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Taxonomic and genetic investigation on sect. *Ricciella* genus *Riccia* in Europe with emphasis on *R. fluitans* and *R. rhenana*

Doctoral dissertation
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Taksonomiczne i genetyczne badania sekcji *Ricciella* z rodzaju *Riccia* w Europie ze szczególnym uwzględnieniem *R. fluitans* i *R. rhenana*

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Abstract

Taxonomy provides a systematic framework for classifying living organisms, which is continuously evolving to accommodate the diversity and dynamics of life. The species, as the fundamental unit of taxonomic classification, has historically been defined based on morphological characters, ecological traits, and geographic distribution. In the pregenetic era, researchers collected specimens, documented their morphology, ecology, and distribution, and published detailed descriptions. Once published and compliant with the rules of the relevant International Code of Nomenclature, a species was formally recognized. However, the scientific community could reject a proposed species, or multiple species epithets might emerge for the same organism across different regions, reflecting the dynamic and iterative nature of taxonomy. Advances in genetic research have significantly enhanced taxonomic science, enabling more precise species delineation and fostering ongoing refinements to classification systems. In a simpler way, taxonomy today is a systematic framework that integrates morphological and genetic traits to classify living organisms. With the species rank being the backbone to the entire taxonomic science, the processes of speciation and their understanding have the fundamental role of moving taxonomy forward. Hybridization is a key mechanism of speciation, producing hybrids through sexual reproduction between individuals of different species. The genetic structure of a hybrid varies depending on the parental species, resulting in either homoploid hybridization, where the hybrid retains the same chromosome number as its parents, or polyploid hybridization, where the hybrid inherits the combined chromosome sets of both parents, forming a polyploid. Polyploidy can also arise through mechanisms other than hybridization, such as somatic doubling, apospory, or apogamy. Polyploids are classified as autopolyploids when chromosome duplication occurs within

a single species, or allopolyploids when resulting from interspecific hybridization. These processes are particularly relevant to the speciation of sect. *Ricciella* genus *Riccia*, where genetic diversity and morphological simplicity drive taxonomic complexity.

The main focus of this work is the genetic relationship between *R. fluitans* and *R. rhenana*. In order this relationship to be revealed, the entire section *Ricciella* is taken into account. Major works dealing with the genus are analyzed and essential taxonomic clarification are made. Important biological aspects are discussed and a new species-specific characteristics identified. Genome sizes, chromosome counts, isozyme data, and barcodes are generated and analyzed. The results obtained during my work strongly support the hybrid origin of *R. rhenana* and by this its taxonomic position as a separate species, and not as a cytotype of *R. fluitans*. I also prove the already existing hypothesis of *R. fluitans* being a complex of closely related cryptic species. Based on the obtained preliminary results, section *Ricciella* does not form a monophyletic clade, within subgenus *Ricciella*. The current species composition of the section is here not supported. A possible section rearrangement could be as follows: sect. *Ricciella* comprised by the aquatic species *R. fluitans*, *R. rhenana*, *R. stricta*, *R. stenophylla* and the cryptic species belonging to them, including the different hybrids, withing the *R. rhenana* complex. A separate section should be established for the *R. canaliculata* group, including also *R. perennis*, *R. duplex* and the potential other hybrid species. Whether *R. huebeneriana* and *R. multifida* should be included together within the *Canaliculata* group at this points is still unclear. At the current stage of knowledge on the genus, definite taxonomic rearrangements cannot be made. The data and interpretations on section *Ricciella* presented herein will be used as foundation for further investigation.

Streszczenie

Taksonomia dostarcza systematycznych ram do klasyfikacji organizmów żywych, które są nieustannie udoskonalane, aby uwzględniać różnorodność i dynamikę życia. Gatunek, jako podstawowa jednostka klasyfikacji taksonomicznej, historycznie definiowano na podstawie cech morfologicznych, cech ekologicznych i rozmieszczenia geograficznego. W erze pregenetycznej badacze gromadzili okazy, dokumentowali ich morfologię, ekologię i rozmieszczenie oraz publikowali szczegółowe opisy gatunków. Po opublikowaniu i dostosowaniu do zasad odpowiedniego Międzynarodowego Kodeksu Nomenklatury, gatunek był formalnie uznawany. Społeczność naukowa mogła jednak odrzucić proponowany gatunek lub dla tego samego organizmu w różnych regionach mogły pojawić się różne nazwy gatunkowe, co odzwierciedlało dynamiczny i iteracyjny charakter taksonomii. Postępy w badaniach genetycznych znacząco wpłynęły na rozwój taksonomii, umożliwiając precyzyjniejsze rozgraniczenie gatunków oraz wspierając ciągłe doskonalenie systemów klasyfikacyjnych. W ujęciu uproszczonym, współczesna taksonomia stanowi systematyczne ramy klasyfikacyjne, łączące dane morfologiczne i genetyczne w celu precyzyjnego porządkowania różnorodności biologicznej. Ponieważ ranga gatunkowa stanowi podstawę całej nauki taksonomicznej, procesy specjacji i ich zrozumienie odgrywają fundamentalną rolę w rozwoju taksonomii. Hybrydyzacja stanowi kluczowy mechanizm specjacji, prowadzący do powstania mieszańców w wyniku rozmnażania płciowego między osobnikami należącymi do różnych gatunków. Struktura genetyczna takich mieszańców zależy od gatunków rodzicielskich, co może skutkować hybrydyzacją homoploidalną, gdy mieszaniec zachowuje tę samą liczbę chromosomów co rodzice, lub hybrydyzacją poliploidalną, gdy mieszaniec dziedziczy połączone zestawy chromosomów obojga rodziców, tworząc poliploid. Poliploidia może również powstawać w

