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Original Research

Discovering the Rare *Tolypella intricata* (Trentepohl ex Roth) Leonh. 1863 (Charales, Charophyceae) in Poland

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Abstract

Tolypella intricata is one of the rarest charophytes from the genus *Tolypella* that can be found both in Poland and in Europe. Rarely found in the last 200 years, it was recently rediscovered in two localities in eastern Poland in the Lublin region. In this article, we present two new locations of *T. intricata* in Poland that make is a significant contribution to a closer understanding of the distribution of the biology and ecology of this species. The article provides basic information on the autecology of *T. intricata* together with the associated macrophyte vegetation. The morphology and ecology data are compared with the literature, along with a critical review of such information found in the literature. The results of molecular tests confirming the new position of *T. intricata* are also presented.

Keywords: biogeography, charophyta, distribution, ecology, *Tolypella intricata*, Poland

Introduction

In general, genus *Tolypella* (A.Braun), belonging to the class Charophyceae (order Charales, family Characeae), is one of the rarest genera in the whole class. Currently, 21 species are accepted taxonomically in the genus under the *Tolypella* species name [1]. Of this genus, species *Tolypella intricata* (Trentepohl ex Roth) Leonh. 1863 seems to be one of the rarest charophytes species in Europe and worldwide [2]. It was first discovered and collected probably in the vicinity of Berlin and Leipzig (Eastern Germany). The available information allows us to state that until the end of the 20th century, only a few sites where *T. intricata* was recorded were known. All currently known information on the distribution of *T. intricata* is provided in Table 1 and Fig. 1.

So far, there are no more recent data on the prevalence of *T. intricata* in Europe. Certainly, *T. intricata* is an extremally rare species that is not easy to find, and this is one reason why no new localities of this specimen have been identified. This study aims to report the two new records of *T. intricata* in Eastern Poland located in the Lublin Region. The discovered populations of *T. intricata* are described in detail and compared with literature data. This new finding of *T. intricata* is discussed as a significant contribution to the knowledge on this species biogeography.

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Materials and Methods

All data presented in Table 1 on the known locations of *T. intricata* were collected from information obtained using queries of herbarium collections (C, GB, GLM, H, JE, KRA, L, LD, NY (C.V. Starr Virtual Herbarium), POZ, WRSL, WAW) and literature data. Acronyms of herbaria are given after NYBG Index Herbariorum [3] Algae scientific names are given after AlgaeBase [1] and IPNI [4] The abbreviated author names for plant names are given as in IPNI [4] following recommendations of ICN [5]. Below, we describe the two more recently found localities of *T. intricata* in Poland. The species was found by accident during many years of monitoring research in the area of over a dozen or so kilometers along the Vistula River. In further studies, no other localitites of *T. intricata* occurrence were found. In the case of the specimen collected near Witkowice, molecular DNA analyses were performed to confirm the discovery of *T. intricata*. The algae from the locality near Witkowice was grown in artificial conditions (greenhouses) in 2019 from soil samples (diaspors) collected in early spring 2018. We have also used determination keys [6, 7] to confirm both findings.

Table 1. Summary of all information known so far on the occurrence of *T. intricata* in Europe

