



Extension of the known northeastern range limits of the agile frog (*Rana dalmatina*) in southern Poland

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Manuscript received: 15 December 2020

Accepted: 17 May 2021 by STEFAN LÖTTERS

Abstract. The precise delimitation of range boundaries is imperative for the conservation of amphibians in the wake of intense habitat conversion and global climate change. Despite decades of study, the distributions of many amphibian species in Central and Eastern Europe are still relatively uncharted. In this contribution, we report 40 new breeding localities of the agile frog (*Rana dalmatina*) in southern Poland, extending the known northeastern range of this species by ca. 100 km. In 2018–2020 we surveyed the Roztocze region in southeastern Poland, a predominantly forested area with extensive agriculture and numerous small water bodies. We focused on the presence of egg clutches of *R. dalmatina* at breeding sites and confirmed species identification using barcoding and a newly developed, PCR-based mitotyping technique that allows for rapid and inexpensive discrimination between *R. dalmatina* and two other, broadly sympatric, European brown frog species, *R. temporaria* and *R. arvalis*. Out of a total of 121 ponds, 40 (33%) contained at least one *R. dalmatina* clutch. Most of the localities were located in the Roztocze National Park (RNP) or its vicinity, and some were located more distantly, close to the Polish-Ukrainian border. The breeding sites of *R. dalmatina* were mostly small water reservoirs located in diverse habitats, but always within or close to forest. Our results indicate the existence of another, possibly isolated, enclave of *R. dalmatina* at its northeastern range limit in southern Poland. Moreover, we show that RNP and its surroundings are an important stronghold of *R. dalmatina* in Poland, providing crucial information for the conservation of peripheral populations of this species.

Key words. Amphibia, Anura, Ranidae, barcoding, geographic range, mitotyping, peripheral populations, Roztocze.

Introduction

Determining precise range limits is of fundamental importance for tracking the responses of species to anthropogenic disturbance and climate change. The range of a species is an expression of its ecological niche. Range shifts track changes in environment, but may also reflect niche evolution over time (SEXTON et al. 2009). Documenting current range boundaries thus provides a foundation for understanding changes in climate and landscape, as well as the eco-evolutionary processes enabling adaptation to new environments (GASTON 2003). Moreover, for species of conservation concern, management decisions are ideally based on detailed knowledge of the population biology and edge characteristics of peripheral populations (GIBSON et al. 2009). Several amphibian and reptile species reach their northern range boundaries in Central Europe. Six amphibian species reach their northeastern distribu-

tion limits in Poland, these being *Bombina variegata*, *Hyla arborea*, *Ichthyosaura alpestris*, *Lissotriton montandoni*, *Rana dalmatina*, and *Salamandra salamandra*; additionally, *Hyla orientalis* has its northwestern limit in this country (GŁOWACIŃSKI & SURA 2018). Of these, the agile frog (*Rana dalmatina* FITZINGER in BONAPARTE, 1838) has an extensive European range, but is declining (KAYA et al. 2009) and is considered rare and at risk in Poland (GŁOWACIŃSKI 2001, SZYMURA & BONK 2018). Its geographical range covers most of central and southern Europe, from the Pyrenees to the Black Sea (Fig. 1). The northern border of its range is marked by isolated populations in northern Germany, Denmark and southeastern Sweden (SILLERO et al. 2014), but the range limits in Poland and Ukraine are much less known. Indeed, the presence of this species in Poland was confirmed only from the late 1980's (RAFIŃSKI et al. 1987, SZYMURA 1994). Since then, further observations have documented two main areas of occurrence: (I) in

south-central Poland along the Odra River valley, and (II) in southeastern Poland (Sandomierz Basin, Carpathian Foothills) (Fig. 1; NAJBAR et al. 2011, BONK et al. 2012, SZYMURA & BONK 2018). A recent observation of an adult *R. dalmatina* in the Roztocze National Park (RNP; Fig. 1; 2015 – R. CYMBAŁA unpubl. data, SZYMURA & BONK 2018), an area ~80–100 km north of the known range of the species in the Carpathian Foothills, suggested that *R. dalmatina* could be more widespread in southeastern Poland than previously thought. Moreover, the presence of this species in what is now southern Poland (including Roztocze) and northwestern Ukraine was noted in studies from the early 20th century (TENENBAUM 1913, FEJERVÁRY 1923, BAYGER 1937). However, these early observations were later deemed unreliable due to the loss of voucher specimens and inconclusive descriptions of observed individuals (JUSZCZYK 1987). A more recent record from extreme southeastern Poland (Bieszczady Mountains; KOWALSKI 1970) was debunked due to the inadequate description of morphological indicators used to differentiate between *Rana* species (BORKIN 1974, JUSZCZYK 1987), despite a photograph of the collected specimen, which in fact depicted the characteristics typical of *R. dalmatina* (KOWALSKI 1970). The irrefutable rediscovery of the species in RNP after more than a century prompted us to survey the Roztocze region specifically for *R. dalmatina*.