wyniku mechanizmów innych niż hybrydyzacja, takich jak podwojenie somatyczne, aposporia lub apogamia. Poliploidia dzieli się na autoploidy, gdy duplikacja chromosomów zachodzi w obrębie jednego gatunku, oraz na alloploidy, gdy powstają w wyniku hybrydyzacji międzygatunkowej. Procesy te są szczególnie istotne dla specjacji w sekcji *Ricciella* z rodzaju *Riccia*, gdzie różnorodność genetyczna i prostota morfologiczna potęgują złożoność taksonomiczną.

Głównym celem niniejszej pracy było określenie pokrewieństwa genetycznego między *R. fluitans* a *R. rhenana*. W tym celu objęto analizą całą sekcję *Ricciella*. Przeanalizowano najważniejsze prace dotyczące tego rodzaju i dokonano niezbędnych wyjaśnień taksonomicznych. Omówiono ważne aspekty biologiczne i zidentyfikowano nowe cechy charakterystyczne dla danego gatunku. W przeprowadzonych badaniach przeanalizowano rozmiary genomów, liczbę chromosomów, dane izoenzymatyczne oraz sekwencje barkodowe DNA. Wyniki uzyskane w trakcie mojej pracy zdecydowanie potwierdzają hybrydowe pochodzenie *R. rhenana*, a tym samym jej pozycję taksonomiczną jako odrębnego gatunku, a nie jako cytotypu *R. fluitans*. Udowodniłem również istniejącą już hipotezę, że *R. fluitans* jest kompleksem blisko spokrewnionych gatunków kryptycznych. Na podstawie uzyskanych wstępnych wyników, sekcja *Ricciella* nie tworzy monofiletycznego kladu w obrębie podrodzaju *Ricciella*. Obecny skład gatunkowy sekcji nie został tutaj potwierdzony. Możliwa rearanżacja sekcji mogłaby wyglądać następująco: sekcja. *Ricciella* obejmuje gatunki wodne *R. fluitans*, *R. rhenana*, *R. stricta*, *R. stenophylla* oraz gatunki kryptyczne do nich należące, w tym różne mieszańce, w obrębie kompleksu R. rhenana. Należy utworzyć osobną sekcję dla grupy *R. canaliculata*, obejmującej również *R. perennis*, *R. duplex* i potencjalne inne gatunki mieszańcowe. Nadal nie jest jasne, czy *R. huebeneriana* i *R. multifida* powinny być włączone razem do grupy *Canaliculata*. Na obecnym etapie wiedzy na temat rodzaju nie jest możliwe dokonanie ostatecznych reorganizacji taksonomicznych. Dane i interpretacje dotyczące sekcji *Ricciella* przedstawione w niniejszej pracy posłużą jako podstawa do dalszych badań.

Declaration

This is to certify that:

(i) This thesis comprises only my original work towards the Doctor of Biological Sciences except where indicated in the preface;

(ii) Due acknowledgments have been made in the text to all other materials used and all literature sources have been exhaustively cited;

Galin Gospodinov

Oświadczenie

Niniejszym zaświadczam, że:

(i) Niniejsza rozprawa zawiera wyłącznie moje oryginalne prace na stopień Doktora nauk biologicznych, z wyjątkiem miejsc wskazanych we wstępie;

(ii) W tekście zamieszczono stosowne podziękowania dla wszystkich pozostałych wykorzystanych materiałów, a wszystkie źródła literaturowe zostały wyczerpująco zacytowane;

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Preface

The thesis contains one chapter that has been submitted in a peer-review journal (Chapter 3), with co-authorship with one of my supervisors - Dr. Rayna Natcheva (Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences). The Chapter is submitted to Comprenhensive Plant Biology, with minor changes. Chapter 4 is in final preparation with co-authorship with Dr. Eva Temsch (University of Vienna, Austria). Chapter 5 is in a final stage of preparation for publication and will be published with my other supervisor dr. hab. prof. UAM Katarzyna Buczkowska-Chmielewska (Department of Genetics at Adam Mickiewicz University in Poznań, Poland). There is therefore some necessary repetition between chapters, which I have attempted to keep to a minimum.

The original idea concept, principal contribution, and primary authorship of all chapters presented in this dissertation are mine. My supervisors -dr. hab. prof. UAM Katarzyna Buczkowska-Chmielewska and Assoc. Prof. Rayna Natcheva PhD - provided guidance and advice throughout.

Dr. Eva Temsch (University of Vienna, Austria) provided the genome size measurements and is a recognized co-author in the paper concerning this matter.

The chromosome counts were provided by Zoya Mitrinska (Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences).

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Finally, I extend my heartfelt gratitude to the entire Department of Genetics at Adam Mickiewicz University in Poznań, Poland. To all my colleagues, thank you for welcoming me from the very first day and making my time there feel like home. It was a privilege to work alongside such a supportive and inspiring group.

