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NOTES ON THE DISTRIBUTION OF *DALMATINELLA SIMONAE* (GASTROPODA: HYDROBIIDAE) IN CROATIA AND THE UTILITY OF BARCODING IN ITS DETERMINATION

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ABSTRACT: *Dalmatinella simonae* is a recently described species from Lake Sladinac and the river Cetina (Croatia). New findings from the rivers Cetina, Neretva and Ruda indicate a wider distribution of this species especially in comparison with its relative *D. fluviatilis,* known only from a short section of the river Zrmanja. Its occurrence is also probable in the river Neretva in Bosnia and Herzegovina. Barcoding analysis, using mitochondrial cytochrome oxidase subunit I (COI), confirmed the occurrence of this species in new sites, and the nearly complete lack of genetic divergence.

KEYWORDS: barcoding; gastropod; habitat; Balkan; Cetina; Neretva

INTRODUCTION

DNA barcoding is a method allowing wide-scale and quick species identification using DNA sequences as molecular species-specific markers. Barcoding studies offer an opportunity to document biodiversity, using a short, standardized region of the genome to differentiate species – the mitochondrial cytochrome c oxidase I (COI) gene in animals. This region is variable enough to distinguish species in most cases, yet short enough to be sequenced cheaply (see BOLD, RATNASINGHAM & HEBERT 2007). This method is particularly useful for the analysis of the distribution of recently described species, for which there is no precise distribution data. For species that are difficult to distinguish by morphology, such as small, aquatic snails in the Hydrobiidae, this method is also helpful in confirming new locations where the species has been morphologically identified.

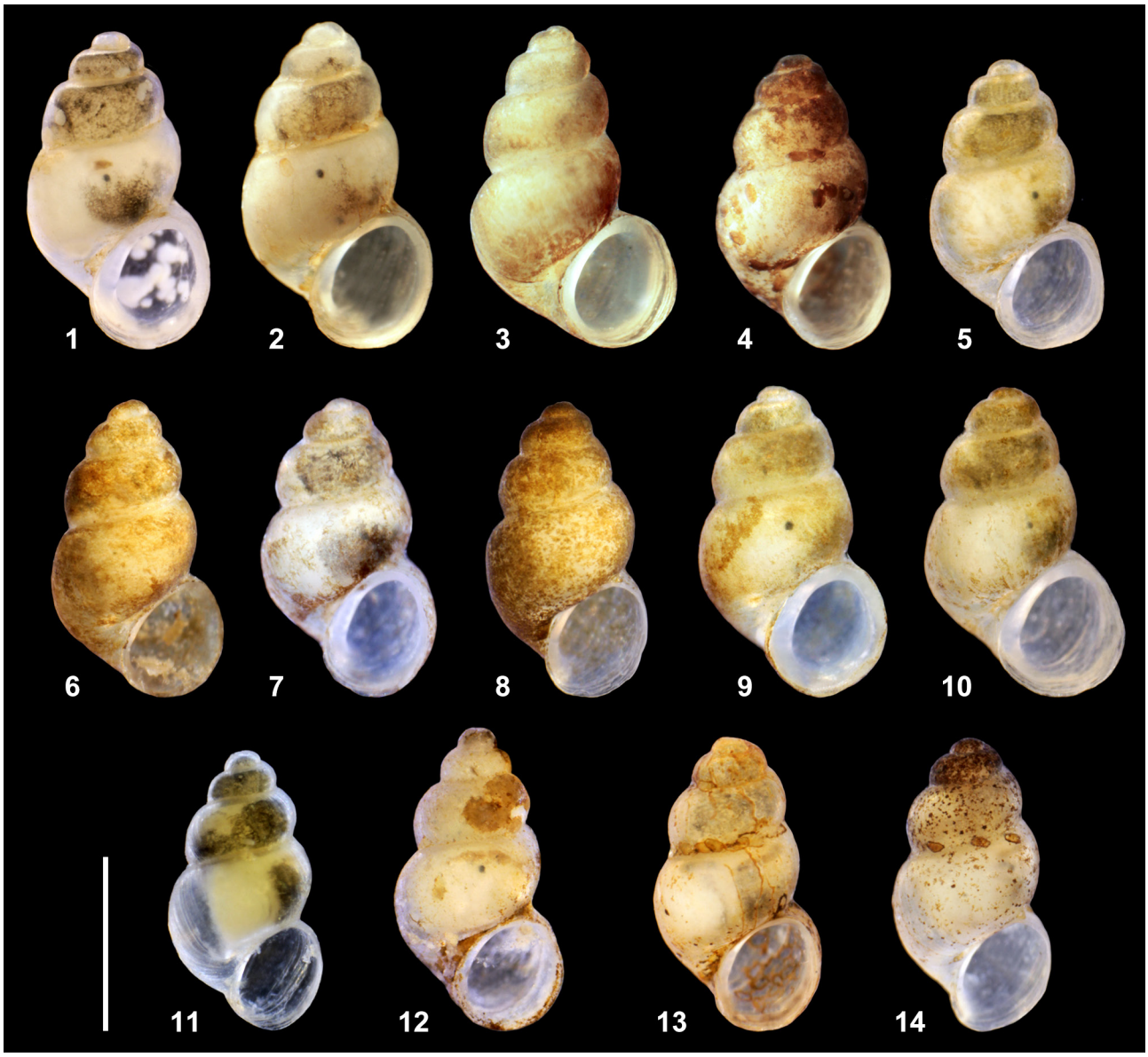
Two representatives of the genus *Dalmatinella* Radoman, 1973 (Hydrobiidae) are known so far. *Dalmatinella fluviatilis* Radoman, 1973 is an endemic species of the river Zrmanja in northern Croatia (FALNIOWSKI & SZAROWSKA 2013, BERAN 2021). *Dalmatinella simonae* Beran et Rysiewska, 2021 was recently described from two sites of the river Cetina and from Lake Sladinac both situated in the southeastern part of Croatia (BERAN et al. 2021). RADOMAN (1983) reported *Dalmatinella* (mentioned as *D. fluviatilis*) also from the river Neretva. These species are difficult to identify without DNA barcoding.