Locality	Herbarium code, References
Belgium	
1) East Flanders: Antwerp	NY
Denmark	
1) Sydsjylland	C, NY, Olsen [35]
France	
1) Centre-Val de Loire, Loir-et-Cher: Romorantin	NY, Corillion [36]
2) Nouvelle-Aquitaine, Gironde: Bordeaux	NY
3) Pays de la Loire, Sarthe: Le Chevain near Alençon, Sarthe Précigné	NY
	NY
Germany	
1) NSG Bislicher Inseln	U.Abts (private collection)
2) Brandenburg: Berlin, Schöneberg, Falkensee, Trebbin b. Berlin, Neuruppin vor Kränzlin	NY
3) Baden-Württemberg: Karlsruhe (Brechtsee)	NY, Weyer [29]
5) Saxen: Bienitz bei Leipzig	NY
6) Niederlausitz: Golßen	NY
6) Nordrhein-Westfalen: Krefeld	NY
Grece	
1) Attica	NY
Hungary	
1) Budapest-Rákosfalva	NY
Ireland	
1) close to Dublin	Moore [37]
Italy	
1) Gargano	WRSL
2) Lombardia	Bazzichelli and Abdelahad [38]
3) Veneto	Bazzichelli and Abdelahad [38]
4) Emilia-Romagna	Bazzichelli and Abdelahad [38]
Netherland	
various localities	Briunsma et al. [39]
Poland	
1) Warmia: Carlshof bei Altfelde	(C, GLM, H, LD, POZ, NY, W)
2) Wielkopolska: Valley of Warta River (Wielkopolska region)	Gąbka [40]
Spain	
1) Castilla - La Mancha: Cuenca, Torca del Agua	NY
Sweden	
1) Gotland:Hangvar, Lerdala, Slite,Visby,	NY
2) Øland	[41]
3) Skåne: Alnarp, Christianstad Lund, Trelleborg, Uppsala,	NY
Serbia	
1) Valjevac and Pačja	[42]
2) Zasavica	[43]

Table 1. Continued.

United Kindgom	
1) England, Cambridgeshire: Harston, Welches Downs, The Wash below Mepal, Sutton	NY, [37] NY
2) England, Yorkshire: Goole	NY
3) England, Bedfordshire: Brammingham near Luton	NY
4) England, Surrey: Egham	NY
5) England: Shortwood Common near Staines	NY
6) England: Gloucestershire	[44]

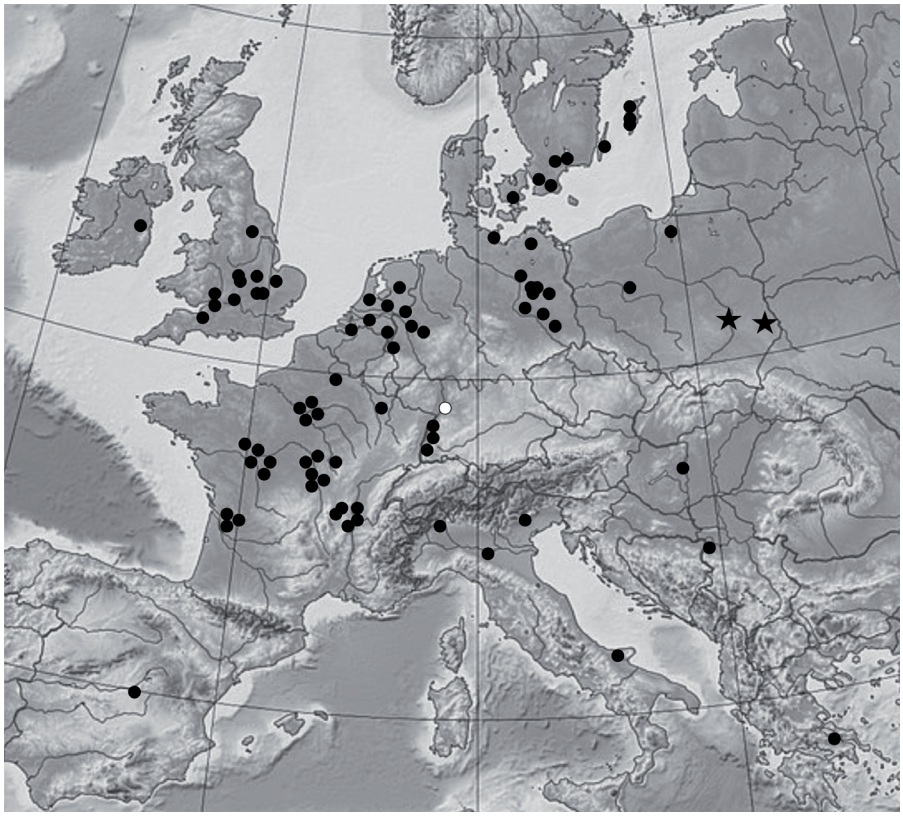

Fig. 1. Distribution of *T. intricata* in Europe - drawing based on Table 1 and contained therein references and data from Herbaria collection. Black dot - historical records<1945, white dot – record found near Karlsruhe, Germany at 2003 (NY), stars - recently found records (this paper).