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Chapter 1

Synopsis

1.1 General introduction

Bryophytes are the second largest group of plants in term of species richness, after the flowering plants (Hodgetts et al., 2023). Three main divisions (phylla) are recognized under the general name "bryophytes": mosses (Bryophyta), liverworts (Marchantiophyta), and hornworts (Anthocerotophyta) (Frey, 2009). The number of species per each group is estimated to be between 11 000 – 13 000 for the mosses, 7 000 – 9 000 for the liverworts, and 200 – 250 for the hornworts (Villarreal et al., 2010). However, recent works suggests that mosses and liverworts form a clade, termed "Setaphyta", after the sporophyte seta, which is a stalk supporting the spore bearing capsule in both mosses and liverworts. The Setaphyta clade is defined phylogenetically, not based on an apomorphy, since comprises the living mosses and liverworts, and their last common ancestor together with all of its descendants (Puttick et al., 2018; Hodgetts et al., 2023).

Liverworts are the oldest evidence of colonization of terrestrial environments by plants (Bowman, 2013; Brown et al., 2015; Rodríguez-López et al., 2021). They are subdivided into two main groups: leafy (Class Jungermanniopsida) and thallose (Class Marchantiopsida), additional small group of rather anomalous plants is recognized, with both leafy and thallose features (Class Haplomitriopsida) (Hodgetts et al., 2023). Bryophytes originated from freshwater algae and started to colonize the land between the Cambrian and Ordovician periods 470-515 million years ago

(Morris et al., 2018; Harris et al., 2020; Fernández-Martínez et al., 2021), and heir most significant diversification is during the Albian-Cenomanian interval (Cooper et al., 2012; Villarreal et al., 2016; Rodríguez-López et al., 2021).

All bryophytes are united by the same general life cycle pattern, which is strikingly opposite to the one found in flowering plants. In flowering plants the sporophyte is the dominant developmental state, as it is the diploid phase forming the main plant body (roots, stems, leaves, and flowers), the gametophyte phase is strongly reduced, and dependent on the sporophyte (Raven et al. 2005). In bryophytes, the opposite is observed: the dominant phase is the haploid gametophyte with short living, codependent on the gametophyte, diploid sporophyte (Schuster, 1992a). Despite the presence of different ploidy levels within the bryophytes, in the gametophyte all alleles are functionally dominant and under direct selection (Anderson, 1963; Crum, 1972; Gemmell, 1950; Sorojsrisom et al., 2022).

Ricciaceae Dumort. is among the most advanced, most widespread, and most highly derived families within the complex thalloid liverworts (order Marchantiales). Its adaptations rely almost entirely on simplification, resulting in extreme niche specialization and its classification, and identification constitute almost a separate subdiscipline of Hepaticology (Schuster, 1992b,b; Perold, 1991). One of their most striking simplifications is the extreme reduction of the sporophyte to the point where it is entirely embedded within the thallus tissue, completely lacking seta. The generally accepted composition of the family is comprised by two genera: *Ricciocarpus* (L.) Corda and *Riccia* (Micheli) L. (Dumortier, 1829). Genus *Ricciocarpus* is a monotypic, with just a single species – *R. natans* (L.) Corda. By contrast genus *Riccia* is one of the most species-rich genera within Marchantiales reaching approx. 200 species (Jovet-Ast, 1986; Damsholt, 2002). The large number of species in the genus are united under two general reproduction strategies, which are heavily affected by their mating systems. The main group of species are monoicous, abundantly reproducing via spores. The other group of species are dioicous, which may or may not rely on sexual reproduction. The monotypic *Ricciocarpos* and members of *Riccia* are

the only aquatic species among the complex thalloid liverworts (Shaw and Goffinet, 2000)

Riccia L. is the most widespread and species-rich of the complex thalloid liverworts (Class Marchantiophyta) (Jovet-Ast, 1986; Damsholt, 2002), diverging at ca. 60 million years ago (the Paleocene) (Villarreal et al., 2016). The genus unites species with different reproductive systems, different habitat preferences and survival strategies. It's cosmopolitan occurrence and diverse ecology, generate different taxonomic treatments, since different authors shape and propose taxonomic arrangements based on different unifying or separating criteria.

The focus of this work is section *Ricciella* A. Braun, subgenus *Ricciella* (A. Braun) Bisch. Since the species arrangement within the section is not well defined, and various compositions are proposed from different authors, the section is here treated as comprised from the species *Riccia fluitans* L. emend Lorb. in Müll. Frib., *R. rhenana* Lorb. ex Müll. Frib., *R. canaliculata* Hoffm., *R. duplex* Lorb. ex Müll. Frib., *R. huebeneriana* Lindenb. and *R. perennis* Steph. The species differ in their life history traits and important species specific features are provided in Table 1.1.

Table 1.1: Summary of important species-specific features of the studied species.

Species	**Ploidy level**	**Sexual system**	**Habitat**	**Reproduction types**
R. fluitans	haploid	dioicous	mixed	asexual
R. rhenana	diploid	not known	mixed	asexual
R. canaliculata	haploid	dioicous (?)	terrestrial	sexual
R. duplex	diploid	monoicous	terrestrial	sexual
R. huebeneriana	haploid	monoicous	terrestrial	sexual
R. perennis	haploid	monoicous	terrestrial	sexual

The morphological resemblance within the haploid-diploid pairs of *R. fluitans*-*R. rhenana* and *R. canaliculata*-*R. duplex* strongly suggests genetic relationship, but such has not been proven by any means, so far. Contributing factor to the problematic identification are also the wide range of morphological descriptions of *R. fluitans* s. lat., from different authors, creating functionally nonapplicable species differentiation (Table 1.2).