The main goal of our work was to confirm the occurrence of *D. simonae* in a much wider area than indicated in the first description, using barcoding methods, which will allow a certain identification.

MATERIAL AND METHODS

New occurrence records were obtained by the first author in the years 2020–2021. The rivers Cetina, Ruda and Neretva (only Croatian part) were studied. The main sampling method used was washing vegetation and sediments using metal sieves (diameter 20 cm, 0.8 mm mesh and/or diameter 10 cm and mesh 0.5 mm) combined with collections by hand from the surfaces of stones, wood and artificial materials (e.g. plastic bags and bottles). Snails were fixed in 80% analytically pure ethanol, replaced two times. Next, the snails were put in fresh 80% analytically pure ethanol and kept in −20 °C temperature in a refrigerator. Selected material of shells and specimens fixed in 80% ethanol is deposited in the first author's collection and in the collection of Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University in Kraków (Poland).

DNA was extracted from whole specimens; tissues were hydrated in TE buffer (3 × 10 min); then total genomic DNA was extracted with the SHERLOCK extraction kit (A&A Biotechnology), and the final product was dissolved in 20 µl of tris-EDTA (TE) buffer. The extracted DNA was stored at −80 °C at the Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University.



Figs 1–14. Shells of the sequenced specimens of *Dalmatinella*: 1–2 – locality 1 (2P56, 2i35), 3–5 – locality 5 (2i29, 2i30, 2P60), 6–7 – locality 6 (2P53, 2P54), 8 – locality 7 (2P61), 9–10 – locality 8 (2P37, 2P38), 11–12 – locality 9 (2P94, 2P95), 13 – locality 10 (2P50), 14 – locality 11 (2P58). Bar equals: 1 mm. Photo: ALEKSANDRA JASZCZYŃSKA