Besides *R. dalmatina*, two other brown frog species, the common frog (*Rana temporaria*) and the moor frog (*R. arvalis*), are broadly sympatric in Central Europe. In

southern Poland and elsewhere, the common frog often co-occurs with *R. dalmatina* (RAFIŃSKI & SZYMURA 1997, BARTOŃ & RAFIŃSKI 2006), but all three species can be syntopic and may breed in the same aquatic habitats (ZAVADIL 1997). Morphological discrimination of the three brown frog species is relatively easy in the case of adult specimens, especially during the breeding season, but is more difficult when it comes to juveniles, tadpoles and egg clutches. However, these species do not hybridize and show strong mitochondrial DNA (mtDNA) divergence, exceeding 11% in the mitochondrial NADH dehydrogenase 1 (ND1) gene (BONK et al. 2012), making molecular identification of DNA samples straightforward. Here, we developed a PCR-based method using differences in amplicon size on agarose gels as a means of differentiating between *R. arvalis*, *R. dalmatina*, and *R. temporaria*.

The aim of this contribution is to identify the extent of occurrence of *R. dalmatina* in the Roztocze region of southern Poland. We present data that significantly expand the known northeastern distribution of this species, and also report observations on the terrestrial and aquatic breeding habitats of these peripheral populations.

Material and methods

Study area

Roztocze is a highland area encompassing ca. 3,400 km² of which about 70% is located in southeastern Poland and

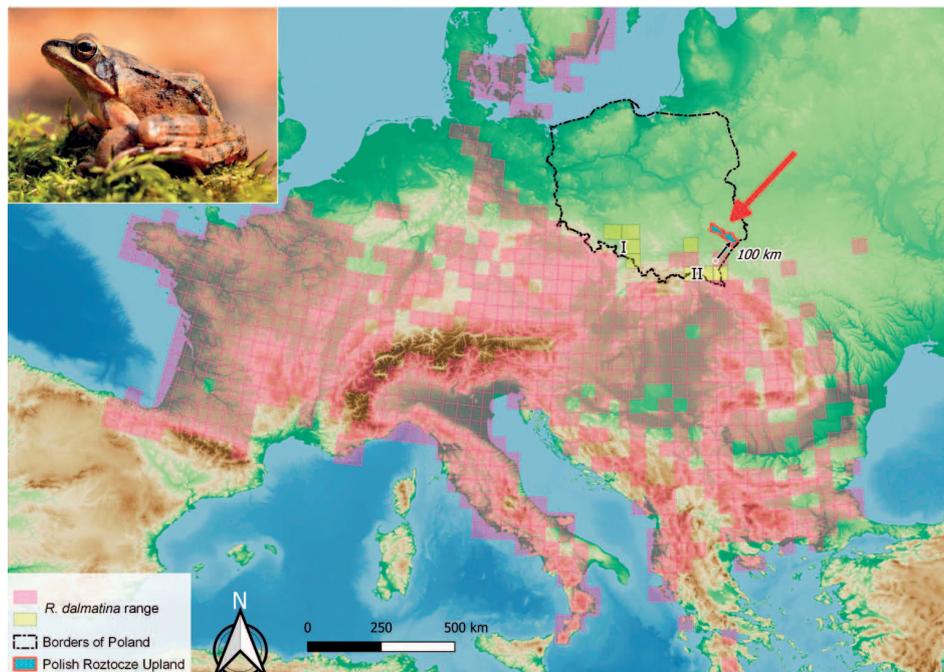


Figure 1. European range of *Rana dalmatina* according to SILLERO et al. (2014). Yellow shading denotes new data for Poland (GŁOWACIŃSKI & SURA 2018) not included in SILLERO et al. (2014). Red arrow and outline delineate the spatial extent of the Roztocze region of southeastern Poland. Black arrow and white point indicate extension of known *R. dalmatina* range (100 km). I and II denote *R. dalmatina* populations close to the Odra River valley and in the foothills of the Carpathian Mts. and adjacent valleys, respectively. Upper left corner – adult *R. dalmatina* found in Roztocze National Park. Photo: P. STACHYRA.

the rest in northwestern Ukraine (WŁAD 2011). Maximum altitudes in the region reach 400 m above sea level (BURACZYŃSKI & ZINKO 2015) and the relief is varied, with many areas hosting a high concentration of deep gorges. The dominating sediment is loess, formed mostly during Pleistocene glaciations. Marl, sand and limestone deposits formed in the upper Cretaceous and Miocene are also present (HARASIMIUK et al. 2015). Climatic conditions are continental with an annual temperature amplitude of 21.5–22°C, a mean annual temperature of 7.1–7.5°C, low degrees of cloud cover in the summer months, and a relatively large number of sunny days per annum (KASZEWSKI et al. 2015). Mean temperatures in January range from -4.1 to -3°C, and in July from 16.5 to 17.7°C (FICK & HIJMANS 2017). Annual precipitation is relatively low, at 600–650 mm (KASZEWSKI et al. 2015). Forests make up about 39% of the area, while agricultural and urban/built-up areas comprise ca. 46% and 6% of the region, respectively (Corine Land Cover 2018). Predominant tree species include pine (*Pinus silvestris*) and beech (*Fagus sylvatica*), often forming homogeneous stands in managed forest, with smaller proportions of lime (*Tilia cordata*), hornbeam (*Carpinus betulus*), oak (*Quercus robur*, *Q. petraea*), and fir (*Abies alba*). Riparian forests (*Alnus*, *Fraxinus*, *Salix*) occur in river valleys and wetlands. Open areas are covered with croplands, meadows, and to a smaller extent peatlands and marshes (LORENS et al. 2015). Land use is mostly extensive with the largest city, Tomaszów Lubelski, hosting 20,000 inhabitants.

