis of trivalents formed by original unfused chromosomes and fusion product in fusion heterozygotes. Due to this meiotic modification, chromosomes of the trivalent segregate correctly [60]. In spite of this, fusions of holokinetic chromosomes also restrict gene flow, and this effect is cummulative (it increases proportionally with the number of chromosome fusions) [61, 62]. Comparison of Innsbruck and Prague lineages of *D. parthenogenetica* indicates that chromosome translocations could be also involved in karyotype differentiation of this species. Translocations are a potent mechanism restricting gene flow, regardless of the structure of chromosomes (e.g., [63, 64]). As stated above, involvement of chromosome changes into formation of reproductive barrier between *D. hungarica* and *D. parthenogenetica* is also indicated by the fact that most derived karyotypes of *D. parthenogenetica* occur at regions, which are close to range of *D. hungarica*. According to our model, crossing of both species has been consequently fully prevented by losing ability to copulate in *D. parthenogenetica*.

The observed meiotic cells of *D. parthenogenetica* sp. nov. belong to the so-called diffuse stage, characterised by a considerable decondensation of bivalents. This period occurs in the prophase of the first meiotic division, usually between pachytene and diplotene. The diffuse stage has evolved in many animal groups. It is more frequent in females due to the need to produce nutritive substances for the development of the embryo [55, 57]. The diffuse stage probably occurs in females of all spiders (Král unpublished). The male diffuse stage is a synapomorphy of synspermiate spiders (including dysderids) and several related clades [55, 65].

Individuals of *D. parthenogenetica* are diploid, although in some spiders, haploid embryos that developed from unfertilised eggs have also been recorded (*Pityohyphantes phrygianus* C.L. Koch, 1836 from the family Linyphiidae [66]). These haploid embryos are, with all probability, unviable. There is no record of haploid adults in spiders karyotyped thus far (based on the database by [67]). The fact that meiotic cells can be found in *D. parthenogenetica* sp. nov. suggests that parthenogenesis in this species is automictic (i.e., involves meiosis). In organisms with automictic parthenogenesis, the restoration of diploidy is secured by mechanisms other than by the fusion of male and female sex cells, which occurs in sexual organisms. One possibility is the duplication of the chromosome set at premeiotic interphase [68, 69]. However, this phenomenon does not occur in *D. parthenogenetica* sp. nov., where the chromosome number during the first meiotic division is the same as in mitosis. Therefore, diploidy is restored by another mechanism, most likely by the fusion of the oocyte with the first or second pole body. While in the case of apomictic parthenogenesis, genetic diversity is generated only by new mutations, in the case of automictic thelytoky the diversity is also generated by meiotic processes (crossing-overs and fusions of the oocyte and the first polar body, respectively) [1].

Interestingly, the karyotype evolution of *D. parthenogenetica* tends to a gradual reduction in chromosome number. If the average number of recombinations per chromosome remains unchanged during this reduction, it could cause a decrease in the overall level of recombinations within the genome and, thus, a certain decrease in genetic diversity.

4.3. Behavioural Incompatibility. Females of *D. parthenogenetica* sp. nov. did not copulate with *D. hungarica* males. The disappearance of the need to mate is an expected consequence of adaptation for obligate thelytokous reproduction. *Dysdera parthenogenetica* sp. nov. possesses reduced copulatory organs. Even if copulation would take place, it would probably not lead to the production of fertile offspring due to karyotype differences.

4.4. Morphology. Parthenogenesis in *D. parthenogenetica* sp. nov. does not seem evolutionarily old, as the females still possess a copulatory organ that is only slightly reduced compared to the copulatory organ of the ancestral sexual species. Reductions in copulatory organs have also been detected in some other parthenogenetic spiders (e.g., *T. minutissima* [70] and *C. troglocaecus* [11]). The copulatory organ of parthenogenetic *T. stenaspis* females is still complex [71]. The low evolutionary age of parthenogenesis in *D. parthenogenetica* sp. nov. is also supported by the automictic mode of reproduction, which is

the ancestral form of thelytoky from an evolutionary point of view [1].

In addition to the reduction of the copulatory organ, *D. parthenogenetica* sp. nov. differs from females of *D. hungarica* by the absence of the transversal groove on the anterior base of the basal cheliceral segment (Figure 6A, compare with *D. hungarica*—Figure 6C). It is possible that the groove on the chelicerae of *D. hungarica* females plays a role in holding partners in the copulatory position. This would explain why it is not present in females of *D. parthenogenetica*, in which it would lack function due to parthenogenetic reproduction.

