

Article

Ecology and Biology of the Rare Endemic Land Leech *Xerobdella anulata* (Xerobdellidae)

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Abstract: The genus *Xerobdella* contains three species of land leeches confined to the Palearctic region, one of which is *X. anulata* (Autrum, 1958), an exceptionally rare endemic of the Dinaric Alps. In the current study, we provide new data and a literature overview on this rarely encountered species, presenting its currently known distribution, providing additional data on its morphology and, for the first time, presenting comprehensive data on its habitat preferences and seasonal dynamics. Additionally, we provide novel DNA barcodes for the Dinaric land leech and compare the obtained sequences with the related *X. lecomtei*. Altogether, 22 specimens of *X. anulata* were collected using pitfall traps in three habitat types: managed forests with adjacent meadows and a primeval forest in the Dinaric Alps of Croatia. We report the first finding of *X. anulata* in open habitats, which harbored most of the specimens. Our findings show that *X. anulata* exhibits surface activity, highlighting the effectiveness of pitfall traps in sampling such elusive taxa, with perspectives for future morphological, phenological and even molecular research.

Keywords: Xerobdellidae; pitfall traps; distribution; habitat preferences; seasonal dynamics



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1. Introduction

Leeches from the genus *Xerobdella* (Frauenfeld, 1868) are endemic to the Palearctic region and are the only known land leeches in Europe. They constitute a separate line from the tropical land leeches Haemadipsidae and represent a distinct family of land leeches (Xerobdellidae), according to Borda and Siddall [1], Kutschera et al. [2] and Trontelj et al. [3]. The genus contains three species: *Xerobdella lecomtei* (Frauenfeld, 1868), *Xerobdella anulata* (Autrum, 1958) and *Xerobdella praealpina* [4,5]. The known distribution of *X. lecomtei* borders the distribution of *X. praealpina*, the former occurring in the Eastern Alps and the latter inhabiting south-eastern parts of the Alps (both are found in Austria, Italy and Slovenia) [6,7]. In contrast, *X. anulata* is confined to the western parts of the Balkans (Dinaric Alps) [7].

Living specimens of the genus *Xerobdella* are brown to black with a pale ventral side, but become pale when preserved (e.g., in alcohol, formalin) [2,7,8]. They are characterized by body segments with five annuli, a pair of oral palps and four pairs of eyespots [4,9]. Since all three species are rather similar in general appearance [6], only a few morphological features can be used for identification. In particular, the species differ in the position and spacing of male and female and/or accessory female gonopores. The spacing of male and female gonopores clearly separates the three species; in *X. lecomtei* they are separated by three-and-a-half (rarely three) annuli, in *X. anulata* by four-five annuli and in *X. praealpina* by six-and-a-half annuli [4,7]. The latter species can be further distinguished by the accessory pore situated only half an annulus behind the female gonopore, while the spacing is three-and-a-half annuli in both *X. lecomtei* and *X. anulata* [7].

Despite being initially described as sanguivorous, e.g., *X. lecomtei* has been considered a parasite on salamanders [9], the blood-feeding habits of *Xerobdella* leeches have been put into question by some authors [7]. The members of this genus seem to have a rather diverse diet, encompassing various soil invertebrates such as earthworms and snails [9]. It was noticed that they can also predate on dipteran larvae (e.g., Chironomidae, Mycetophilidae) that feed on the leaf litter where the *Xerobdella* species burrow during the day [10]. These leeches can also be found under stones and tree bark, preferring microhabitats with higher moisture and lower temperatures (optimal 10–15 °C) [10,11]. They are active at night but are rarely encountered on the surface [10].

The Dinaric land leech (*X. anulata*) was the second species described in the genus, from a specimen found in Sarajevo, Bosnia and Herzegovina [5,12]. It is exceedingly rare [4,7,13] and, consequently, there is a lack of data on its biology, ecology and distribution. Therefore, the main objectives of this paper are: (1) to present the currently known distribution of *X. anulata*, (2) to give additional information on its morphology, (3) to provide first comprehensive data on its habitat preferences and seasonal activity and (4) to explore the phylogenetic relationships between *X. anulata* and *X. lecomtei*.

2. Materials and Methods

2.1. Study Area

The study was carried out in the managed Dinaric beech–fir forests (ass. *Omphalodo-Fagetum*, alliance *Aremonio-Fagion*) and adjacent meadows in the Gorski Kotar region, near the Ravna Gora settlement (5,499,767, 5,022,169; 945 m a. s. l., Figure 1a) and in the primeval Dinaric beech–fir forest in the Lika region, Čorkova Uvala (5,539,788, 4,975,054; 967 a. s. l., Figure 1b). These forests are developed on limestone and dolomite bedrock [14]. The Dinaric beech–fir mixed forests, covering 12% of total forest area in Croatia (CRONIF), are managed by selective logging. Uneven-aged forest management, known as a “close-to-nature” management system, naturally establishes a selection stand structure, supporting permanent vegetation cover, preventing soil erosion and preserving plant diversity [15,16]. Čorkova Uvala, one of the best-preserved beech–fir forests in Croatia, is located in the Plitvice Lakes National Park, covering an area of 29,685 ha [17]. The managed forests and adjacent montane meadows of alliance *Arrhenanthesion elatioris* are developed on leached soils (ass. *Alchemillo-Trisetetum* and ass. *Festuco-Agrostietum*). Both studied regions are characterized by a temperate humid climate with warm summers, with a mean annual temperature of 7.2 °C and mean annual precipitation of 1840.9 mm (data for Parg in the Gorski Kotar region) [18,19].