Fieldwork

Our survey focused on the central and eastern parts of Polish Roztocze in the spring seasons (mostly March and April) of 2018–2020, encompassing an area approximately 60 × 15 km. We first identified potential breeding habitats (small water bodies and wetlands) using satellite imagery and topographic maps (Google Earth Pro, geoportal.gov.pl). We typically surveyed a particular breeding site only once; however, some sites in Roztocze National Park were checked repeatedly. At a study site, all species of amphibians encountered as well as habitat characteristics were documented.

Rana dalmatina can be found in terrestrial habitats throughout the vegetative months, but is most conspicuous during the breeding season (~ two weeks in late March/early April) due to its distinctive egg clutches and deposition sites. Freshly laid clutches are composed of tightly connected eggs, always in single batches of globular shape, and are often attached to submerged branches or aquatic plants (BONK 2012, BONK et al. 2012). After several days, they will float to the surface and may turn greenish due to the formation of algae in the gelatinous envelopes, remaining more or less round in shape throughout embryonic development (Fig. 2; BAUMGARTNER et al. 1996, BONK et al. 2012). In contrast, common and moor frogs typically lay clutches in aggregations near the edge of a pond, in shallow water or on submerged vegetation. During their development, these clutches become disassembled, irregular in shape, and often merge with adjacent egg clumps, while al-

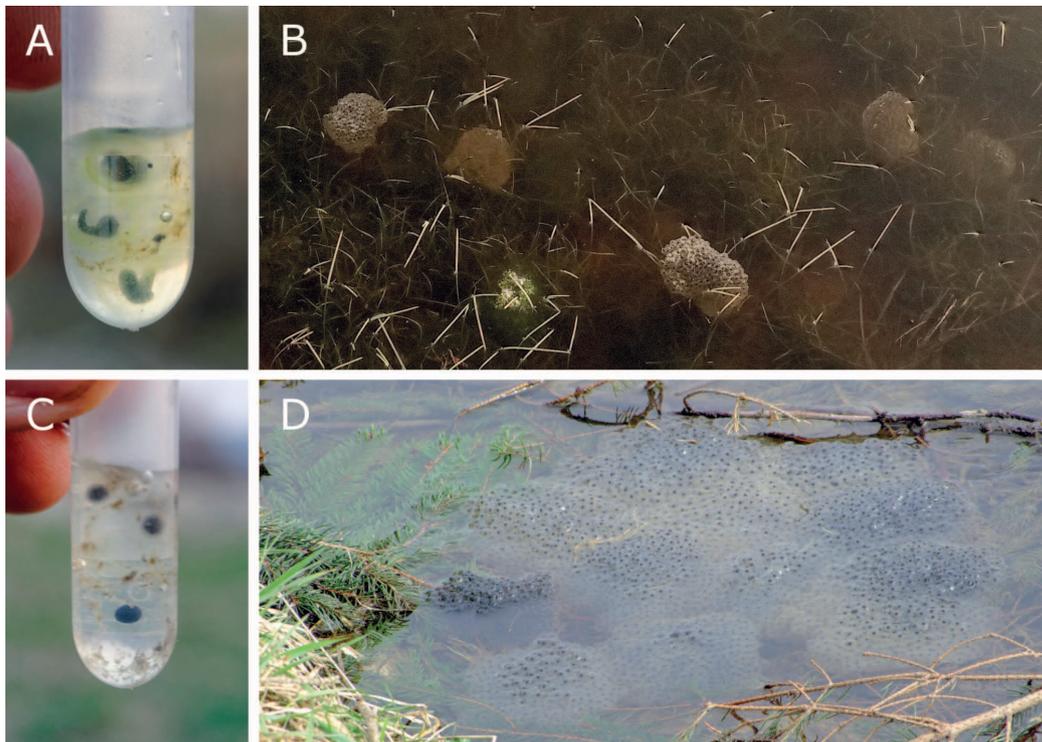


Figure 2. Comparison of eggs/embryos (A, C) and egg clutch deposition (B, D) of *Rana dalmatina* (top row) and *Rana temporaria* (bottom row). Note green layer of algae surrounding the embryos of *R. dalmatina*. Photos: A, C, D by B. ZAJĄC, B by P. STACHYRA.

gae rarely proliferate in the egg envelopes (BAUMGARTNER et al. 1996). Our spring surveys were timed as to coincide with the end of the breeding season of *R. dalmatina*, increasing the probability of detecting egg clutches of this species. We collected 1–3 eggs from clutches at study sites in which frog spawn was detected. Eggs were fixed in tubes with 95% ethanol.

Molecular identification

We extracted genomic DNA from a total of 59 samples. First, we rehydrated the eggs in deionised water and then manually removed the gelatinous capsule. The embryos were then digested singly in digestion buffer with proteinase K at 55°C under shaking. Genomic DNA was further purified using either a standard phenol/chloroform method or the solution-based Wizard Genomic DNA Purification Kit of Promega.