4.5. Taxonomy. We consider *D. parthenogenetica* sp. nov. to be a separate species. Thelytokous lineages do not fulfil the criteria for biological species, as there is no intraspecific exchange of genetic information. However, they may fulfil, as a monophyletic group with a shared history, the definition of evolutionary (cohesion) species [72]. In general, most asexual animals seem to be monophyletic, as their genetic diversity is low; they can usually be traced back to a single or a few closely related maternal founders [73]. The variation of analysed DNA markers of *D. parthenogenetica* sp. nov. is markedly low compared to the ancestral *D. hungarica* (identical in COI, low diversity in ITS2). The pattern of *D. parthenogenetica* sp. nov. distribution also does not provide any support for polyphyly. It is a single area that almost does not overlap with the area of the ancestral species.

In *Dysdera*, species-rich complexes of morphologically similar species are common [18, 19]. The species complex of *D. hungarica* seems to be such a case. It has two species centres. The first centre is in the northwestern Balkans and northern Italy, where *D. adriatica* and *D. pristiphora* occur. The second centre is located in the Caucasus and Transcaucasia regions, where three subspecies of *D. hungarica* have been distinguished (*D. h. hungarica*, *D. h. atra* and *D. h. subalpina*). These taxa likely evolved later than the species from the northwestern Balkans and northern Italy, as they are morphologically less diversified. The possible cryptic diversity of *D. hungarica* in the Caucasus and Transcaucasia regions will require further taxonomic study. Only the nominate subspecies of *D. hungarica* and *D. parthenogenetica* sp. nov. expanded far north from southern species centres.

By separating the parthenogenetic lineages as a separate species, the mother species *D. hungarica* becomes paraphyletic. Paraphyly is common in speciation, whereby an ancestral species (a paraspecies) gives rise to a daughter species without itself becoming extinct (e.g., through peripatric speciation; [74, 75]). Frequent mechanisms of such speciation are polyploidisation, hybridisation, or, as in this case, parthenogenesis. The resulting species are often characterised by little or no differentiation of genetic markers from the parent species. Available data indicate that as many as 20% of animal species and between 20% and 50% of plant species are paraphyletic [76, 77]. Most of the well-documented examples are from vertebrates due to more detailed studies (e.g., fishes [75, 78, 79]; amphibians [80]; birds [81]; mammals [82, 83]), but there are also well-documented examples from insects such as butterflies and aphids [84, 85].

Because of parthenogenetic reproduction, *D. parthenogenetica* does not fulfil the biological species concept sensu Mayr [86]. But it inhabits an adaptive zone distinct from the parent species and thus fulfils the ecological species concept sensu Van Valen [87]. Moreover, in phylogenetic analysis, it appears as the smallest apomorphic lineage and thus satisfies the evolutionary species concept sensu Simpson [88], genealogical species concept sensu Baum and Shaw [89], and phylogenetic species concept sensu Nixon & Wheeler [90].

Our proposition to give a parthenogenetic taxon a separate species name is justified. The nomenclature of most similar examples has been solved in the same way (e.g., crayfish *Procambarus virginalis* [91], invasive *Daphnia* [92], brine shrimp *Artemia parthenogenetica* [93], almost 400 species of bdelloid rotifers [94], four species of nematods of the genus *Meloidogyne* [95], almost 30 ostracods from the group Darwinulidea [96], almost 200 oribatid mite species [97, 98], stick insects [99], 17 species of myrmecophilous aphids of the genus *Trama* and *Neotrama* [100], grass thrips [101], Caucasian rock lizards [102]).

4.6. Distribution. A comparison of ranges of *D. hungarica* and *D. parthenogenetica* sp. nov. suggests that the thelytoky of the latter species evolved from geographic thelytoky. This hypothesis is further supported by a specific pattern of distribution of particular cytotypes of *D. parthenogenetica* (see the Karyology section above). Geographic thelytoky (sensu Vandel [103]) is used to be present on the edges of the distribution area of sexually reproducing populations, it is defined by the ecological limits. Typically, in species inhabiting the Northern Hemisphere, thelytokous lineages occur on the northern border of the distribution, where the climatic conditions are borderline cold. In young geographic parthenogenesis, parthenogenetic individuals often coexist with sexually reproducing individuals. Such cases include the millipede *Polyxenus lagurus* Linnaeus, 1758 [104] or the grasshopper *Saga pedo* (Pallas, 1771) [105]. However, in *D. hungarica*, no mixed lineages have been detected. Thelytokous lineages occur on the western edge of sexual populations in the same climatic zone. Here, the border of distribution is instead formed by the border of two types of landscapes: the Carpathian mountain range with forests generally suitable for *Dysdera* spiders and, towards the west, the Pannonian lowland with predominantly open habitats that are not suitable for sexually reproducing *Dysdera* spiders. *Dysdera parthenogenetica* sp. nov. probably naturally colonised the northwestern part of Pannonia (western Hungary, western Slovakia, southeastern Czechia). Isolated locations in western Czechia (Prague and its surroundings) and western Austria (Innsbruck) are probably a result of accidental transport by humans. This hypothesis is supported by two facts. First, spiders of the genus *Dysdera* cannot disperse over long distances: ballooning or phoresis has never been observed among them. Second, the habitats that are inhabited by *D. parthenogenetica* in these isolated locations are mostly man-made. Karyotypes of lineages from Prague and Brno are very similar, which suggests that colonisation of the Prague region occurred from the southeast part of Czechia.