Table 1.2: Morphometrics of *R. fluitans* from different authors. (A) Aquatic, (T) terrestrial plants.

Author	**Length (mm)**	**Width (A)**	**Width (T)**	**Furcation**
Jovet-Ast (1986)	10-15	0.8-1	1.5	-
Damsholt (2002)	10-50	(0.3-) 0.5 -1	2.8	45-50°
Paton (1999)	20(30)	0.3-1(1.2)	1.5	60-90°

In addition to the currently accepted species from the genus in Europe (*sensu* Hodgetts et al. (2023)) the occurrence of *Riccia stenophylla* Spruce is suspected by Damsholt (2002). However, at this point there is no confirmation for this species in Europe and the sole reason for its suspected occurrence is based on the reports of fertile *R. fluitans*-like plants. Being exclusively sterile species in Europe, Damsholt (2002) classify all fertile reports of *R. fluitans* as *R. stenophylla*.

1.2 Aim

The primary objective of this thesis is to elucidate the genetic relationships between *R. fluitans* and *R. rhenana*, with a specific focus on the diploidization process in *R. rhenana*. Given the taxonomic uncertainty and suspected cryptic speciation within the *Ricciella* section, this study encompasses a comprehensive analysis of the entire section. Due to the potential origin of the diploid *Riccia rhenana* multiple times at multiple locations, the sampling for the purposes of this study is restricted to Europe, aiming to reveal the genetic composition of the European species concept of the polyploid. Nonetheless, the study addresses the *Ricciella* subgenus and its section *Ricciella* in a broader context, including relevant commentary on species outside Europe.

1.3 Objectives

To achieve the aim of this study, the following objectives were made:

1. Summarizing available literature on subgenus *Ricciella*, focusing mainly on the species from the section *Ricciella*

2. Revision of herbaria collections from different herbaria across Europe.

3. Field expeditions and sample collection of the species of interest for the purposes of genetic and morphological studies.

4. Investigation of the chromosome counts and the genome size of the haploid-diploid species pairs *R. fluitans* - *R. rhenana* and *R. canaliculata* - *R. duplex*.

5. Investigation the potential genetic relationship between the haploids and the diploids using isozyme analysis.

6. Resolving the genetic relationships between the species within the section by phylogenetic reconstruction, based on both chloroplast and nuclear markers.

7. Investigation of the subgenus position in the context of the entire genus based on phylogenetic reconstruction.

1.4 Significance

The genus *Riccia*, particularly sect. *Ricciella*, remains understudied in Europe and globally. While Cargill et al. (2016) provided a preliminary phylogeny for Australian *Riccia* species, their work does not address sect. *Ricciella* in detail. Similarly, earlier studies, including the monograph by Jovet-Ast (1986) and works by Perold (1991, 1995) and Schuster (1992b), offer limited insight into European species and lack genetic analyses. This study addresses these gaps by investigating the ploidy dynamics of *R. rhenana*, elucidating mechanisms of speciation within the genus for the first time. DNA barcoding reveals phylogenetic relationships within sect. *Ricciella* and its position within the genus. Furthermore, the mating systems of haploid and diploid *Riccia* species position them as valuable models for studying evolutionary processes, particularly speciation through polyploidy, enhancing our understanding of taxonomic complexity in liverworts.

1.5 Literature review

Brief taxonomic history

The beginning of binomial taxonomy is marked by the publishing of the fundamental work Species Plantarum, by Carl Linnaeus in 1753. In this work he systematically applies binomial nomenclature, formalizing the use of two-part Latin names (genus and species epithet) for species identification. Before this milestone for the modern taxonomy, plants have also been observed and attempts for their classification and summarization have been made. In fact, *R. fluitans* can be traced back to the work of Petiver (1695) - "Musei Petiveriani centuria prima-[decima] rariora naturae; continens: viz. animalia, fossilia, plantas, ex variis mundi plagis advecta, ordine digesta, et nominibus propriis signata". There, under number 253, *R. fluitans* is addressed as "Lactuca aquatica" and described "tenuifilia segmentis bifidis. Found in a ditch near Deptford-Dock". Most likely this is one of the very first records of *R. fluitans* in the literature. Later, it was also discussed in the work of Ray (1724),Vaillant et al. (1727), and Dillenius (1742). Linnaeus discovered *R. fluitans* at one of his journeys to the region of Scania, which he later published as "Skånska resa". Based on this finding, he included *R. fluitans* in Species Plantarum, summarizing also the records from Vaillant et al. (1727) and Dillenius (1742). After the description of *R. fluitans* from Linnaeus, Hoffmann and Palm (1796) described *R. canaliculata*, and in Lindenberg (1836) *R. huebeneriana*. *Riccia perennis* is described by Stephani (1898), and Müller (1941) described the two diploid species *R. rhenana* and *R. duplex*. For a short period of time a separate taxon existed, morphologically intermediate between *R. fluitans* and *R. rhenana*. Described by Klingmüller (1958), *R. media* is rejected by Bapna (1961). It is beyond the scope of this work to discuss if the recognition of *R. media* as a separate taxon is valid or not, but it points out some of the problems within the group, concerning the over simplified morphology and general phenotypic plasticity.