Phylogenetic Research

The genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen; Hilden Germany), according to the manufacturer's protocol using Mixer Mill MM400 (Retsch; Haan, Germany) for plant tissue disruption. We have used *psb*C chloroplast DNA region that is used for phylogenetic studies in plants at all taxonomic levels that allowed confirmation of the newly sequenced cpDNA fragment from collected *T. intricata*, with present data available at NCBI (Gene Bank). The used region: *psb*C is often considered evolutionary conservative but employed in phylogeny and taxonomy, and some studies have found intraspecific variation in this biogeographically informative gene regions that has been contradicted [7]. PCR reaction mix included (in the total volume of 20 µl): 1U Taq recombinant polymerase (Thermo-Fisher Scientific), 10X Taq Buffer, 1 mM $MgCl_2$, 0.5 µM of each primer, 0.4 mM dNTP and 1 µl DNA template. PCR cycle was performed with a Veriti Thermal Cycler (Life Technologies, Carlsbad, CA, USA) with the following parameters: 8 min at 95ºC, followed by 30 cycles of 45 s at 95ºC, 45 s at annealing temperature (51.2ºC) and 1min at 72ºC, followed by a final extension step of 10 min at 72ºC. Prior to sequencing, PCR products were purified using GeneMATRIX PCR/ DNA Clean Up Purification Kit (Eurx, Gdańsk, Poland). Sequencing, post-reaction purification and reading were done by Genomed (Warsaw, Poland) using an ABI 377XL Automated DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA). All sequences are available in GenBank (accession numbers: SP114263-SP114265). All molecular analyses has been done at Department of Botany

and Plant Ecology Wrocław University of Environmental and Life Sciences). The obtained *psb*C sequences were aligned using DNA Baser Sequence Assembler v4 [8] and checked for nucleotide variation using Mega 11 software [9]. The cpDNA sequences were aligned using Muscle software implemented into Mega 11 [9, 10] before the phylogenetic analyses. We performed maximum likelihood (ML) to infer the phylogenetic relationships among selected individuals of Charophyte nucleotide collected from NCBI. Bootstraps analyses are based on 1000 replications of full heuristic searches. The results based on the new data and data collected from GeneBank are presented in Table 2.

Results and Discussion

Collection

The new localities of *T. intricata* were located at:

(1) a small river lake close to the Vistula river near the village Witkowice (Figs 2-3), near Stalowa Wola

Table 2. Voucher details and GenBank psbC gene accession numbers of taxa used in this study. An-dash (-) indicates unavailable information; new sequences are written bold.