We amplified a fragment of the mitochondrial ND1 gene using the primers Pel6367F (5'-CACTCTATC-CAGCGAGCTTC-3') and ND1daH (5'-AAAATCAGCG-GGTRAATATCAC-3'). This reaction produces amplicons differing in the number of base pairs of amplified mtDNA for *R. dalmatina*, *R. temporaria* and *R. arvalis*. The species-specific amplification patterns can be scored on agarose gels, without the need for sequencing (Fig. 3). The PCR reaction contained 2.5 µl 10× polymerase buffer (Fermentas), 2.5 µl of 25 mM MgCl₂, 0.5 µl of 10 mM dNTP, 1 µl of 10 µM primers, and 0.2 µl of Taq polymerase; 1 µl of diluted genomic DNA suspension (1:50) and PCR-grade water was added to 25 µl in total volume. Positive controls for all three brown frog species, as well as negative controls with no DNA, were used for amplifications. The PCR encompassed an initial denaturation at 95°C for 3 min., and each of 35 PCR cycles entailed denaturation at 94°C for 30 sec., annealing at 56°C for 30 sec., extension at 72°C for 40 sec., and a final extension step at 72°C for 10 min.. Electrophoresis was conducted on 2% agarose gels at 80 V and 20 mA for 20–30 minutes. We confirmed the agarose-based identification of frogs by Sanger-sequencing the ND1/ND2 genes according to BONK et al. (2012) in a subset of samples.

Results

Our PCR method based on scoring differences in band sizes reliably differentiated between the three brown frog species, producing amplicons of 193 bp for *R. dalmatina*, 209 bp for *R. temporaria*, and 208 bp for *R. arvalis*, in addition to two larger fragments (Fig. 3). The difference between *R. dalmatina* and the two other species could be discerned by eye on agarose gels. Sanger-sequencing of ND1/ND2 in 14 of the samples fully confirmed the scoring based on band patterns. The obtained sequences were identical to homologous sequences of *R. dalmatina* from other regions of southern Poland (Sandomierz Basin, Bieszczady Mountains; sequences JX481918–JX481930 in NCBI GenBank).

We used the PCR-based method to verify species identity of egg samples from brown frog clutches in the Roztocze region. *Rana dalmatina* egg clutches were detected in 40 out of 121 surveyed water bodies (Table 1, Fig. 4). The number of egg clutches in particular ponds varied from 1 to 75 (mean: 6). Most of the breeding sites of *R. dalmatina* were located within the Roztocze National Park (RNP, N = 30), along the Wieprz River valley and close to Bór village (sites 13–27, 43, 45, 47–55). Other sites (28–30, 32–33) were mostly 3–10 km east of RNP (villages of Adamów, Suchowola and Zaboreczno). More distant sites (104, 108, 109, 116) were found in proximity (1.2–3.5 km) to the Polish-Ukrainian border, near the villages of Hrebenne and Werchrata.

The majority of *R. dalmatina* breeding sites were situated in mixed forests (67%). Others were found in meadows and cropland, in the proximity of forest (mean: 44 m from the forest edge, max. distance: 330 m). Most of the water bodies containing *R. dalmatina* eggs were small: 19 out of 40 breeding ponds were smaller than 100 m², and only 5 were larger than 1000 m² (range: 10–20,000 m²). Four types of *R. dalmatina* breeding sites, representative of the variety present in the Roztocze region, are depicted in Figure 5. Co-occurring amphibians (Table 1) includ-

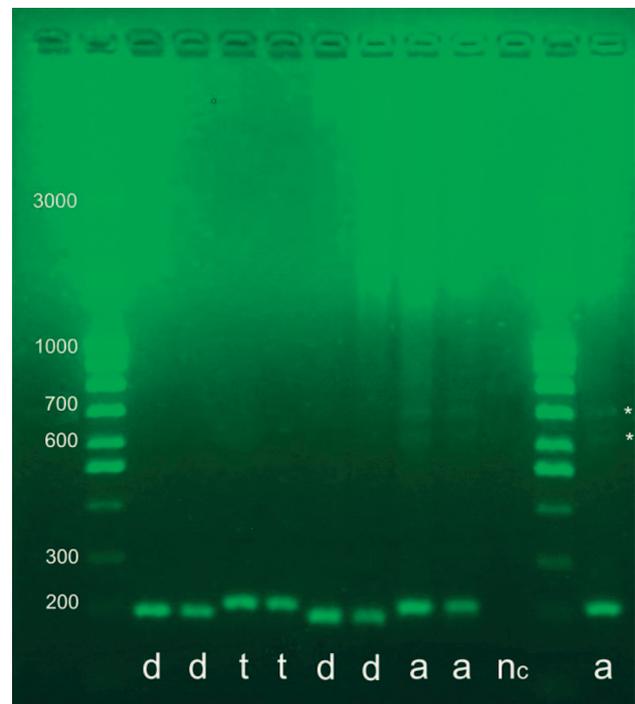


Figure 3. Photograph of an agarose gel preparation illustrating the size differences in PCR amplicons of a fragment of the mitochondrial ND1 gene used for rapid molecular identification of three European brown frog species. Lanes are labelled according to species: d – *Rana dalmatina*, t – *R. temporaria*, a – *R. arvalis*, n_c – negative control. Two lanes contain the GeneOn Rainbow DNA ladder with the sizes (in base pairs) of selected bands labelled. Asterisks depict two additional weakly but consistently amplified products in *R. arvalis*. Gel stained with Midori Green (Nippon Genetics).