Figure 1. (a) managed Dinaric beech–fir forest and adjacent meadow, (b) the primeval Čorkova Uvala Dinaric beech–fir forest, (c) pitfall trap installed at one of the sampling sites in the Dinaric beech–fir forest, Croatia.

2.2. Sampling and Identification

Land leeches were sampled with pitfall traps primarily set for soil invertebrates in managed and primeval forests. Additionally, adjacent meadows were sampled in the area of the managed forests, with a total of 18 sampling sites (for details about the sampling

design please see Brigić et al. [20]). In the primeval forest, four sampling sites were set. At each sampling site, five plastic traps (volume 0.5 dm³; depth 13.5 cm; diameter 9.4 cm) were placed 5 m apart in a linear transect. In total, 110 pitfall traps were set. The traps were partially filled with a solution of wine vinegar, ethanol and water (1:1:1), which served as an attractant and a preservative. To reduce the surface tension of the liquid, a few drops of neutrally smelling detergent were added. The traps were dug into the soil up to their rims and a brown plastic roof was placed above each trap to protect them from litter and rain (Figure 1c). The traps were emptied once a month, between April and November 2009 and 2010.

Leeches were identified to the species level according to Autrum [8] and Minelli [4]. We measured total body length (mm) and maximum width (mm) using an eyepiece micrometer on a Zeiss Stemi 2000 microscope, Carl Zeiss Microscopy, Jena, Germany. Additionally, in each specimen we counted the annuli separating the male and the female gonopores. Photographs of the specimens were taken using a Zeiss Axiocam 208 color attached to a Discovery V8 stereomicroscope, Carl Zeiss Microscopy, Jena, Germany. All sampled specimens were kept in 75% ethyl alcohol and deposited in the Varaždin City Museum, Natural History Department, Varaždin, Croatia.

2.3. Molecular Identification

Small parts of tissue (about 1 mm³) were excised from the flanks of the bodies of 22 specimens. Total DNA was isolated using the Chelex method and protocols described in Casquet et al. [21]. A polymerase chain reaction (PCR) was performed to amplify the DNA barcode region of the mitochondrial COI gene, using “universal primers” LCO 1490 and HCO 2198 [22]. PCR amplifications were conducted using DreamTaq PCR Master Mix (2X; includes DreamTaq DNA Polymerase, 2X DreamTaq buffer, dATP, dCTP, dGTP and dTTP (0.4 mM each) and 4 mM MgCl₂), 200 nM of each primer and 1 µL of DNA template. The total volume of the PCR mixture was 15 µL. PCRs were carried out with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 46 °C for 1 min, elongation at 72 °C for 1 min and a final elongation step at 72 °C for 10 min. The PCR products were checked using (1%) agarose gel electrophoresis. The enzymatic purification of the PCR products was conducted using Antarctic Phosphatase and Exonuclease I (New England Biolabs, Ipswich, MA, USA). Sequencing of the PCR products was performed by Macrogen Inc. (Seoul, South Korea) using the same primers that were used for the gene amplification. The purified PCR products were sequenced on an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence trace files were analysed using SEQUENCHER (version 5.3; Gene Codes Corp., Ann Arbor, MI, USA).

2.4. Vegetation and Soil Analysis

Vegetation was sampled using phytosociological relevés according to the standard Central European methodology [23], with expanded cover-abundance scale [24]. Plant nomenclature follows Euro + Med PlantBase [25].

On each monthly visit to the study sites, we measured, following environmental variables, near each pitfall trap: (1) soil temperature (°C) at 5 cm and 10 cm depth, using a P300 Dostmann electronic thermometer (Dostmann Electronic, Wertheim, Germany) and (2) soil moisture (%) using a FieldScout TDR100 soil moisture meter (Spectrum Technologies, Inc., Bridgend, UK). In the laboratory, we measured soil pH in water with a ratio of 1:2.5 (*w/v* = 10 g substrate/25 mL H₂O) using a WTW pH 330i meter (Xylem Analytics, Weilheim, Germany).

2.5. Data Analysis

2.5.1. Distribution

Data on the distribution of *X. anulata* were obtained from field studies and the literature data. For each record, the following data were provided: country, biogeographical region,

locality with the name of the geographical region in brackets (e.g., Gorski Kotar, Zagorje), date and data source. For certain records, exact dates are missing as they were not provided in the cited literature. Additionally, we provided the habitat type and number of specimens documented in the literature. Distribution data are presented and discussed in terms of three biogeographical regions: Alpine, Continental and Mediterranean (according to EEA [26]). All records were georeferenced using the Register of Geographical Names [27] and Google Maps. Distribution maps were created in QGIS 3.4.6.

2.5.2. Soil Properties

Soil properties measured both in situ (temperature at 5 and 10 cm depth and moisture) and in the laboratory (pH) were displayed as mean values with standard deviation per habitat type.

2.5.3. Seasonal Activity

Seasonal distribution is based on a two-year sampling study using pitfall traps (see Section 2.2) and data obtained from the literature, which are based on hand collecting. The number of individuals were pooled per month.

2.5.4. Phylogenetic Analysis

COI sequences were used to infer DNA-based species' identification of *Xerobdella* specimens collected in the present study. Obtained sequences in the present study were analyzed together with COI sequences of *Xerobdella* and outgroup leech species from the GenBank base (<https://www.ncbi.nlm.nih.gov/genbank/>) (accessed on 17 July 2022). Newly obtained sequences in the present study were deposited in the GenBank base under accession numbers (Acc. No.) OP270246–OP270248.