Taxa	Isolate, Voucher	Locality	*psbC* gene NCBI accession number
Chara foliosa	KGK0233, Proctor138	-	MG880182.1
Chara foliosa	KGK0333, NY02146579	-	MG880181.1
Chara foliosa	KGK0341, NY00739274	-	MG880178.1
Chara globularis	KGK1574, NY02282230	-	KX430968.1
Chara tomentosa	- , -	-	KJ395866.1
Lamprothamnium heraldii	KGK0069, -	-	KJ395867.1
Lamprothamnium macropogon	- , -	-	KJ395868.1
Lychnothamnus barbatus	KGK4840, NY02020578	Lithuania	MF166879.1
Lychnothamnus barbatus	KGK3082, NY02146274	-	MF166876.1
Nitella axiliaris	KGK2327, NY00739255	-	KX430974.1
Nitella flexilis	KGK1769, NY02137614	-	KX430978.1
Nitella hyalina	KGK0190, NY02282228	-	KX430979.1
Nitella hyalina	KGK0059b, -	-	KJ395873.1
Nitella opaca	F146, -	-	KJ395876.1
Nitellopsis obtusa	KGK2013, NY02146765	-	KX430980.1
Nitellopsis obtusa	F131B, -	USA	KJ395878.1
Tolypella canadensis	WP0270, NY02026386	USA	KJ395828.1
Tolypella glomerata	WP0168, NY01475172	USA	KJ395848.1
Tolypella glomerata	WP0273, NY02026388	Canada	KJ395841.1
Tolypella glomerata	WP0263, NY02026383	Canada	KJ395840.1
Tolypella glomerata	WP0285, NY01003601	USA	KJ395831.1
Tolypella intricata	WP0147, NY01475169	-	KX430983.1
Tolypella intricata	WP0040, NY01475199	USA	KJ395844.1
Tolypella intricata	WP0058, NY01474971	USA	KJ395842.1
Tolypella intricata	WP0073, NY01474970	USA	KJ395829.1
Tolypella intricata	JB1477, - this paper	Poland	SP114263.1
Tolypella intricata	JB1478, - this paper	Poland	SP114264.1
Tolypella intricata	JB1479, - this paper	Poland	SP114265.1
Tolypella prolifera	WP0260, NY02026384	USA	KJ395856.1
Tolypella prolifera	WP0060, NY01003496	Canada	KJ395843.1

Fig. 2. Occurrence of *T. intricata* near Witkowice.

Fig. 4. Occurrence of *T. intricata* near Kotlice.

Fig. 3. Small river lake close to Vistula river near village Witkowice where *T.intricata* was found.

Fig. 5. Small temporary pond among arable fields near Kotlice village.

(Central Poland, Nadwiślańska Valley, Sandomierska Basin), 50°45′38″N, 21°50′46″E, 08-06-2009. *T. intricata* was collected using grapnels, then dried and stored in the collection of dry specimens in the Department of Botany and Plant Ecology Wrocław University of Environmental Sciences.

(2) a temporary pond among arable fields near Kotlice village (Figs 4-5), near Hrubieszów (Kotlina Hrubieszowska, Wyżyna Wołyńska), 50°40′54″N, 23°35′28″E. *T. intricata* was grown in artificial conditions (greenhouses) in 2019 from soil samples (diaspors) collected in early spring 2018.

Description of *T. intricata* Specimens from the Two New Localities Found in Poland

Both newly found specimens of *T. intricata* were medium-size, 25-40 cm-high plants, branched with plant main axis 1.0-1.5 mm in diameter. Plants were greyish green and moderately to heavily encrusted (Witkowice, Figs 6-8).

The internodes were up to three times as long as the branches. We have observed 7-8 fertile branchlets in a whorl in very dense heads, divided once or twice with nodes bearing 3-6 lateral rays. Secondary rays with 1-2 nodes bore 5-7 celled tertiary rays. The end segment was 3-4 cells, with the last cell conical up to acute. About 7-8 sterile branchlets in a whorl were long, simple, and divided once or twice with one node bearing 2-3 lateral rays. The end cell was conical to acute. Lateral rays were elongated, composed of 4-6 cells with the end cell conical and acute. Dense heads consisted of two or more reduced fertile whorls on the upper shortened part of branch axes. All studied *T. intricata* specimens were monoecious plants with gametangia at fertile branchlet nodes at the base of fertile branchlets. Oogonia were numerous, aggregate, 2-4 at a node, 385-460 µm long, 280-465 µm wide. Oospores were dark brown 305-360 µm long and 220-360 µm wide. Antheridia were generally solitary and were about 310-360 µm in diameter (data are based on measurements of approximately 15-20 oospores and 5-7 antheridia).