The genus *Riccia* is first established by Micheli (1729) and then validated by Linnaeus. The subgenus *Ricciella* is originally described as a separate genus by Braun (1821), including the species with ventrally protuberant sporangia vs. dorsally protuberant in *Riccia*. It is proposed for the reception of *R. fluitans* and *R. canaliculata* by Evans (1907). This rather simple separation is initially accepted by taxonomist such as Stephani, Dumortier and Revisan, but Stephani himself who accepts the genus, proposes a new refined classification, based on thallus morphology and reclassify *Ricciella* as a subgenus, and for a type species to the subgenus accepts *R. fluitans* (Stephani, 1898). It should be also mentioned that Warnstorf and Warnstorf (1903), treated *Ricciella* as a separate genus. The section *Ricciella* is recognized by Frye and Clark (1937), based on the original criteria from Braun (1821). As a type species, Grolle (1976) selects *R. fluitans*, and later *R. canaliculata* is suggested to be better choice, being known fertile, from Schuster (1992b). However, *R. fluitans* remains the type species for the section.

Taxonomic history of subgenus Ricciella

Table 1.3 summarizes the taxonomic history provided in Perold (1995). Given are only the taxonomic concepts having relation to Europe.

In the work of Meijer (1951) the subgenus *Ricciella* are described as having the ability to adopt an amphibian life style. It is not precisely clear how "amphibian life style" is here used, since the species of this group are strictly connected to water. If the term is here used to describe that all species are capable of living indefinite both as aquatic or terrestrial, should be considered questionable. Despite the numerous analyzed samples, I was not able to find aquatic forms of *R. canaliculata*, *R. huebeneriana*, or *R. crystallina*.

Müller (1951) describes the thalli of *R. fluitans, R. canaliculata, R. rhenana* and *R. duplex* as rarely forked, while based on my observations, all *R. fluitans* and *R. rhenana* are always dichotomously branched.

Table 1.3: General taxonomy of sebgen. *Ricciella* according to different authors.

Author	Subgenus	Section	Species
Meijer (1951)	*Ricciella*	-	*fluitans/rhenana/canaliculata/crystallina/huebeneriana*
Müller (1951)	*Ricciella*	-	*fluitans/rhenana/canaliculata/duplex/huebeneriana/frostii/crystallina/cavernosa*
Arnell and Nyholm (1956)	*Ricciella*	-	*fluitans/rhenana/canaliculata/crystallina/huebeneriana*
Grolle (1976, 1983)	*Ricciella*	*Ricciella*	*fluitans/rhenana/canaliculata/crystallina/huebeneriana*
		Spongodes	
Volk (1983)	*Spongodes*	*Ricciella*	*fluitans/canaliculata/stricta*
		Spongodes	*crystallina/cavernosa/volkii*
Jovet-Ast (1986)	*Ricciella*	*Ricciella*	*fluitans/canaliculata/duplex/huebeneriana/perennis*
		Spongodes	*crystallina/cavernosa/frostii*
Damsholt and Hallingbäck (1986)	*Ricciella*	-	*fluitans/canaliculata/huebeneriana/cavernosa*
Smith (1990)	*Ricciella*	*Ricciella*	*fluitans/rhenana/canaliculata*
		Spongodes	*crystallina/cavernosa/huebeneriana*
Melick et al. (1991)	*Ricciella*	*Ricciella*	*fluitans/canaliculata*
		Spongodes	*cavernosa/huebeneriana*
Schuster (1992b)	*Ricciella*	*Ricciella*	*fluitans/stenophylla/rhenana/canaliculata/duplex/huebeneriana*
		Cavernosae	*cavernosa/crystallina*
		Frostii	*frostii*

The definition of the species as aquatic (or capable of being so), needs additional clarification. If a population of given species is found growing as freely floating, it should be considered as exposed to such conditions for a prolonged period of time. The secondary adaptation to aquatic conditions mentioned by Damsholt and Hallingbäck (1986) should be considered as valid only to the truly aquatic species such as *R. fluitans*, *R. rhenana*, *R. stenophylla* ect. As aquatic species from the European *Riccia*, the only proven such are *R. fluitans* and *R. rhenana*. Here as "aquatic" I define only those species, which can exist and develop floating or submerged for indefinite periods of time. Sporadically submerged species, which can tolerate periods of flooding, such as *R. canaliculata*, *R. duplex*, *R. huebeneriana*, *R. cavernosa*, and *R. frostii* I do not consider "aquatic" or "capable of developing aquatic forms".

In Grolle (1983) the two sections *Ricciella* and *Spongodes* are recognized for the first time. The authors for the subgenus *Ricciella* are stated to be (A. Br.) Rchb. and *R. fluitans* is newly selected as a lectotype for the subgenus and the *Ricciella* section, while *R. crystallina* is selected as a lectotype for section *Spongodes*. Nevertheless, species are not assigned in the new sections or any descriptions provided.

Volk (1983) recognizes as a subgenus only *Spongodes*, while *Ricciella* is recognized only as a section, with sporangia bulging and opening ventrally. He also includes *R. stricta* and adds "etc." probably indication the inclusion of species with this general habitus.