Extension of the range of *Rana dalmatina* in southern Poland

Table 1. Descriptions of breeding sites and numbers of observed egg clutches of *Rana dalmatina* found in the Roztocze region of southeastern Poland in 2018–2020. Site numbers refer to localities depicted in Figure 5 (only those hosting *R. dalmatina* are listed). Amphibian species: *Bb* – *Bufo bufo*, *Bbo* – *Bombina bombina*, *Ho* – *Hyla orientalis*, *Lv* – *Lissotriton vulgaris*, *Pe* – *Pelophylax esculentus* complex, *Pf* – *Pelobates fuscus*, *Ra* – *Rana arvalis*, *Rd* – *Rana dalmatina*, *Rt* – *Rana temporaria*, *Tc* – *Triturus cristatus*. * – first documented observation after WW II, one adult individual caught near road during spring migration.

Site	Latitude (N)	Longitude (E)	Clutches	Amphibian species	Aquatic habitat (ca.)	Terrestrial habitat
13	50.6371	23.0426	1	<i>Rd, Rt</i>	pond (30 m ²)	mixed forest
14	50.5718	23.0358	1	<i>Rd</i>	pond (120 m ²)	mixed forest
15	50.6311	23.0557	8	<i>Rd, Bb, Lv, Pe, Rt</i>	pond (700 m ²)	meadows, mixed forest
16	50.6278	23.0514	5	<i>Rd, Bb, Rt</i>	peat pond (400 m ²)	mixed forest
17	50.6257	23.0591	30	<i>Rd, Bb, Lv, Pe, Pf, Rt, Ra, Tc</i>	marsh	mixed forest
18	50.6276	23.0690	12	<i>Rd, Bb, Bbo, Lv, Rt, Tc</i>	pond (60 m ²)	meadows, mixed forest
19	50.6264	23.0671	40	<i>Rd, Bb, Lv, Ra, Rt, Tc</i>	marsh	mixed forest
20	50.6233	23.0655	30	<i>Rd, Bb, Lv, Pe, Pf, Ra, Tc</i>	marsh	mixed forest
21	50.6228	23.0647	6	<i>Rd, Bb, Lv, Pe, Pf, Ra, Tc</i>	peat pond (800 m ²)	mixed forest
22	50.6223	23.0657	18	<i>Rd, Bb, Lv, Pf, Ra, Rt, Tc</i>	peat pond (60 m ²)	mixed forest
23	50.6221	23.0664	10	<i>Rd, Bb, Rt</i>	peat pond (800 m ²)	mixed forest
24	50.6167	23.0389	24	<i>Rd, Bb, Lv, Pf, Rt, Tc</i>	peat pond (100 m ²)	mixed forest
25	50.6167	23.0451	1	<i>Rd</i>	pond (200 m ²)	mixed forest
26	50.6168	23.0468	1	<i>Rd, Bb, Lv, Pe, Pf</i>	peat pond (50 m ²)	mixed forest
27	50.6187	23.0975	1	<i>Rd</i>	peat pond (60 m ²)	mixed forest
28	50.6089	23.1462	2	<i>Rd</i>	pond (10 m ²)	mixed forest
29	50.5999	23.1565	75	<i>Rd</i>	pond (110 m ²)	crops, meadows, mixed forest
30	50.6005	23.1567	50	<i>Rd, Rt</i>	pond (160 m ²)	crops, meadows, mixed forest
32	50.5841	23.2309	10	<i>Rd</i>	peat pond (100 m ²)	meadows, village, mixed forest
33	50.5898	23.2439	2	<i>Rd</i>	pond (150 m ²)	crops, meadows, village
34	50.5634	23.3236	40	<i>Rd</i>	pond (700 m ²)	mixed forest
43	50.5988	23.0633	8	<i>Rd</i>	pond (70 m ²)	meadows, river valley, pine forest
45	50.5905	23.0598	10	<i>Rd</i>	pond (80 m ²)	meadows, pine forest
46	50.5855	23.0775	1	<i>Rd</i>	pond (40 m ²)	meadows, mixed forest, village
47	50.5854	23.0590	6	<i>Rd, Bb, Lv</i>	pond (400 m ²)	meadows, mixed forest
48	50.5830	23.0577	3	<i>Rd, Bb, Lv</i>	pond (30 m ²)	mixed forest, shrubs, meadows
49	50.5821	23.0566	26	<i>Rd, Bb, Lv</i>	pond (50 m ²)	mixed forest, shrubs, meadows
50	50.5700	23.0496	2	<i>Rd</i>	pond (60 m ²)	mixed forest
51	50.5753	23.0493	38	<i>Rd, Bb, Lv, Pe, Pf</i>	pond (80 m ²)	mixed forest, meadows
52	50.5749	23.0497	2	<i>Rd, Bb, Lv, Pe, Pf</i>	pond (60 m ²)	mixed forest
53	50.6346	23.0438	3	<i>Rd</i>	pond (60 m ²)	meadows, mixed forest
54	50.5803	23.0505	3	<i>Rd</i>	pond (40 m ²)	mixed forest
55	50.5769	23.0260	6	<i>Rd</i>	pond (200 m ²)	mixed forest
67	50.5619	22.9993	10	<i>Rd, Bb, Ho, Lv, Pe, Pf</i>	pond (6400 m ²)	mixed forest
70	50.5492	23.0249	3	<i>Rd</i>	pond (70 m ²)	mixed forest
71	50.5438	22.9989	6	<i>Rd, Bb, Tc</i>	peat pond (800 m ²)	mixed forest
104	50.3445	23.4365	5	<i>Rd</i>	wheel rut	mixed forest
108	50.3010	23.4024	3	<i>Rd, Pe</i>	ditch (10 m ²)	peatland, mixed forest
110	50.2993	23.5547	1	<i>Rd, Ra</i>	marsh	alder carr
116	50.2449	23.5061	23	<i>Rd, Bb, Pe, Rt</i>	pond (1000 m ²)	meadow, railway station
	50.6224	23.0577	*	<i>Rd</i>	–	mixed forest, shrubs, crops