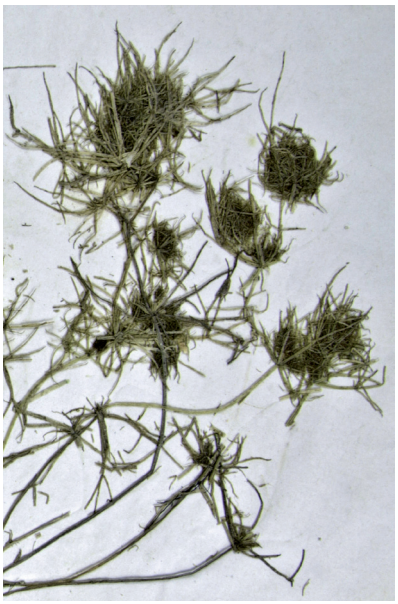

Fig. 6. General appearance with reduced fertile whorls of *T. intricata*.

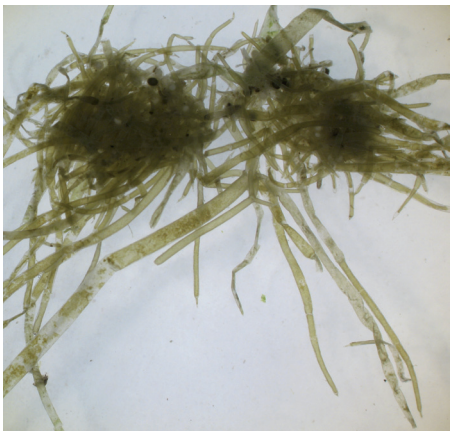

Fig. 7. Branchlets fertile in a whorl in dense heads of *T. intricata*.

Fig. 8. Fertile branchlets with oogonia and visible end segment with the last cell conical, acute of *T. intricata*.

Phylogenetic Analyses

As similar as in case phylogenetic analysis presented prevoiusly [11, 12] genus *Tolypella* was monophyletic in performed analysis and both sections: sect. *Tolypella* to which belong *T. glomerata*, as well as sect. *Rothia* (*T. intricata*, *T. prolifera*, *T. canadensis*) were strongly supported in the ML bootstrap analysis (Fig. 9). Additionally, in a sect. *Rothia* all studied sequences that origin from two species *T. intricata* and *T. prolifera* formed two separate clades with strong bootstrap support. One of them contained seven individuals consisted of sequences that come from *T. intricata* (three newly sequenced and four other collected from NBCI), whereas second was formed by two specimens of *T. prolifera*. Single sequence of *T. canadensis* formed a sister clade to them, but weaker supported. Species from the genus *Nitella* (inside tribe *Nitellae*) were paraphyletic to *Tolypella* and as similar as in results obtained by [13, 14] was a sister clade to *Chareae* (*Chara, Lamprothamnium, Lychnothamnus* and *Nitellopisis*).

In general, the newly collected in Poland sequences from *T. intricata* samples together with samples from NCBI was separated from *T. prolifera*. Both species formed monophyletic clades strongly supported by bootstrap analyses ≥99 (Fig. 9).

Notices about the Occurrence of *T. intricata*

It seems that *T. intricata* in SE Poland can be found in two types of habitats. The first type near the town of Witkowice is small river lakes formed on the active floodplain of a large river, such as the Vistula or the Warta rivers. These are astatic ponds periodically drying out partially or completely. The substrate is dominated by a fine-grained mineral fraction. The aquatic vegetation in such reservoirs is poorly developed and not very rich in species; the species found are often pioneering species. The following species of vascular plants include *Potamogeton trichoides* Cham. & Schltdl., *Ceratophyllum demersum* L., *Elodea canadensis* Michx., *Myriophyllum spicatum* L., *Potamogeton natans* L. and *Nuphar lutea* (L.) and *Chara globularis* Thuil. Marsh vegetation is more well developed and built by patches of *Glyceria maxima* (Hartm.) Holmb., *Phalaris arundinacea* L., *Bulboschoenus maritimus* (L.), Palla *Oenanthe aquatica* (L.) Poir., and *Rorippa amphibia* (L.) Besser. Important components are also pioneering annual species growing at the edge of waterside habitats, such as *Polygonum* L. spp. and *Bidens* L. spp.; *Agrostis stolonifera* L., *Ranunculus sceleratus* L. and *Alopecurus aequalis* Sobol.; and pleuston species, such as *Lemna minor* L., *Spirodela polyrhiza* (L.) Schleid., and *Salvinia natans* (L.) All.