Thelytoky supports colonisation abilities. As each adult specimen can produce eggs, thelytokous reproduction is twice as fast as sexual reproduction, where half of the population is

male [106]. Moreover, new localities can be colonised more quickly by a single sex, and even juvenile individuals or eggs can give rise to a new lineage [107]. In addition to *D. parthenogenetica* sp. nov., enhanced dispersal abilities (preadaptations to being introduced by humans to new locations) have also been recorded in another parthenogenetic spider, *T. minutissima*. This species occurs in tropical rainforest floor litter. Most species of such habitats are endemic, but *T. minutissima* nowadays occurs across continents [6].

4.7. Habitats. The habitats characteristic of *D. parthenogenetica* sp. nov. include, in addition to the natural habitats, semirural woods and bushes, often within cities. This species also occurs in afforested habitats, unusual locations for other *Dysdera*, such as wetlands with reeds (*Phragmites australis*), salt marshes, wet meadows, and agroecosystems (orchards, vineyards, and fields). Low abundances are characteristic of such habitats. These observations support the hypothesis that thelytoky enables lineages to survive even in suboptimal habitats, which are unsuitable for harbouring the high abundance necessary for sexual reproduction. A similar increase in habitat tolerance with parthenogenesis was recorded in the terrestrial isopod crustacean *Trichoniscus pusillus* in Great Britain. The parthenogenetic lineages of this forest species were also observed to colonise open habitats [108].

D. hungarica lives essentially only in dry forests or scrubland (Figure 8). *D. hungarica atra* is known from mountainous *Quercus*, *Carpinus* and *Fagus* forests [47]. *D. hungarica subalpina* is known for inhabiting mountainous forests and the subalpine zone [47].

5. Conclusion

Analysis of the parthenogenetic lineages of the spider *D. hungarica* (Araneae: Dysderidae) led us to recognise them as a distinct taxon, *D. parthenogenetica* sp. nov. This species differs from *D. hungarica* morphologically by slightly reduced copulatory organs. The diversity of DNA markers in *D. parthenogenetica* sp. nov. is low with respect to the ancestor *D. hungarica* (single COI haplotype and two closely related ITS2 haplotypes), as shown by molecular marker analysis and it nested within *D. hungarica*. Our findings imply that *D. parthenogenetica* sp. nov. exhibits automictic thelytoky. The distribution pattern of this species and its cytotypes suggests that geographic thelytoky led to speciation of this taxon. The distribution areas of *D. parthenogenetica* sp. nov. and *D. hungarica* nearly do not overlap; *D. parthenogenetica* sp. nov. colonised regions west of the ancestral sexual species. Compared to *D. hungarica*, *D. parthenogenetica* sp. nov. has been discovered in a far wider range of habitats, including agroecosystems. Parthenogenesis in this species is linked to its ability to colonise suboptimal, often disturbed environments compared with other *Dysdera* species. *Dysdera parthenogenetica* sp. nov. exhibits a considerable karyotype diversity despite having very low variation of the analysed nuclear and mitochondrial DNA markers. The transition to parthenogenesis has been accompanied by a reduction in the number of chromosome pairs via chromosomal fusions. Due to their divergence, the karyotypes of *D. parthenogenetica* sp. nov. are probably no longer compatible with those of *D. hungarica*. Between these two taxa, there is also an interspecific behavioural barrier.

Data Availability Statement

The data are contained in the paper. The resulting consensus DNA sequences are available in the NCBI GenBank database under the accession numbers KJ941210–KJ941224, KJ941226–KJ941234, KJ941236–KJ941244, KJ941246–KJ941248, KJ941261–KJ941270, KJ941274–KJ941275, KJ941277–KJ941279, KJ941281–KJ941298, KJ941300–KJ941302, KJ941309–KJ941310, KJ941312–KJ941315, and KJ941319–KJ941322.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information 1. File S1: List of material used for phylogenetic analyses: specimen codes, species, localities, and accession numbers.

Supporting Information 2. File S2: List of all studied specimens of *Dysdera parthenogenetica* sp. nov. from Austria, Czechia, Hungary, and Slovakia, not included among the type material. Codes of individuals used for molecular analyses are in bold.

Supporting Information 3. File S3: List of the sequences used in the phylogenetic analysis that were not generated in the course of the present study.

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