ed *R. temporaria* and *R. arvalis*, which were present in 44 and 40 sites, respectively. We also observed common toads (*Bufo bufo*), water frogs (*Pelophylax esculentus* complex), smooth newts (*Lissotriton vulgaris*), great crested newts (*Triturus cristatus*), common spadefoots (*Pelobates fuscus*),

and fire-bellied toads (*Bombina bombina*). In many cases (26), *R. dalmatina* was the only brown frog species occurring at a site. In eight ponds, *R. dalmatina* coexisted with *R. temporaria*, in three with the moor frog; all three brown frog species were detected in three ponds in RNP.

Discussion

Our findings constitute a northeastern extension of the known range of *R. dalmatina* by ca. 100 km, covering the Roztocze region of southeastern Poland. Our fieldwork resulted in the discovery of 40 previously unknown breeding sites, showing that *R. dalmatina* is widespread in this area, and may even be abundant in some places. For instance, Roztocze National Park (RNP) contains at least 30 breeding sites, rendering it a regional stronghold for this species. Moreover, we found breeding sites of *R. dalmatina* outside of RNP, scattered across the Roztocze region and reaching the Ukrainian border. These findings suggest that *R. dalmatina* may be more widespread in the surrounding regions (e.g., western Ukraine) than currently recognized. The relatively short study period and the atypically dry weather in the spring seasons of 2019 and 2020 lowered the chances of our detecting this species, and therefore our results may underestimate the distribution of *R. dalmatina* in this area.

Revised distribution of *R. dalmatina* at its northeastern range boundary

The closest current records of *R. dalmatina* are located in the foothills of the Carpathian mountains approximately 80 km south of Roztocze (RAFIŃSKI et al. 1987, C. DUFRESNES & T. SUCHAN unpubl. data), in lowlands ca. 110 km to the southwest near the town of Kolbuszowa (M. BONK unpubl. data), and ca. 130 km to the southeast near the towns of Stryj, Kałusz and Bursztyn in Ukraine (SMIRNOV 2013, SMIRNOV and BUCHKO 2018). An earlier observation from 120 km to the east of Roztocze, in Pieniaki village in Ukraine (BAYGER 1937), has never since been confirmed (SMIRNOV 2013).

It is unknown at present whether the Roztocze population is an extension of the contiguous range of *R. dalmatina* in the Carpathian foothills of southeastern Poland and western Ukraine, or whether it is a peripheral, isolated enclave of this species. Unfortunately, mitochondrial sequence variation of *R. dalmatina* is nearly non-existent at its northeastern range boundary (BONK et al. 2012, this study), low across the entire distribution of this spe-