The second type of habitat where *T. intricata* was found is a temporary pool with a diameter of 70-80 m that occasionally appears (in the years with abundant

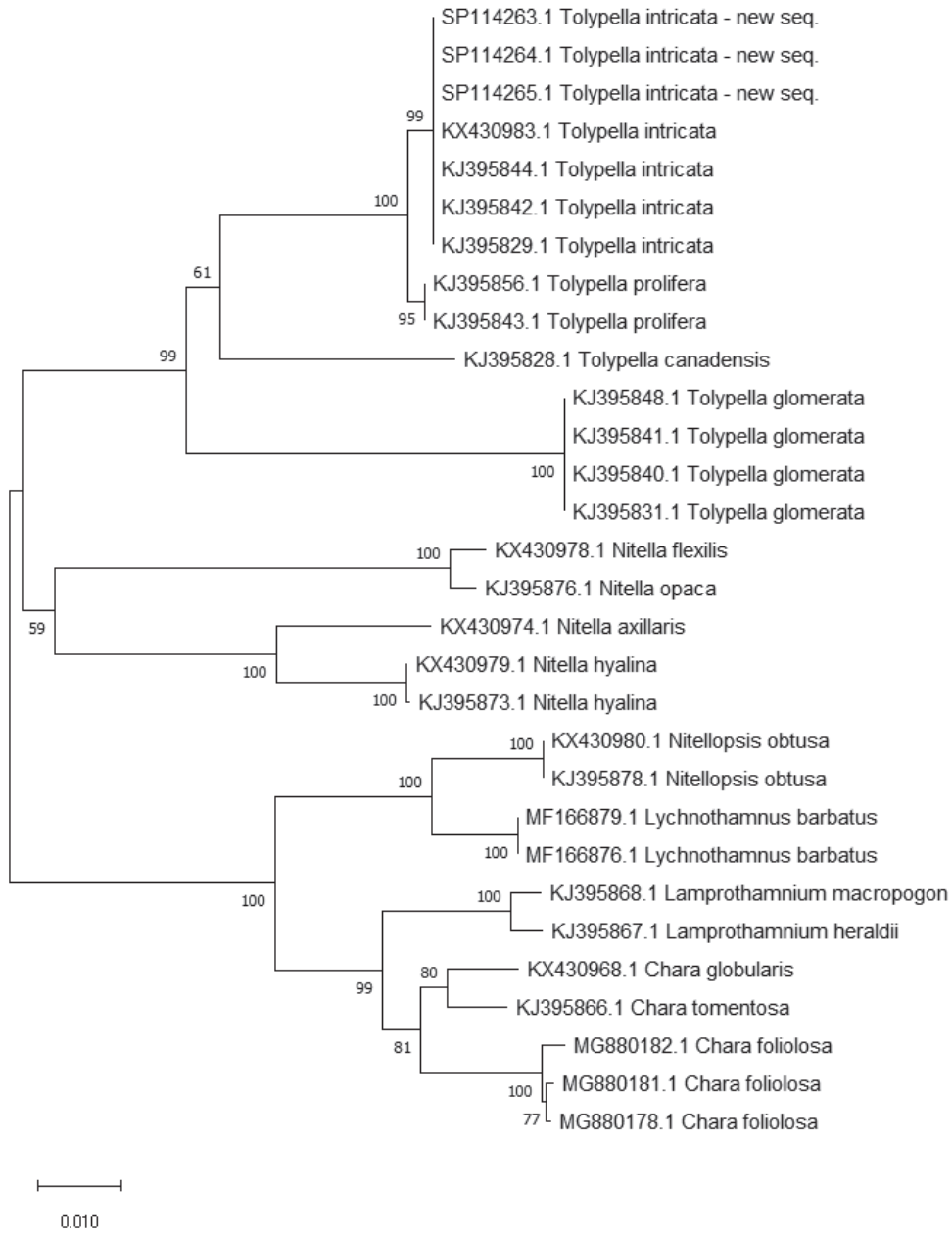

Fig. 9. Maximum Likelihood tree of the *psb*C gene analyses performed to confirm the discovery of *T. intricata.* Bootstrap analyses with values above 50 are included. The scale bar indicate 2% divergence.

rainfall) in a water body in the agricultural landscape [15, 16]. Such a shallow water body developed in the Lublin region in karst depressions in arable fields. In this case, the substrate was clay soil covering shallow chalk rocks, and the formation of wetlands in this place was last observed in 2013 [17]. The vegetation found in this location was dominated by amphibious plants – dwarf ephemeral wetland species and low growing marsh species such as *Alisma lanceolatum* With., *Alisma plantago-aquatica* L., *Alisma gramineum* Lej., *Isolepis supina* (L.) R.Br., *Limosella aquatica* L., *Lythrum hyssopifolia* L., *Elatine alsinastrum* L., *Potentilla supina* L., *Rorippa palustris* (L.) Besser, *Polygonum amphibium* L., *Polygonum lapathifolium* L., *Callitriche verna* L., *L. minor*, *Juncus bufonius* L., *Oenanthe auqatica* (L.) Poir., *Veronica beccabunga* L., *Plantago intermedia* Gilib., *Sparganium erectum* L., and *Typha angustifolia* L. As mentioned earlier, *T. intricata* was not recorded here but grew out of the collected sediment in the laboratory together with *Chara globularis* Thuill., *Chara vulgaris* L..

Present Distribution of and Threats of *T. intricata*

Currently, it is very difficult to unambiguously determine the distribution of *T. intricata* and the species conservation protection status in Europe [18-23]. Due to the sporadic records of the species and its periodical findings, almost all information concerning its distribution in concerns historical sites and only three