For species verification a fragment of COI was sequenced, as this fragment is using for animal barcoding studies (see BOLD, RATNASINGHAM & HEBERT 2007). Details of PCR conditions, primers used and sequencing were given in SZAROWSKA et al. (2016). Sequences were aligned with the MUSCLE algorithm (EDGAR 2004) in the program MEGA 7 (KUMAR et al. 2016) and then checked in BioEdit 7.1.3.0 (HALL 1999). Uncorrected p-distances were calculated in MEGA 7. The estimation of the proportion of invariant sites and the saturation test for entire data sets (XIA 2000, XIA et al. 2003) was performed using DAMBE (XIA 2013). In the phylogenetic analysis, *Montenegrospeum bogici* (FALNIOWSKI et al. 2014) was used as outgroup, and all known *Dalmatinella* sequences (FALNIOWSKI & SZAROWSKA 2013, BERAN 2021) were added. The GeneBank numbers of used sequences were given on the phylogenetic tree. The data were analysed using approaches based on Bayesian Inference (BI) and Maximum Likelihood (ML). In the BI analysis, the GTR + I + Γ model of nucleotide substitution was applied. This model was selected using MrModelTest 2.3 (NYLANDER 2004). The Bayesian analyses were run using MrBayes v. 3.2.3 (RONQUIST et al. 2012) with default of most priors. Two simultaneous analyses were performed, each with 10,000,000 generations, with one cold chain and three heated chains, starting from random trees and sampling the trees every 1,000 generations. The first 25% of the trees were discarded as burn-in. The analyses were summarised as a 50% majority-rule tree. Convergence was checked in Tracer v. 1.5 (RAMBAUT & DRUMMOND 2009). The Maximum Likelihood analysis was conducted in RAxML v. 8.2.12 (STAMATAKIS 2014) using the 'RAxML-HPC v.8 on XSEDE (8.2.12)' tool via the CIPRES Science Gateway (MILLER et al. 2010). We applied the GTR + I + Γ model, whose parameters were estimated by RaxML.

RESULTS

In addition to the three known sites (BERAN et al. 2021) specimens of genus *Dalmatinella* (Figs 1–14) were found at a further eight new sites. All known sites are shown in Figure 15 and listed in the Appendix 1. Five new sites were found in the river Cetina (sites 1, 2, 5–7, Figs 16, 17) while one new