Jovet-Ast (1986): recognizes the Grolle's lectotypification of section *Spongodes* and section *Ricciella*. Her description of *R. canaliculata* as having ventral apex of lobes covered white scale, while in other species not seems unclear. The ventral scale in *R. canaliculata* is a descriminative trait, which is used to separate it from *R. duplex*, where in the former species, the scale is soon splitting (2-ranked), whereas 1-ranked and semi-lunate in the latter one (Damsholt (2002); Schuster (1992b). Also, as pointed out in Paton (1999), Jovet-Ast (1986) "clearly illustrates two rows of half-scales" for both. Based on this, Jovet-Ast (1986) rejects a major species-discriminative morphological trait. No comments are given on this matter in her work.

Damsholt and Hallingbäck (1986) does not recognize any sections within the subgenus *Ricciella*. The subgenus is described and air pores are said to be used as a floating mechanism. The comment about the air pores raises some questions, since of the listed species floating is only *R. fluitans*. Even if terrestrially growing plants are flooded, they are not expected to float, since all terrestrial individuals develop rhizoids, which fix them to the substrate.

Smith (1990) recognizes the subgenus *Ricciella* with its two sections *Ricciella* and *Spongodes*. Bellow I provide his entire description of the sections, since I conceder it as most relevant to the European species concept.

Subgenus *Ricciella*: thallus with chambers; epidermis breaking down to form lacunae in older parts of thallus or not; margins without cilia.

Section *Spongodes*: plants terrestrial; dorsal epidermal cells in older parts of thallus breaking down to form lacunae, rendering thallus spongy in appearance capsules

embedded in thallus, not protruding strongly on ventral side.

R. cavernosa, *R. ctystallina*, *R. huebeneriana*. Plants terrestrial; not strap-like; older parts spongy in appearance; capsules not protruding strongly on ventral side of thallus.

Section *Ricciella*: plants aquatic or terrestrial; epidermal cells on dorsal side of thallus not breaking down, older parts of thallus not spongy in appearance; capsules protruding very strongly on ventral side.

R. canaliculata, *R. fluitans*, *R. rhenana*. Thallus not channeled, except sometimes in older parts; not reticulate or often reticulate; capsules common or rare.

Melick et al. (1991) does not accept *R. duplex* and *R. rhenana* as a good species, because of the lack of constant diagnostic characters to differentiate them from *R. canaliculata* and respectively *R. fluitans*. By this, he automatically treats the polyploid plants as cytotypes. Comments on the sexual systems of the *R. canaliculata* and *R. duplex* are not made.

Schuster (1992b) provides probably the most complex arrangement of the group. He recognizes three sections: *Ricciella, Cavernosae* and *Frostii*. The *Ricciella* section is significantly larger in his treatment, including the two subspecies of *R. huebeneriana* and *R. stenophylla*. *Riccia perennis* is placed in synonymy with *R. canaliculata* (as in Müller (1951)), but later it is rejected in Jovet-Ast (1994). In my current work, *R. perennis* is genetically proven to be a sister species to *R. canaliculata* and *R. duplex*.

Review of the literature concerning Ricciella genetics

The first work discussing in detail the genetics of *R. rhenana* is probably the work of Lorbeer (1934). There, the chromosome arrangement of "diploid" *R. fluitans* is discussed. In his work is also the first mention of the name *Riccia (Ricciella) Rhenana*, on which later Müller (1941) describes formally the species. In his description of the chromosomes behavior, Lorbeer points out that the pairs are not homologous "Die

Gemini sind untereinander nicht homolog". Probably the most important comments on this matter are those about the reasons for such non homologous behavior, which he suspect might be in result of accumulation of mutations in a homologous chromosome set or in result of hybridization. Nevertheless, a final conclusion is not made.

Some years later, Berrie (1964) performs colchicine chromosome duplication on thalli of *R. fluitans* and *R. canaliculata* in order to compare them with the diploid *R. rhenana* and *R. duplex*, respectively. The hypothesis which he tests is that *R. rhenana* is a diploid form of *R. fluitans* and *R. duplex* is a diploid from of *R. canaliculata*. In this work, he concludes that the experimental diploid of *R. fluitans* is identical to *R. rhenana* in all diagnostic characters, and similar in its range of variation. *Riccia rhenana* is presumed originated in nature, perhaps several times at different localities, following somatic doubling of the chromosome complement in thalli of *R. fluitans*. Because of this, and in view of the difficulty in distinguishing haploid and diploid when growing under natural conditions, it is suggested that the diploid should not be given the status of a separate species, but should be regarded as diploid *R. fluitans*.

It should be noted that Berrie does not refer to Lorbeer's work in any way, particularly regarding chromosome behavior in relation to homology. The chromosome sets in colchicine-induced polyploids, being duplicates of the original set, imply homology, yet Berrie provides no observations or comments. No comments are made and on the m_1 and m_2 chromosomes described by Lorbeer in wild diploid *R. rhenana*. Furthermore, no comparisons are made between chromosome behavior in artificial polyploids and wild *R. rhenana*, despite both being available for study.