including both described in this paper were found in 21 century (Fig. 1). *T. intricata*, on the other hand, is much more common in the USA [13, 14, 24]. This is probably related to the biology of the species, which is found periodically in ephemeral sites and is often not recorded every year, as, for example, in the case of Kotlice. In most of the red lists of protected species, *T. intricata* has an endangered status [25-28] and is considered a very rare species [29]. It must also be remembered that the other species belonging to the genus *Tolypella* are also rare [11]: *T. glomerata* Leonh. 1863, *T. canadensis T.Sawa 1973*, *T. hispanica* Nordst. ex T.F. Allen 1888, *T. normaniana* (Nordst.) Nordst. 1868*, T. salina Corill. 1960.* Only *T. nidifica* (O.F.Müller) A.Braun 1857 is a locally quite common species found in the Baltic Sea. Fig. 1 and Table 1 show the places where *T. intricata* has been found and the two described sites from Poland. It is clear that there are not many locations. This is certainly influenced by the biology of the species and its sporadic finding and by the decrease in field research in recent years. It is often assumed that the distribution of plant species and the decline in biodiversity are influenced by the progressive anthropopression and destruction of habitats [19, 20, 30]. Furthermore, all known descriptions and locations of the occurrence of this species come from very specific sites, such as small water reservoirs, pits, canals and in-field reservoirs. They are, of course a subject of anthropopression, but the distribution of *T. intricata* is also influenced by a poor understanding of these specific habitats and the knowledge that this species can be found in such places [31-34].

This study reported the first record of *T. intricata* in Poland in over 150 years and also the most recent reliable record of the species in Europe. Results of our study enrich our knowledge distribution and the habitat characteristics and overall ecology of this rare Charophyte species. Our finding significantly contributes to the species biogeography, which is reviewed and discussed.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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