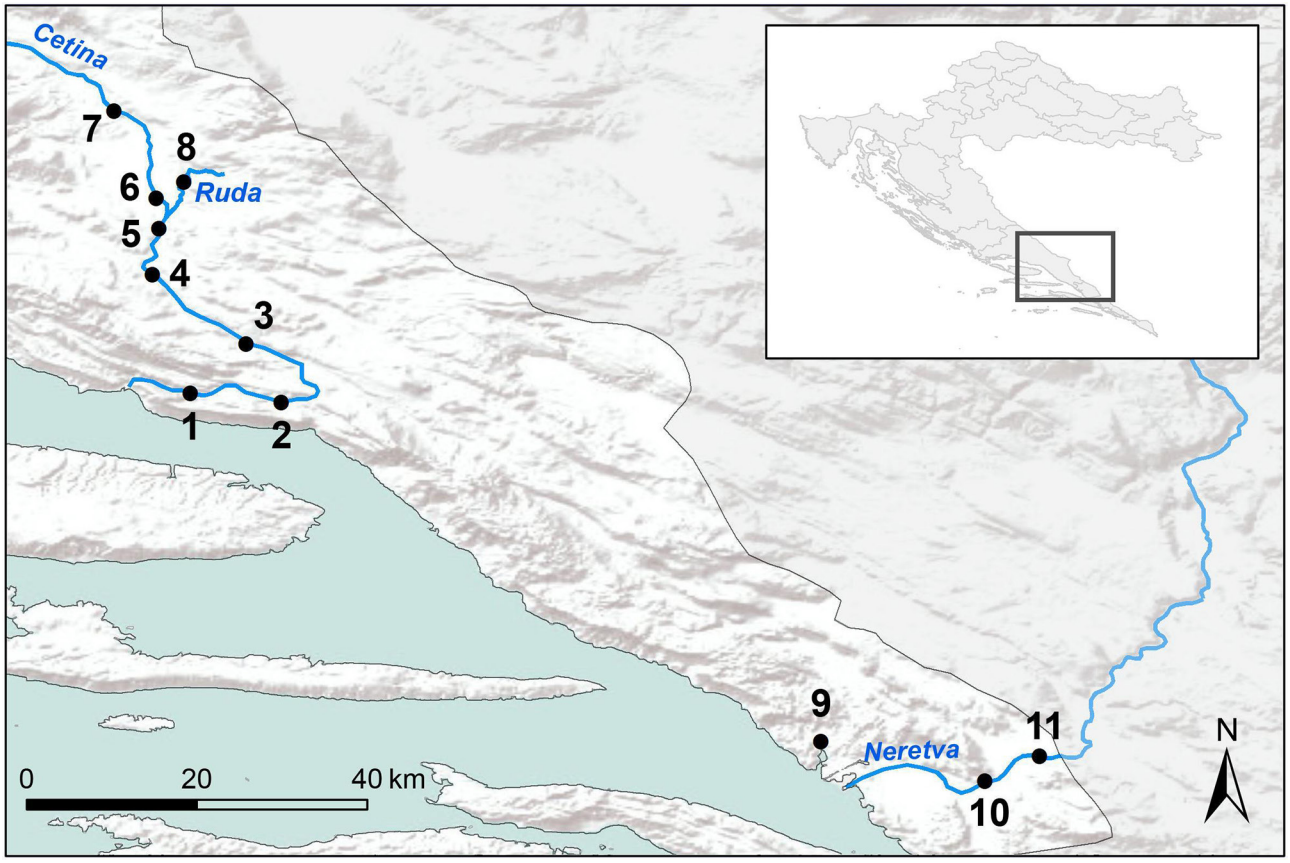


Fig. 15. The map of the southeastern part of Croatia with the geographical distribution of the sampling sites. Drawing H. MEDKOVÁ

Fig. 16. The river Cetina near Podgrađe (locality 2). Photo: LUBOŠ BERAN

Fig. 17. The Cetina upstream of Obrovac Sinjski (locality 7). Photo: LUBOŠ BERAN

Fig. 18. The river Ruda (locality 8). Photo: LUBOŠ BERAN

Fig. 19. Lake Sladinac (locality 9), the type locality of *D. simonae*. Photo: LUBOŠ BERAN

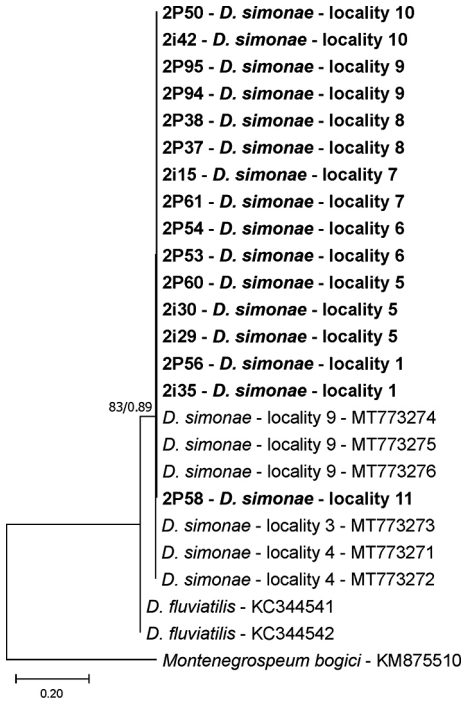


Fig. 20. Simplified COI tree of *Dalmatinella*: maximum likelihood (ML) phylogram based on COI. Bootstrap support and Bayesian posterior probabilities are shown, when bootstrap supports >65%. The sequences obtained in this study are marked in bold, for each isolate the number is given, as reported in the GenBank. For reference sequences the GenBank numbers are given (KC344541-KC344542 – FALNIOWSKI & SZAROWSKA 2013; KM875510 – FALNIOWSKI et al. 2014; MT773271-MT773276 – BERAN et al. 2021)