Evolutionary analyses were conducted in MEGA7 [28]. Estimates of evolutionary divergence between the sequences were calculated using the Kimura 2-parameter model (K2P) [29]. The rate variation among the sites was modeled using a gamma distribution (shape parameter = 1). Divergence is presented by the number of base substitutions per site between the sequences. Standard error estimates were obtained by a bootstrap procedure (100 replicates).

The phylogenetic reconstruction was inferred using the Maximum Likelihood (ML) method based on the K2P and the Neighbor-Joining (NJ) method based on the evolutionary distances computed using the K2P model [30]. ML initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with a superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among the sites (five categories (+G, parameter = 0.6856)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

3. Results and Discussion

3.1. Distribution

Xerobdella anulata occurs in two biogeographical regions of Croatia: Alpine and Continental (Figure 2a, Table A1 (in the Appendix A)). In total, it has been recorded at seven localities and most findings are confined to the Alpine biogeographical region. In the Continental biogeographical region of Croatia, the species was found at a single locality, 56 years ago (Table A1) [31].

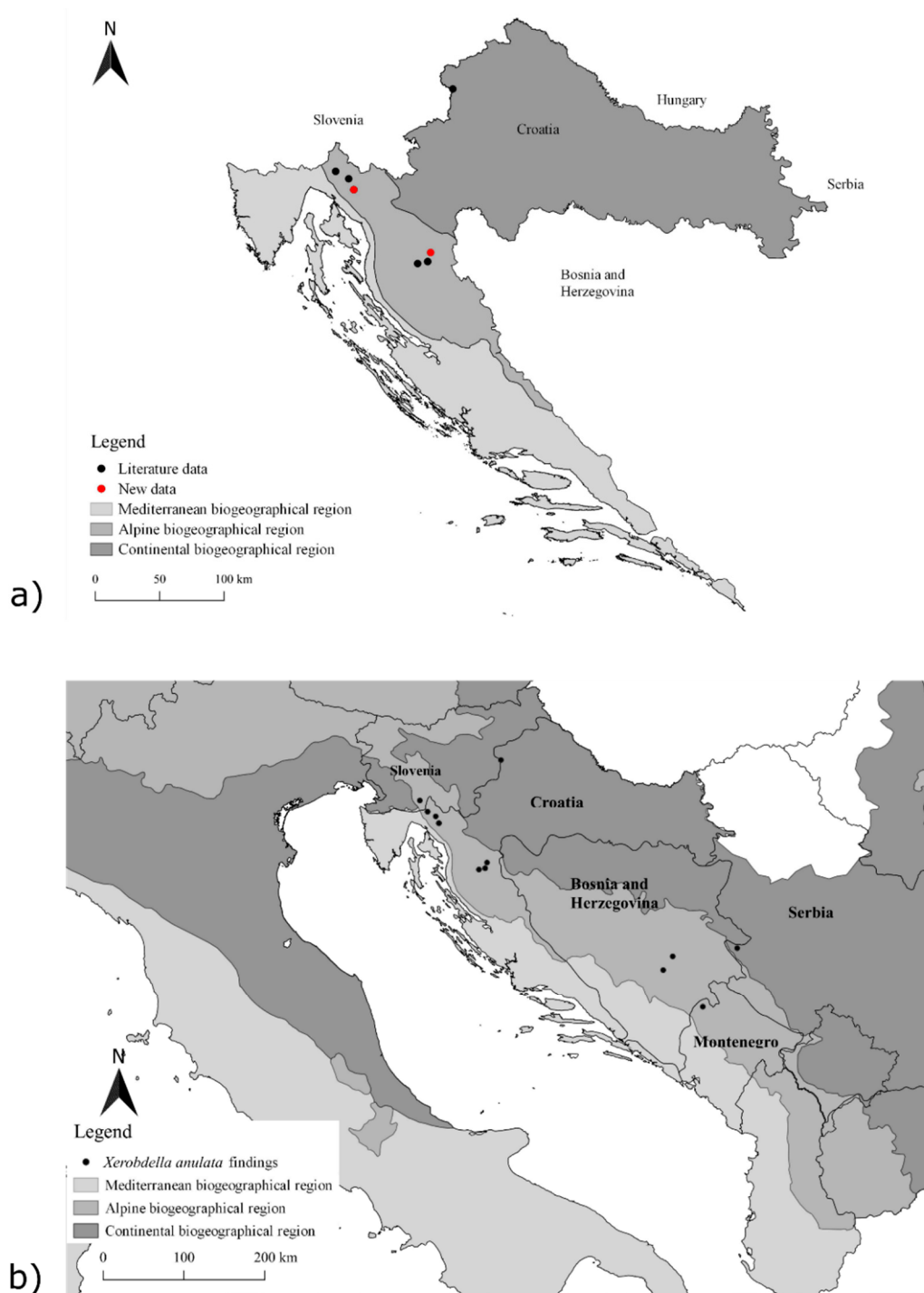


Figure 2. (a) Distribution of the Dinaric land leech (*Xerobdella anulata*) in Croatia, based on the historic literature data and new findings; (b) currently known distribution of the Dinaric land leech (*X. anulata*) in Europe, with respect to biogeographical regions.

According to present knowledge, *X. anulata* is distributed in five countries: Slovenia (Figure 2b, Table A1) [11], Croatia [8,31], Bosnia and Herzegovina [11], Serbia [7] and Montenegro [32]. In terms of biogeography, most findings are documented for the Alpine biogeographical region, with only two records from the Continental biogeographical region (Figure 2b, Table A1).

The species was recorded from 462 to 1505 m a. s. l. [7,8,31], with most records confined to higher altitudes between 700 m and 1100 m a. s. l.