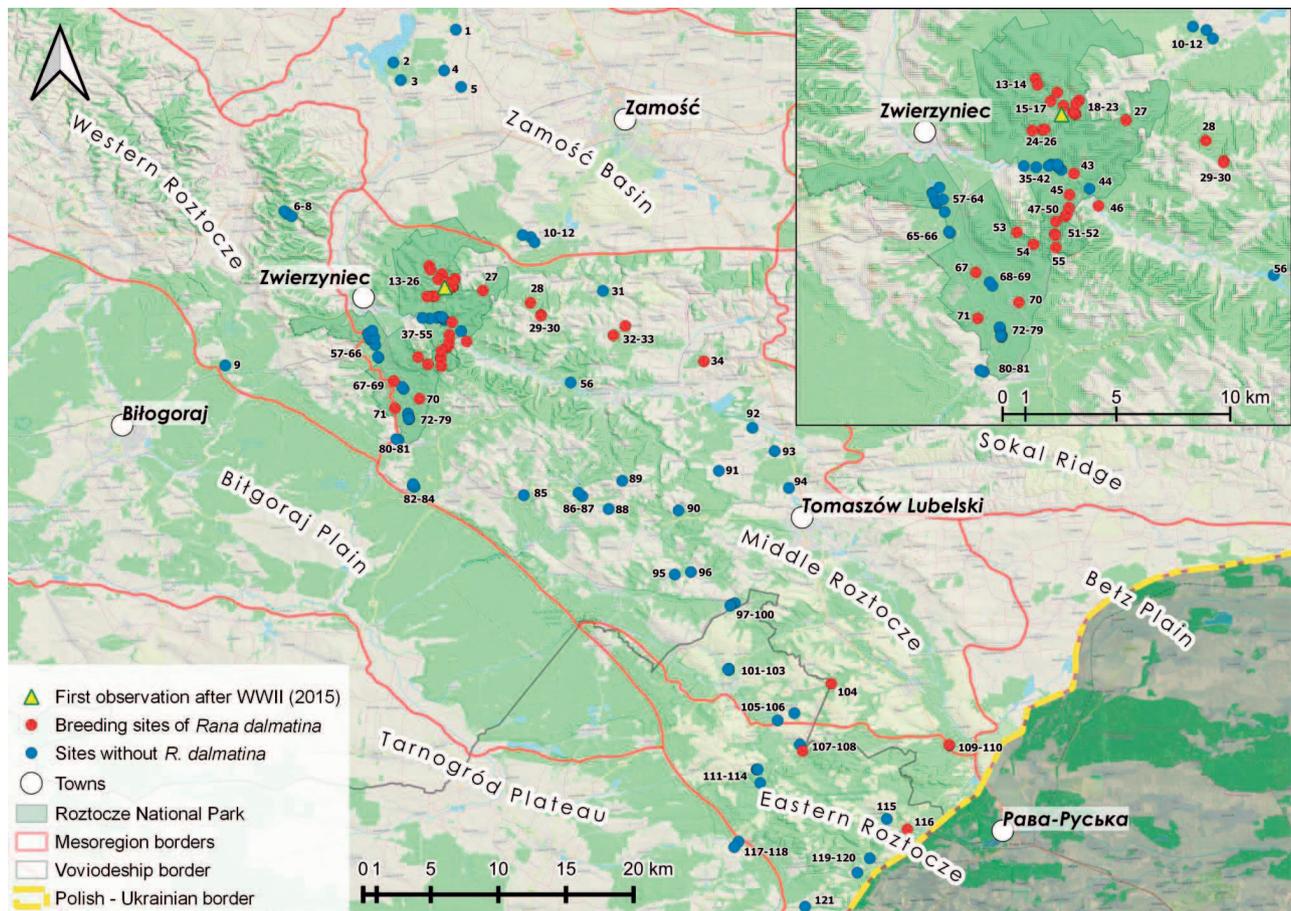


Figure 4. Map of Roztocze and adjacent regions in southeastern Poland depicting the locations of water bodies surveyed in this study. Inset shows the density of the surveying effort in the vicinity of Roztocze National Park at a finer scale. Mesoregion borders depicted according to SOLON et al. (2018).

cies (VENCES et al. 2013), and thus is unhelpful in differentiating between these scenarios. Amphibian surveys in wooded areas immediately south of the new localities (B. ZAJĄC & J.M. SZYMURA unpubl. data) have failed to detect this species, suggesting that the Roztocze *R. dalmatina* population is indeed spatially detached from other inhabited areas. Currently, a wide (ca. 40 km) expanse of intensively cultivated land between the Carpathian Mountains and the Roztocze region and neighbouring forests probably imposes a significant migration barrier for this species, as is suggested by the lack of *R. dalmatina* populations in this area. We conclude that the northeastern periphery of the range of *R. dalmatina* is spatially fragmented into three centres of distribution: (I) Upper Odra River Valley in the Czech Republic and southern Poland, (II) the foothills north and east of the Carpathian Mountains in Poland and Ukraine, respectively, along with adjacent depressions (i.e., Sandomierz Basin of southeastern Poland), and (III) the Roztocze region. Within these centres, *R. dalmatina* breeding sites are concentrated around larger forest complexes with some smaller subpopulations being scattered across the landscape, reflecting the fragmented forest

habitat (BONK et al. 2012). Similarly, most of the breeding sites of *R. dalmatina* in the Roztocze region were detected within the heavily forested RNP, with only a few being located outside of the park in smaller habitat patches. This pattern is characteristic for range peripheries (SEXTON et al. 2009) and is reminiscent of other amphibian range borders in southern Poland (e.g., that of the alpine newt [PABIJAN & BABIK 2006] and of the fire salamander [NAJBAR et al. 2015]), as well as for *R. dalmatina* populations in other parts of Europe, e.g., in Sweden (AHLÉN 1997).

Environmental features of *R. dalmatina* breeding habitats

Common features of *R. dalmatina* breeding sites included proximity to forest, small surface area of water bodies, relatively low depth, a lack of fish, and dependence on rainfall (Table 1). Typical *R. dalmatina* breeding habitats in the Roztocze region include vernal pools completely or partially surrounded by forest or woodland, inundated peat excavation sites, larger water bodies such as marshes, as



Figure 5. Examples of *Rana dalmatina* breeding sites in the Roztocze region of southeastern Poland: (A) Vernal pool at the edge of the forest (site 43); (B) temporary pond in a meadow (site 47); (C) vernal pool in mixed pine-fir-beech forest (site 51); (D) wheel rut on forestry road in mixed beech-pine-fir forest (site 104). Photos: A by S. BANACH, B, C by P. STACHYRA, D by B. ZAJĄC.

well as small wheel ruts on forest roads temporarily filled with water. Our data as well as a previous study (BONK et al. 2012) suggest that the presence of large stands of mixed or deciduous forest may be decisive in shaping the distribution of this species in southern Poland. In this region, open agricultural land seems to be largely avoided by *R. dalmatina*, at least for breeding. Likewise, HARTEL et al. (2009) identified a strong positive effect of connectivity and proximity to forest on the numbers of egg masses at breeding sites in Romania. In central France, PONSÉRO & JOLY (1998) found that most egg masses of *R. dalmatina* were deposited within 100 m from forest edges, while LESBARRÈRES & LODÉ (2002) found evidence for higher fecundity at breeding sites within, or close to, woodland compared to those located in non-forested habitats. Breeding in close proximity to woodland may benefit *R. dalmatina* in several ways. First, woodland may buffer extreme weather events, particularly in early spring for breeding adults and in early summer for emerging froglets. Forested areas also provide cover for dispersing amphibians (ROTHERMEL & SEMLITSCH 2002). Third, woodland contributes to a more heterogeneous habitat with refuges for frogs during their active season and hibernation habitats during the cold months. Moreover, woodland may influence the abiotic properties of vernal pools, such as water chemistry and temperature, as well as the biological communities inhabiting the water bodies, with potentially important effects on frog eggs and tadpoles (TESAR et al. 2008). For instance, the presence of emergent vegetation at breeding sites has a strong positive effect on the number of *R. dalmatina* clutches; however, clutch numbers decline in ponds with > 50% macrophyte cover (HARTEL et al. 2009). We suggest that a distribution modelling approach is needed to improve our understanding of *R. dalmatina* and its habitat at the northern periphery of its range.