Based on the obtained *R. canaliculata* colchicine diploids, Berrie comments that when thalli of *R. canaliculata* are treated with colchicine, the diploid thalli produced resemble *R. duplex* in all diagnostic features except sexuality. *R. duplex* is monoecious (Müller, 1941), but the experimental diploids are dioecious. Living material of *R. duplex* was not available to him, so he does not provide a detailed

comparisons. Also a satisfactory comparisons of the microchromosomes of male and female thalli of *R. canaliculata* is not provided. He hypothesizes also that if the chromosmes should prove to be dimorphic and constitute a sex chromosome mechanism, this might explain why *R. duplex* has microchromosomes of two sizes. The natural diploid would then contain both male and female genomes, and would be monoecious for this reason. No definitive conclusion is made for *R. duplex* if it is simply a diploid form of *R. canaliculata* containing male and female genomes which remains undecided, but from the morphological evidence he speculates that this is so.

As I have mentioned above, the somatic polyploidization results in direct doubling of the chromosome sets of a given cell (cells). Berrie observes that *R. canaliculata* is a dioicous plant, having males and females as different individuals. By this, we could expect that a potential autosomal diploid will have the same sex, as the parent, since it will inherit its chromosomes. Berrie proves that *R. canaliculata* is a dioicous species, since the obtained artificial diploids are also dioicous. This experiment fails to reveal the origin of *R. duplex*, but strongly supports the dioicous sexual system of *R. canaliculata*, on which uncertainty exists. Jovet-Ast (1986) describes *R. canaliculata* as a monoicous species, without question mark, meaning that there is no doubt about it, while Müller (1941) in his work where *R. duplex* is described, addresses the sexual system of *R. canaliculata* as questionably dioicous. Damsholt (2002) describes it as "Monoicous or dioicous" and Paton (1999) as monoicous. The work of Paton should be taken into account with some additional comments. In her work "The Liverwort Flora of The British Isles", she does not give *R. duplex* as occurring on the British Isles, and respectively does not provide a description. Instead, she comments it in the *R. canaliculata* section, explaining that the later is comprised by two morphologically indistinguishable cytological races, where the diploid race is not referable to *R. duplex* Lorb. She also states, that this diploid race of *R. canaliculata* is reported from Sweden, Spain and Portugal. I have failed to obtain any additional information or any specimens from the mentioned diploids from Paton (1999).

Another important work is the one of Cargill et al. (2016), which deals with the entire genus *Riccia* in Australia. Nevertheless, the section *Ricciella* is not well represented, and species such as *R. canaliculata*, *R. rhenana*, and *R. duplex*, are not genetically investigated. Based on the included species, the position of *Ricciella* as a subgenus is questioned and its synonymization with subgenus *Riccia* is made.

1.6 Chapter Summaries and Conclusions

In **Chapter 2** I discuss main aspects of the biology of genus *Riccia* and more precisely the main reproductive strategies. The focus is on the seasonal behavior of the aquatic *R. fluitans/rhenana* and how it affects them and serves as a reproductive mechanism. The main conclusion of this Chapter is that *R. fluitans/rhenana* exhibit unique behavior, where a survival strategy benefits reproductive outcome.

Chapter 3 deals with the occurrence of *R. fluitans* and *R. rhenana* in Europe. Occurrence data is gathered from GBIF, representing the European distribution and compared with the collected and cytologically proven data for the species occurrence in Bulgaria. Based on the Bulgarian occurrence and the European occurrence, comparison of the frequencies of both species is made. Based on the collected field data we present specific trends with respect to the habitat origin and microhabitat preferences. In conclusion, the data indicates that *R. rhenana* is significantly unrecognized and overlooked species.

Chapter 4 provides data on the 1C values of the species *R. fluitans, R. canaliculata, R. rhenana*, and *R. duplex* s. lat. Comparisons between diploids and haploids are made. In conclusion, the variation in the 1C values between the different *Ricciella* species, suggests that this method can be a valuable tool for species delimitation.

Chapter 5 is devoted to the origin of *R. rhenana*. Here I combine isozyme analysis,

together with DNA barcoding in order to shed light on the mechanism of speciation of this diploid species. The isozyme results and the DNA barcoding revealed duplicated homeologous loci in all investigated populations. I prove the allopolyploid origin of *R. rhenana* and confirm its taxonomic rank as a species.

In **Chapter 6** I report preliminary results on the position of section Ricciella within the subgenus *Ricciella* and the genus *Riccia*. Two phylogenetic trees are generated - one tree based on two chloroplast markers *trnL* and *rps4*, representing the section species composition and another tree, based on the *rbcL* chloroplast region, illustrating the genus topology and the position of the subgenus and the section. In conclusion, section *Ricciella* does not form a monophyletic clade and subgenus *Ricciella* also does not form a monophyletic clade within the genus. Based on this, subgenus *Ricciella* and section *Ricciella* are not supported.

Chapter 2

Difficult sex and how to avoid it: survival strategy as reproductive outcome in *Riccia* with emphasis on *Riccia fluitans* and *Riccia rhenana*

2.1 Abstract

Plants have developed a variety of reproductive mechanisms and survival strategies to endure harsh conditions and environments. Most of these strategies rely on sophisticated processes that ensure successful reproduction during periods of favorable conditions. The genus *Riccia* comprises a large and complex group of liverworts adapted to diverse habitats. Among them, the aquatic species *R. fluitans* and *R. rhenana* have developed a distinct strategy that enables them to survive and reproduce differently from most other liverworts. During their resting period, these plants undergo rapid multiplication, resulting in significantly increased number of separate individuals emerging after the dormancy period.