3.2. Morphology

In our sample, the total body length of adult specimens was 24.6 mm on average, ranging from 17.4 to 36.2, whereas the maximum body width was 3.0 mm on average, with a range of 1.7 to 4.7 mm. In juveniles, the average total body length was 15.7 mm, with a range of 10.8 to 23.3 mm, while the maximum body width was 1.0 mm on average, ranging from 0.9 to 1.2 mm. The specimens could be unequivocally identified as *X. anulata* based on the number of annuli separating the male and female gonopores [31]; male and female gonopores were separated by four (in six individuals), four-and-a-half (in three individuals) to five annuli (in nine individuals) (Figure 3a). In all specimens with a four-annuli separation, both male and female gonopores were situated in the furrows between the annuli, whereas in specimens with a four-and-a-half annuli separation, the male gonopore was situated in the middle of an annulus and the female gonopore in the furrow. On the other hand, most specimens with a five-annuli separation had both male and female gonopores situated on their respective annuli (thus separated by $\frac{1}{2}$ annulus + four whole annuli + $\frac{1}{2}$ annulus), but in at least one specimen the gonopores were situated in furrows (separated by five whole annuli). An anterior body part with four pairs of eyespots and a pair of oral palps is shown in Figure 3b and Figure 6.

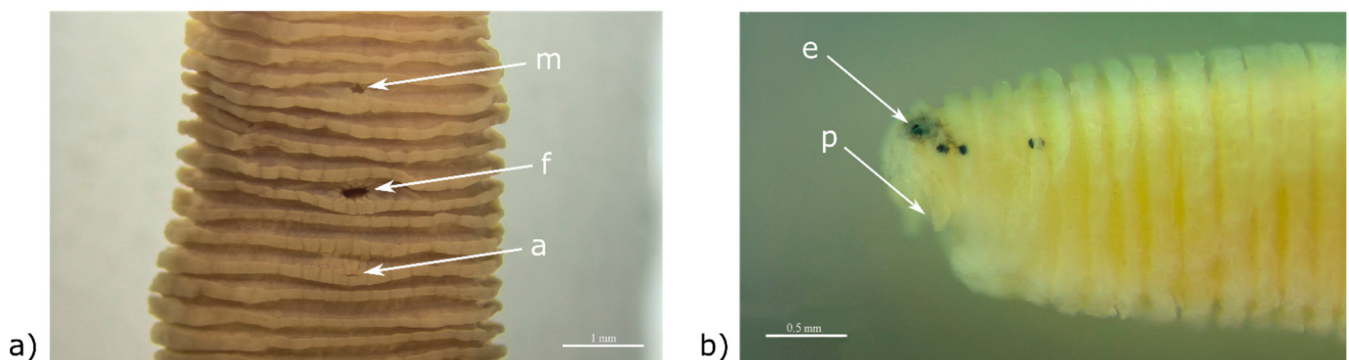


Figure 3. (a) Gonopores in the Dinaric land leech (*Xerobdella anulata*) from Croatia, (b) lateral view of the anterior body part. Legend: m—male gonopore, f—female gonopore, a—accessory female gonopore, e—eyespot, p—oral palp.

3.3. Habitat

In total, 22 specimens of *X. anulata* (19 adults and three juveniles) were sampled within two years of sampling (Table A1). Most specimens were sampled in the meadows, followed by managed Dinaric beech–fir forests, while only one specimen was caught in the primeval Dinaric beech–fir forest (Table 1, Table A1). Species-rich meadows are maintained by forest logging and have been used for a long period of time as hay meadows or pastures (Table 1). Contrary to our findings, *X. anulata* has previously been found solely in forests belonging to various plant associations (Table A1) [31]. Some forests are humid, such as *Lamio orvalae-Fagetum sylvaticae*, *Omphalodo-Fagetum* and *Blechno-Abietetum*, while others, such as *Helleboro nigri-Pinetum sylvestris*, develop on a dry shallow rendzina on the dolomite substrate. Recently, it was found in Serbia for the first time, above a stream in a mixed deciduous–coniferous forest, in the part dominated by beech trees [7]. Thus, our findings from the Dinaric beech–fir forests corroborate previous findings [8,31], while our records from the surrounding meadows also show that *X. anulata* is not strictly restricted to the forests.

Table 1. Results of the vegetation analysis in the studied habitats in Gorski Kotar and Lika regions (Croatia). Numerical values for plant species richness were obtained from a total of two sites in the managed forest, three sites in the meadow and four sites in the primeval forest.