Despite the presence of three brown frog species in the study area, we only occasionally observed two or more species breeding in the same water body (Table 1), suggesting a degree of habitat selectivity by the particular species. BARTOŃ & RAFIŃSKI (2006) showed that *R. dalmatina* can tolerate a wide spectrum of ecological conditions in breeding ponds. *Rana temporaria* is less willing to breed in ditches, ponds with high acidity (peat ponds), or in shallow, vernal pools located inside woodland. Moreover, interactions between species within breeding ponds (the breeding periods of *R. dalmatina* and *R. temporaria* partly overlap in Central Europe) may limit their syntopic occurrence. Males of both species may attempt to mate with females of the other species, with *R. temporaria* males dominating *R. dalmatina* (HETTYEY et al. 2009). *Rana temporaria* males can even reduce the reproductive success of *R. dalmatina* due to reproductive interference (HETTYEY et al. 2014). Competition between species can also occur during larval development as a consequence of limited trophic resources in small ponds. Larval development of *R. dalmatina* may be twice as long as that of *R. temporaria* (RIIS 1991, BAUMGARTNER et al. 1996), making *R. dalmatina* tadpoles more susceptible to poor conditions and desiccation.

Thus, the wider ecological spectrum of *R. dalmatina* may not be caused by breeding habitat preferences, but rather by competition and displacement from optimal ponds by *R. temporaria*.

Regional threats and protection measures

Rather fortuitously, we documented 30 previously unknown breeding sites of *R. dalmatina* within the Roztocze National Park, a protected area subject to strict conservation policies safeguarding the future of this population group, and 10 sites outside of the park. Considering the peripheral status of these populations and their possible role under various climate change scenarios (ARAÚJO et al. 2006), our field survey provides crucial information for the conservation of this species at both national and range-wide levels. Moreover, our results emphasize the significance of protected areas as amphibian population reservoirs. RNP is characterized by extensive forest (93% of its area) with limited impact of silviculture practices, and with many parts being close to natural old-growth forests (up to 200 year-old stands). Because *R. dalmatina* is dependent on deciduous or mixed forest, factors such as intensive forest use and habitat conversion are detrimental to its populations (ZAVADIL 1997, PONSÉRO & JOLY 1998, LESBARRÈRES et al. 2006, HARTEL et al. 2009, BONK et al. 2012), but are negligible in RNP. Currently, RNP is the only national park in Poland with a sizeable population of *R. dalmatina* (this species was also recently confirmed in Magura National Park in the Carpathian Mts. of southern Poland; B. ZAJĄC, unpubl. data). Regional threats to *R. dalmatina* include road mortality and drainage or conversion of non-protected habitats outside of RNP, as well as increasingly severe droughts decreasing the hydroperiod of water bodies throughout this area of Central Europe. The widespread use of agrochemicals in crop cultivation in southeastern Poland (SZPYRKA et al. 2015) may also compromise amphibian health and persistence in this region. Recently, agricultural land use was linked to a high incidence of sex ratio reversal (female to male) in *R. dalmatina* populations (NEMESHÁZI et al. 2020) that has the potential of reducing effective population sizes with consequences for population viability.

Conclusions

Our study demonstrates that *R. dalmatina* is present and surprisingly common in the Roztocze Upland. This discovery emphasizes the necessity of reviewing and reconfirming old records of rare species in Central Europe, even if they seem improbable. Our findings extend the known range of *R. dalmatina* by ca. 100 km and add a northeastern cluster of peripheral populations that are most likely isolated from the continuous range of the species farther south. It remains uncertain whether these are relict populations or a result of a recent range extension,

however the existence of (unconfirmed) older localities suggests the former. Future studies should focus on surveying other potential areas of occurrence of this species in southeastern Poland and northwestern Ukraine to identify the extent of population fragmentation of this species. Moreover, our observations support the important role of strict protection of natural habitats in preserving amphibian populations, as most *R. dalmatina* breeding sites were concentrated within and around Roztocze National Park.

Acknowledgements

We thank ROBERT CYMBAŁA for providing information on his sighting of *Rana dalmatina* in 2015. We thank the Students' Naturalists Society (Koło Przyrodników Studentów UJ) for help in the field, the Roztocze National Park administration for logistical assistance during fieldwork, and the Student Association Council of Jagiellonian University for financing our field trip in 2019. Funding was obtained from statutory resources of Jagiellonian University (K/ZDS/008060), the Voivodeship Fund for Environmental Protection and Water Management in Lublin (Wojewódzki Fundusz Ochrony Środowiska i Gospodarki Wodnej, 39/2019/D/OP), and Roztocze National Park statutory resources. Permits for amphibian surveys and collection of tissues were granted by the Ministry of Environment (decision DZP-WG.6401.02.12.2016.dł.4), and the General Directorate for Environmental Protection (decision DZP-WG.6401.02.6.2018.kp) in Poland, as well as by the Director of Roztocze National Park.

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