site was documented from its tributary Ruda (site 8, Fig. 18) and two from the Croatian section of the river Neretva (sites 10 and 11). Altogether, with published data, its occurrence was confirmed at seven sites on the river Cetina situated in an approximately 60 km long section between Obrovac Sinjski and the end of the freshwater part of this river north of Podašpilje near Omiš and also from its tributary the river Ruda. Only a very short section of the river Neretva is situated in Croatia. Two sites were found in this short section. The first site (site 10) corresponds to historic record of *D. fluviatilis* (RADOMAN 1983) while the second site (site 11) is situated near the border with Bosnia and Herzegovina.

In all cases this species was found on stones in the water. These stones were usually on the banks or in shallow places and it was often found together with other molluscs e.g. *Theodoxus fluviatilis* (Linnaeus, 1758), *Emmericia patula* (Brumati, 1838), *Radomaniola curta* (Küster, 1852), *Horatia klecakiana* Bourguignat, 1887. *D. simonae* was found predominantly in running waters but there are also records from stagnant waters such as lake Sladinac (type locality, site 9, Fig. 19) or the Cetina in the Prančevići dam reservoir (site 4).

The species delimitation, initially determined on the basis of morphology of the shells, was confirmed using molecular methods. The 16 new sequences of COI (457 bp, GenBank accession numbers ON682918-ON682933) confirmed that all the specimens studied belonged to *D. simonae* and that within the species there is nearly no haplotype diversity, the p-distance between the two observed haplotypes (one from the locality 3 and 4 and the other from rest localities) was only 0.003 (Fig. 20). However, the distinctiveness from *D. fluviatilis* is clear, and p-distance between these two species is 0.039.

DISCUSSION

New findings in the rivers Cetina, Ruda and Neretva expand the known geographic distribution of *D. simonae*. This species is thus much more widespread than the related species *D. fluviatilis*, which is known only from a short section of the river Zrmanja (BERAN 2021). New molecular data from the Neretva (sites 10 and 11) confirmed that the *Dalmatinella* specimens found by RADOMAN (1983) belong to *D. simonae* and not to *D. fluviatilis*. FALNIOWSKI & SZAROWSKA (2013) already assumed that specimens from the Neretva probably belong to a different species, however they did not have material from the Neretva available. With regard to the findings of *D. simonae* in the lower section of the Neretva in Croatia, its occurrence can also be expected upstream in the territory of Bosnia and Herzegovina and probably also in some tributaries of this river. In addition to small differences in shells and anatomy between both species described in BERAN et al. (2021) there are also habitat related differences. While *D. simonae* was found only on stones on banks or shallow places, *D. fluviatilis* seems to prefer vegetation and often occurs in deeper places (BERAN 2021). *D. fluviatilis* is known only from running waters while *D. simonae* also lives in stagnant waters.

Our research is a simple example of using the DNA barcoding. This method can be an effective tool for species discovery as well as specimen identification, which results in better knowledge of the species distribution. However, this method, using the COI only, has limitations, especially in the case of gene flow or introgression, or where species have only recently diverged. To solve this problem, a detailed molecular study using nuclear markers would be necessary (e.g. LIU et al. 2017), but in the case of *Dalmatinella*, the nuclear markers we usually use have too little variability. However, our previous research has clearly shown a relationship between differences in COI and differences resulting from shell biometry (BERAN et al. 2021) and therefore there is a high probability that COI barcoding indicates interspecies differences. Such relationships are often found (e.g. CHAN et al. 2014, LOPEZ-VAAMONDE et al. 2021). Using barcoding for species identification is possible when within species variation is lower than between species variation. The studies on the hydrobiid snails clearly indicate that this condition is met (e.g. WILKE et al. 2010, SZAROWSKA et al. 2016, FALNIOWSKI et al. 2021). In our study p-distance between *D. simonae* and *D. fluviatilis* is 0.039, within *D. simonae* 0.003.