Vegetation Analysis	Managed Forest	Meadow	Primeval Forest
Plant associations	<i>Omphalodo-Fagetum</i> /Tregubov 1957/Marinček et al. 1993	<i>Alchemillo-Trisetetum</i> Horvat 1962, <i>Festuco-Agrostetum</i> Horvat 1962	<i>Omphalodo-Fagetum</i> /Tregubov 1957/Marinček et al. 1993
Tree layer			
Dominant species	<i>Fagus sylvatica</i> L. <i>Abies alba</i> Mill. <i>Picea abies</i> (L.) H. Karst. <i>Acer pseudoplatanus</i> L. <i>Sorbus aucuparia</i> L.		<i>Fagus sylvatica</i> L. <i>Abies alba</i> Mill. <i>A. pseudoplatanus</i> <i>Fraxinus excelsior</i> L.
Plant species richness (total)	5		10
Shrub layer			
Dominant species	<i>F. sylvatica</i>		<i>Acer pseudoplatanus</i> L. <i>A. alba</i> <i>Rhamnus alpinus</i> L. spp. Fallax <i>Rubus hirtus</i> Waldst. et. Kit. <i>Daphne mezereum</i> L. <i>Lonicera xylosteum</i> L. <i>Lonicera nigra</i> L. <i>Rubus idaeus</i> L. <i>Daphne laureola</i> L.
Plant species richness (total)	14		26
Herb layer			
Dominant species	<i>Omphalodes verna</i> Moench <i>Allium ursinum</i> L. <i>Galium odoratum</i> (L.) Scop. <i>Oxalis acetosella</i> L. <i>Anemone nemorosa</i> L. <i>Cardamine trifolia</i> L. <i>Lamium orvala</i> L.	<i>Festuca pratensis</i> Huds. <i>Festuca rupicola</i> Heuff. <i>Brachypodium pinnatum</i> (L.) P. Beauv. <i>Dactylis glomerata</i> L. <i>Galium mollugo</i> L. <i>Briza media</i> L. <i>Centaurea nigrescens</i> Willd. <i>Achillea millefolium</i> L.	<i>Dryopteris filix-mas</i> (L.) Scott. <i>Cardamine kitaibelii</i> Becherer <i>Stellaria nemorum</i> L. ssp. <i>glochidiosperma</i> Murb.
Plant species richness (total)	38	92	39

3.4. Soil Properties

The main feature of the soil cover in the studied areas is the highly pronounced spatial variability, with different soil types. Thus, black soil on limestone (calcomelanosol) and brown soil on limestone and dolomite (calcocambisol) are formed [14].

Mean soil temperature, at both 5 and 10 cm below the surface, increases in all studied habitats from May to August and its values decrease again in the subsequent months (Figure 4a,b). The opposite pattern is documented for soil moisture in all studied habitat types (Figure 4c). The greatest difference between the maximum summer and the minimum autumn soil temperature at 5 cm below the surface was recorded in the meadow (18.2 °C). These differences were less pronounced in the studied forests and the temperature ranges recorded in the managed and primeval forests were 13 °C and 9 °C, respectively. Similar patterns are documented for soil temperatures at 10 cm depth (Figure 4b). The greatest difference between the maximum autumn and the minimum summer soil moisture was documented in the meadow (75.9%), followed by the managed forest (54.6%). In the primeval forest, the difference between the maximum autumn and the minimum autumn soil moisture was less pronounced (38%), most likely due to skeletal soil. In May, when most of the specimens of *X. anulata* were recorded, the mean soil temperature at 5 cm depth reached 10 °C in all studied habitat types and the soil was relatively moist, above 40%, with the exception of the primeval forest (28%; Figure 4c).

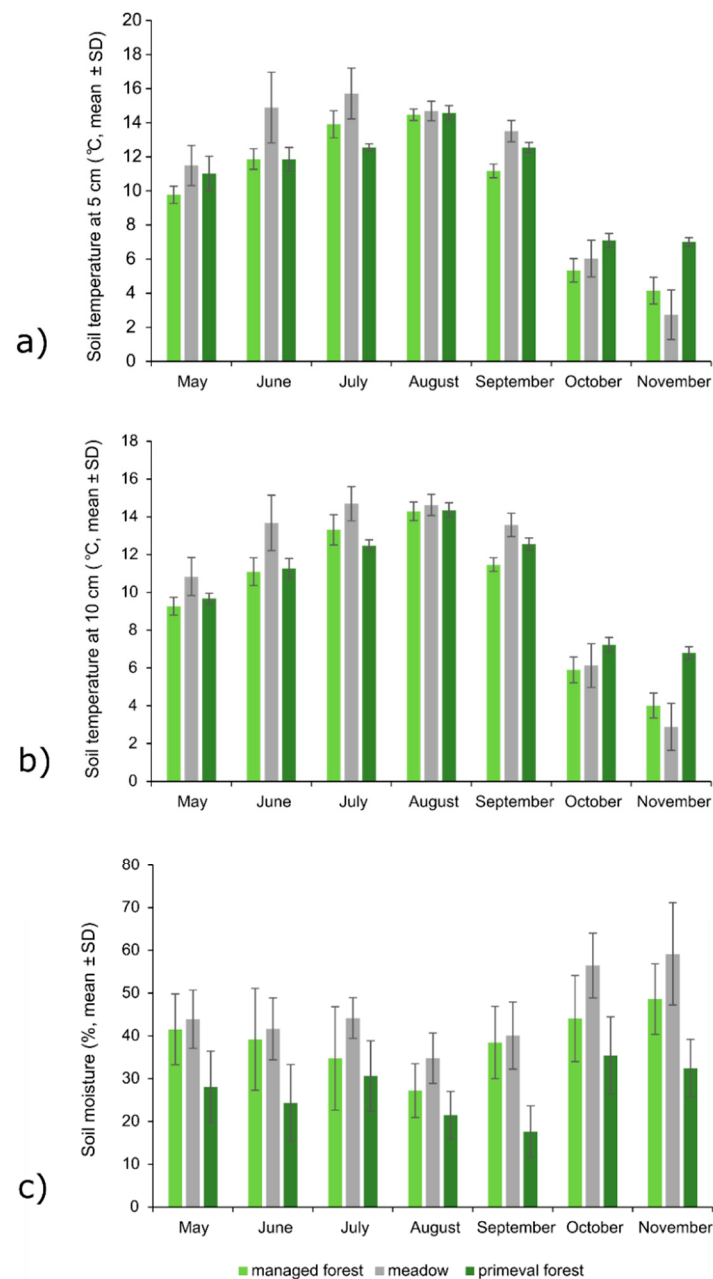


Figure 4. (a) Soil temperature measured at 5 cm below the surface, (b) soil temperature measured at 10 cm below the surface, (c) soil moisture measured at 15 cm below the surface. Soil properties were measured during the vegetation season of 2009, in the Gorski Kotar and Lika regions (Croatia), with the following number of measurements per habitat type: n (managed forest) = 126, n (meadow) = 168, n (primeval forest) = 84.

Soils in managed forests (mean $\pm$ SD, 5.86 ± 0.78 ; range: 4.95–6.80) and meadows (5.98 ± 0.57 ; range: 5.21–7.15) were acidic, with mean values above 5.5. However, in the primeval forest (4.48 ± 0.50 ; range: 4.00–5.00) the soil was strongly acidic, with a mean value below 5.5. Thus, it seems that *X. anulata* can tolerate acidic conditions, related to dense leaf litter layers in the primeval forest and decomposition.

3.5. Seasonal Distribution

The maximum seasonal activity was observed in May, while the number of individuals strongly decreased later in the season, with no specimens recorded after August (Figure 5). The plot is based on the literature data, with altogether 16 hand-collected specimens over a 50-year period, and two years of consecutive field studies using pitfall traps with in total 22 individuals sampled. These results highlight two important facts: (1) land leech *X. anulata* exhibits surface activity and (2) pitfall traps are more effective in land leech sampling than hand collecting. The surface activity of *X. anulata* is expressed early in the season (May), while it is extremely difficult to encounter the species later in the season, despite favorable climatic conditions (lower temperature, higher humidity; Figure 4). An additional search for the species, conducted by the first author at the beginning of July 2014 at the sites where it had been previously recorded, met with no success even after eight hours of soil sieving. It is possible that the species hides beneath the stones and tree bark later in the year, a behavior previously reported by Reisinger [10] at the beginning of the vegetation season. Additionally, the species might hide in deeper soil layers, as it was found in the soil at 5 cm depth while setting up pitfall traps (Brigić, personal observation). Continuous sampling, one of the greatest advantages of pitfall traps, makes them particularly useful for sampling invertebrates occurring in low densities [33]. In the current case, this method proves to be successful in sampling taxa rarely found in nature, which are thus particularly prone to under sampling.

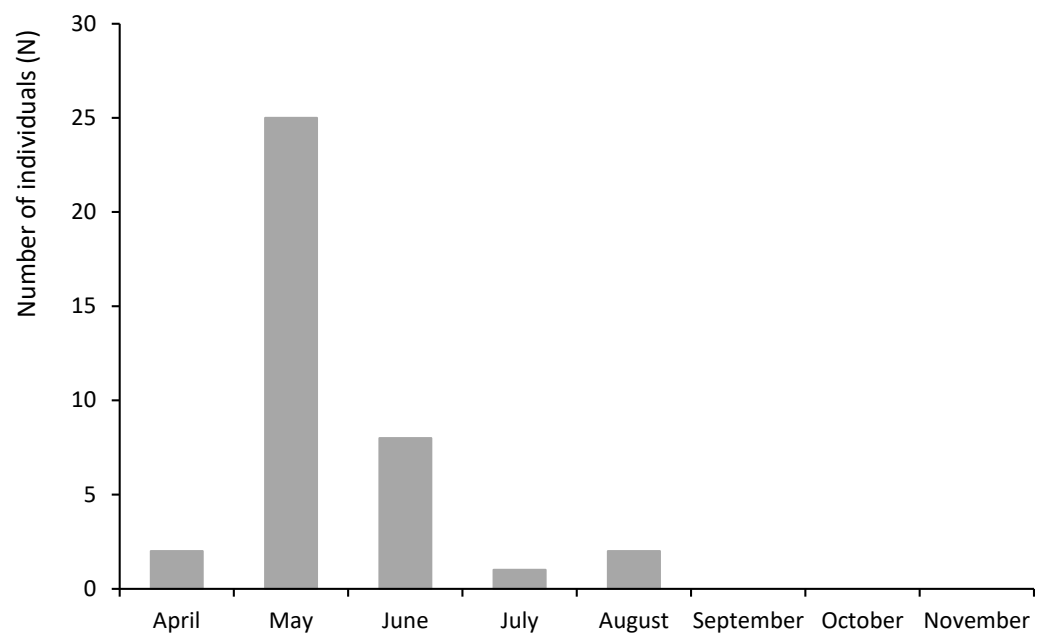


Figure 5. Seasonal dynamics of the Dinaric land leech (*Xerobdella anulata*) based on the literature data and field studies using pitfall traps. The numbers of individuals are pooled per month.

3.6. Feeding Habits

In one of the pitfall traps, we found a specimen of *X. anulata* feeding on microdrile oligochaete of the family Enchytraeidae (Figure 6). Similar feeding habits have been documented in other studies, where land leeches were reported feeding on earthworms [2,9,10]. With a diet encompassing primarily such ground-dwelling invertebrates [2], it is likely that these leeches emerge from their burrows and exhibit surface activity (see *Seasonal distribution*, above) in the evening to forage, when the surface activity of their preferred prey is the highest [34]. Therefore, active searches for *X. anulata* during the night, in combination with pitfall trapping, might additionally increase sampling efficiency and result in larger datasets required to expand our knowledge on this elusive species and its habits.



Figure 6. Dinaric land leech (*Xerobdella anulata*; right) feeding on a microdrile oligochaete of the family Enchytraeidae.