Despite all the limitations, in most cases COI barcoding results in successful species identification and the COI has become the most commonly used marker for animal DNA barcoding (LIU et al. 2017 and literature mentioned therein). Many DNA barcodes are now available in international databases, such as the Barcode of Life Data Systems BOLD (RATNASINGHAM & HEBERT 2007, 2013), but for many species data is still limited and restricted only for part of the species' range, so each study of this kind can boost biodiversity inventories and environmental monitoring as well as constitute a useful tool in taxonomy, ecology, agriculture and conservation. The range of many Hydrobiidae genera appears to be limited, as in *Dalmatinella*; only some have a wider distribution, like *Bythinella* (WILKE et al. 2010), *Radomaniola* (DELICADO & HAUFFE 2022) or *Montenegrospeum* (FALNIOWSKI et al. 2021). Our

data may indicate that the ranges of other genera are also not limited, and barcoding is an extremely useful method for identifying individuals, regardless of their morphology, life stage, size, etc.

Barcoding can also be applied to the protection of biodiversity. *D. simonae* is a recently described species that is not yet listed in the IUCN Red List. Although it is more widespread than *D. fluviatilis* and appears to be less endangered, we propose that it should be included among the endangered species in the further version of the IUCN Red List. The species meets criterion B of the IUCN criteria (IUCN 2021). Its extent of occurrence is less than 5000 km^2 (including expected occurrence in Bosnia and Herzegovina) and its area of occupancy is less than 500 km^2. At the same time, its known distribution is fragmented.

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APPENDIX 1

LIST OF LOCALITIES OF *DALMATINELLA SIMONAE*

Data in the list are as follows: site number, geographical co-ordinates, name of the nearest settlement, description of the site and habitat, number of recorded specimens, date of investigation, citation in the case of published data. Sites are depicted at Fig. 15

Cetina

1 – 43°26'14.3"N, 16°45'27.3"E, Podašpilje, the Cetina to the north of Podašpilje, stones in the shallow places by the river bank, 12 specimens, 19.8.2020;

2 – 43°25'39.8"N, 16°51'12.8"E, Podgrađe, the Cetina approx. 800 m downstream of the bridge, stones in the shallow places by the river bank, 14 specimens, 8.7.2021;

3 – 43°29'22.6"N, 16°48'59.8"E, Trnbusi, the Cetina to the east of hill Gradina (308 m a. s. l.), stones in the shallow places by the river bank, 1 specimen, 22.8.2019 (BERAN at al. 2021);

4 – 43°33'44.9"N, 16°43'03.8"E, Donja Rošca, the Cetina in the Prančevići dam reservoir, stones on the bank of the dam reservoir, about 40 specimens, 29.8.2018 (BERAN et al. 2021);

5 – 43°36'40.5"N, 16°43'28.4"E, Trilj, the Cetina downstream of Trilj, stones in the shallow places of the river, about 30 specimens, 18.8.2020;

6 – 43°38'34.9"N, 16°43'18.3"E, Košute, the Cetina to the northeast of Košute, stones in the shallow places by the river bank, 26 specimens, 16. 8. 2020;

7 – 43°44'06.8"N, 16°40'37.8"E, Obrovac Sinjski, the Cetina upstream of Obrovac Sinjski, stones in the shallow places by the river bank, 6 specimens, 18.8.2020.

Ruda

8 – 43°39'35.9"N, 16°45'03.6"E, Grab, the river Ruda by a bridge to the north of Grab, stones in the shallow places of the river, 27 specimens, 8.7.2021.

Baćina lakes

9 – 43°04'11.8"N, 17°25'22.2"E, Baćina, the south part of lake Sladinac, one stone on the bank of the lake, approx. 30 specimens, type locality, 22.8.2019 (BERAN et al. 2021).

Neretva

10 – 43°01'41.6"N, 17°35'45.6"E, Kula Norinska, the Neretva between Krvavac and Kula Norinska, stones on the river bank, 12 specimens, 17.8.2020;

11 – 43°03'17"N, 17°39'13.4"E, Metković, stones on the river bank, 7 specimens, 17.8.2020.