3.7. Phylogenetic Relationships

We obtained three identical sequences from three *X. anulata* specimens collected by three different pitfall traps (separated by 25–50 m) at the same site in the Dinaric beech–fir forest near Ravna Gora, Gorski Kotar region. The newly generated sequences present the first DNA barcodes of this species. Only two COI sequences of genus *Xerobdella* were available in the GenBank base and both were designated as *X. lecomtei* (Acc. No. EF125040, Kutschera et al. [2]; Acc. No. EU100099, Borda et al. [9]). In total, the phylogenetic reconstruction involved five sequences of *Xerobdella* and two sequences of outgroup species. The trees were rooted with sequences of *Mesobdella gemmata* (Acc. No. EU100097, Borda et al. [9]) and *Hirudo medicinalis* (Acc. No. EU100093, Borda et al. [9]).

The K2P values between the morphologically identified species *X. anulata* and *X. lecomtei* ranged from 3.26% to 4.88%. The maximum distance of $4.88 \pm 1\%$ was observed between *X. lecomtei* from Austria (Acc. No. EF125040) and *X. anulata* from Croatia (sequenced in the present study). The lowest K2P distance of $2.63 \pm 0.7\%$ was observed between *X. lecomtei* from Austria (Acc. No. EF125040) and *X. lecomtei* from Slovenia (Acc. No. EU100099). The ML tree with the highest log likelihood (−2160.07) is shown in Figure 7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches [35]. There was a total of 698 positions in the final dataset.

Three new sequences belong to a single haplotype and form a supported group on the phylogenetic tree (Figure 7). The observed K2P difference of 3.26% to 4.88% between *X. anulata* and *X. lecomtei* in our dataset is at the lower margin of the interspecific threshold of 4–8% K2P distance for freshwater *Glossiphonia* leeches in the Western Balkans, as defined by Jovanović et al. [36]. Nevertheless, in the same study, morphologically well-defined species *G. balcanica* and *G. complanata* differed with a mean K2P interspecific distance of only 3.17%, possibly resulting from the small number of *G. balcanica* sequences used [36]. Accordingly, molecular data obtained from only a few specimens of *X. anulata* from a single population are likely insufficient to clarify the taxonomic status of this species and resolve the phylogenetic relationships within the genus *Xerobdella*. Such an undertaking should encompass a larger series collected from numerous populations of all three *Xerobdella* species (*X. anulata*, *X. lecomtei*, *X. praealpina*) throughout their respective distribution areas, most importantly including type material or fresh specimens collected from type localities.

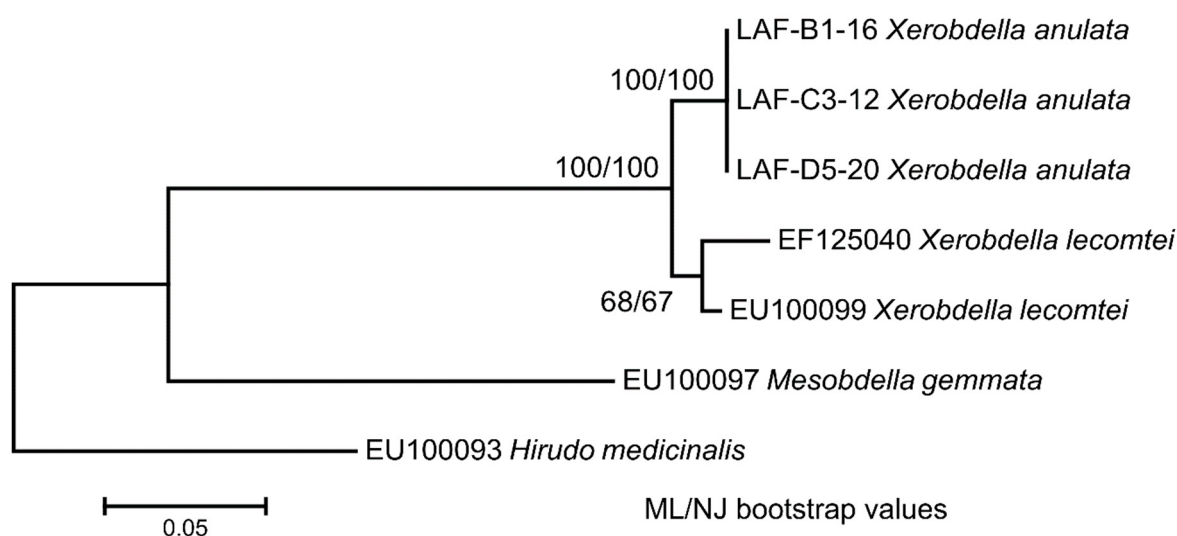


Figure 7. Molecular phylogenetic analysis using the maximum likelihood method. The ML tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support of ML and Neighbor-Joining (NJ) trees are shown next to the branches.

4. Conclusions

In conclusion, *X. anulata* most commonly inhabits humid forests, but our results show that it also occurs in humid montane meadows and less humid forest habitats. It is more likely to occur in microhabitats with higher moisture contents and in acidic to neutral soils. Dinaric beech–fir forests cover approximately 150,000 ha in the Dinaric mountains of Croatia [14], thus it is likely that the species has a much wider distribution than previously thought. Further research is required to validate the full range of this species' habitat preferences. Our findings also indicate that future efforts should focus on collecting *X. anulata* in the spring, considering the observed activity peak in May and June. Importantly, we provide the first evidence that *X. anulata* exhibits surface activity, probably associated with foraging for the preferred prey such as oligochaetes; accordingly, pitfall trapping with a sufficient sampling effort and duration (i.e., a high number of traps exposed continuously throughout the season) proves to be an efficient method for sampling these rare leeches, with great potential for obtaining substantially larger datasets in the future. The current study also shows that, although COI sequences were successfully obtained from only a few specimens (3 out of 22), even the samples collected over 10 years ago in a solution of wine vinegar, ethanol and water (1:1:1) and afterwards stored in 75% ethanol at room temperature can still be suitable for molecular analysis. This is an encouraging prospect for phylogenetic research on rare and under-sampled taxa such as *Xerobdella* leeches.

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Appendix A

Table A1. List of known records of the Dinaric land leech (*Xerobdella anulata*).

Country	Biogeographical Region	Location	Habitat Type	Plant Association, Based on Historic Literature Data	Valid Name of Plant Association	Number of Individuals	Date	Data Source
Croatia	Alpine	Babin potok, Vrhovine (Lika region)	Forest	<i>Helleboreto-Pinetum</i>	<i>Helleboro nigri-Pinetum sylvestris</i> Horvat 1958	1	11.5.1962	[31]
Croatia	Alpine	Babin potok, Vrhovine (Lika region)	Forest	<i>Helleboreto-Pinetum</i>	<i>Helleboro nigri-Pinetum sylvestris</i> Horvat 1958	2	30.5.1963	[31]
Croatia	Alpine	Babin potok, Vrhovine (Lika region)	Forest	<i>Piceetum dolomiticum</i>	Suballiance Vaccinio-Piceeion	1	11.5.1962	[31]
Croatia	Alpine	Babin potok, Vrhovine (Lika region)	Forest	<i>Piceetum dolomiticum</i>	Suballiance Vaccinio-Piceeion	1	26.6.1962	[31]
Croatia	Continental	Cesergradska Gora, Klanjec (Hrvatsko zagorje region)	Forest	<i>Fagetum croaticum montanum</i>	<i>Lamio orvalae-Fagetum sylvaticae</i> (Horvat 1938) Borhidi 1963	1	19.5.1962	[31]
Croatia	Alpine	Bijeli Vrh, Vrhovine (Lika region)	Forest	<i>Fagetum croaticum abietetosum</i>	<i>Omphalodo-Fagetum</i> (Tregubov 1957/Marinček et al. 1993)	2	26.4.1963	[31]
Croatia	Alpine	Bijeli Vrh, Vrhovine (Lika region)	Forest	<i>Fagetum croaticum abietetosum</i>	<i>Omphalodo-Fagetum</i> (Tregubov 1957/Marinček et al. 1993)	1	29.5.1963	[31]
Croatia	Alpine	Frk, Crni Lug (Gorski kotar region)	Forest	<i>Blechno-Abietetum</i>	<i>Blechno-Abietetum</i> Horvat /1938/1950	1	17.7.1964	[31]
Croatia	Alpine	Snježnik (Gorski kotar region)	Forest	<i>Fagetum croaticum subalpinum</i>	<i>Ranunculo platanifolii-Fagetum</i> Marinček et al. 1993	1	19.8.1964	[31]
Croatia	Alpine	Snježnik (Gorski kotar region)	Forest	<i>Pinetum mugho croaticum</i>	<i>Hyperico grisebachii-Pinetum mugi</i> (Horvat 1938) ex Zupančič et al. 2004	2	28.5.1964	[31]
Croatia	Alpine	Snježnik (Gorski kotar region)	Forest	<i>Pinetum mugho croaticum</i>	<i>Hyperico grisebachii-Pinetum mugi</i> (Horvat 1938) ex Zupančič et al. 2004	1	18.8.1964	[31]
Croatia	Alpine	Bukovica, Ravna gora (Gorski kotar region)	Managed forest		<i>Omphalodo-Fagetum</i> (Tregubov 1957/Marinček et al. 1993)	3	13.5.2009	New data
Croatia	Alpine	Bukovica, Ravna gora (Gorski kotar region)	Managed forest		<i>Omphalodo-Fagetum</i> (Tregubov 1957/Marinček et al. 1993)	6	29.5.2010	New data
Croatia	Alpine	Bukovica, Ravna gora (Gorski kotar region)	Meadow		<i>Alchemillo-Trisetetum</i> Horvat 1962, <i>Festuco-Agrostetum</i> Horvat 1962	6	10.6.2009	New data
Croatia	Alpine	Bukovica, Ravna gora (Gorski kotar region)	Meadow		<i>Alchemillo-Trisetetum</i> Horvat 1962, <i>Festuco-Agrostetum</i> Horvat 1962	3	13.5.2009	New data

Table A1. Cont.

Country	Biogeographical Region	Location	Habitat Type	Plant Association, Based on Historic Literature Data	Valid Name of Plant Association	Number of Individuals	Date	Data Source
Croatia	Alpine	Bukovica, Ravna gora (Gorski kotar region)	Meadow		<i>Alchemillo-Trisetetum</i> Horvat 1962,	3	29.5.2010	New data
Croatia	Alpine	Čorkova uvala, NP Plitvice Lakes (Lika region)	Primeval forest		<i>Festuco-Agrostetum</i> Horvat 1962 <i>Omphalodo-Fagetum</i> (Tregubov 1957/Marinček et al. 1993)	1	12.6.2009	New data
Serbia	Continental	Rastište, Tara Mt.	Forest		-	2	24.5.2019	[7]
Slovenia	Alpine	Snežnik	Forest		-	-	-	[11]
Bosnia and Herzegovina	Alpine	Bjelašnica	Forest		-	-	-	[11]
Bosnia and Herzegovina	Alpine	Sarajevo	Forest		-	-	-	[11]
Montenegro	Alpine	Plužine, Piva River	-		-	-	8.5.2018	[32]

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