2.2 Introduction

When discussing the survival mechanisms of bryophytes, we often focus on their ability to survive drought. As poikilohydric organisms, almost all bryophytes desiccate completely, but upon rehydration rapidly resume photosynthesis and growth (Proctor, 1979; During, 1979). Surviving in harsh environments has driven the evolution of multiple adaptations that ensure vital processes such as reproduction, population maintenance, and dormancy. For example the ventral scales of *Targionia* L. or *Reboulia* Raddi. have a protective role as the thalli dehydrate, the scales curve around, shielding the chlorophyllous tissue (Watson, 1914).

Periods of stress are generally defined by a transition from an active state of development to a state of dormancy, limiting the major biological and physiological processes, and completely pause or significantly slowdown the development of the affected individuals. To survive such periods, bryophytes employ diverse mechanisms to ensure clone survival. Among those, different mating systems are combined with different ways of asexual propagation, specialized structures (gemmae, tubers, protonemal brood cells, deciduous organs) and variety of other mechanisms, securing the successful establishment, maintenance, and survival of the clone.

2.3 Survival strategies in genus *Riccia*

Ricciaceae Dumort. is among the most advanced, most widespread, and most highly derivative families within the Marchantiales. Its adaptations rely almost (if not entirely) on simplification, resulting in extreme niche specialization. According to some authors, *Ricciaceae* classification and identification constitute a separate subdiscipline of Hepaticology (Schuster, 1992a; Perold, 1991). One of their most striking simplifications is the extreme reduction of the sporophyte to the point where it is entirely embedded within the thallus tissue. The generally accepted composition of the family comprises two genera: *Ricciocarpus* (L.) Corda and *Riccia* (Micheli) L. (Dumortier, 1829). *Ricciocarpus* is a monotypic, with just a single species – *R. natans.* (L.) Corda. While genus *Riccia* is one of the most species-rich genera within Marchantiales reaching approx. 200 species (Jovet-Ast, 1986; Damsholt, 2002). The species in the genus are united under two mating systems. Most species are monoicous, and abundantly reproducing via spores. Fewer species are dioicous, which may or may not rely on sexual reproduction.

2.4 The Monoicous System

The monoicous species *R. trichocarpa* Howe. and *R. cavernosa* Hoffm. for example, despite sharing the same sexual system, employ quite distinct survival and population maintenance strategies. *Riccia trichocarpa* is a xerophytic, abundantly fertile species, which has been proven to survive desiccation periods up to seven years (Volk, 1984). Its population maintenance is supported by both long-lived individuals producing spores over extended periods, and new individuals emerging from those spores. The case with *R. cavernosa* is quite the opposite, since it inhabits temporary wet habitats that are regularly flooded for prolonged periods or completely dried out. The species has almost none desiccation tolerance. Its survival and population maintenance strategy relies on rapid thallus growth, with rapid spore production and deposition. After the spore deposition the thalli are severely fragmented and dying off. This strategy relies entirely on the spore deposits from

the previous generations, and on spore longevity for spore germination in the next favorable season.

2.5 The Dioicous System

The other sexual system – the dioicous one, that can be found among the *Riccia* species seems to be more challenging, considering its complications. Two species exhibit two contrasting strategies – *R. crustata* Trab. and *R. frostii* Austin. *Riccia crustata* is a rare dioicous species, inhabiting temporary wet habitats (Jovet-Ast, 1986; Bischler, 1988). It rarely reproduces sexually, with no records of fertile individuals for Bulgaria and France (Petrov, 1975; Hugonnot et al., 2014). The sterile populations rely entirely on vegetative propagation and thallus longevity for population maintenance. *Riccia frostii* is a heterothallic species with larger females and smaller males. Inhabits alluvial sites, especially recently exposed after flooding (Schuster, 1992a). Like *R. cavernosa*, it has ephemeric nature, rapid development with spore production and quick dying off. The next generation is entirely dependent on the spore deposit, theoretically with initially balanced population sex ratio. The complete dependence on spore production predefines the sex ratio of the gametophytic population. In ideal theoretical conditions, the sex ratio is 1:1, since the spore tetrads are comprised of two male and two females spores.

One of the main challenges facing the dioicous species is the availability of the opposite sex. Another one is the minimum physical proximity between the male and the female, in order for fertilization to occur. The absolute lack of the opposite sex determines unisexual populations relying entirely on vegetative propagation. Such populations can be established in different ways. One way is via a single spore, developing into a unisexual thallus reproducing vegetatively. Another way is by translocation of a fragment or any other type of regenerative tissue. Third option is the Competitive Exclusion Principle, which means that if a population represented by both male and female individuals persist for a given period of time without sexual reproduction, the superior sex will eventually suppress and exclude the lesser com-

petitive one from the given niche (Hardin, 1960). The sex ratio reset by means of spore production, benefiting the less competitive sex (Haig, 2016), is a valid principle, but without the context of longevity of a given species it might insert some bias. Sex competition is expected within long living species and in time, the superior sex is expected to overgrow by clonal reproduction the other, and by spatial isolation to gradually minimize the sexual reproduction events. By this, the reset of the sexes by spores is gradually eliminated.

An ideal theoretical assumption is that all individuals are fertile and freely producing gamets. A representative of this "ideal case" is *R. gougetiana* Durieu & Mont., which is a heterothallic, dioicous species, freely reproducing sexually. It has also desiccation tolerant, tuberiferous thalli, ensuring the survival of the individual. The survival strategy of *R. gougetiana* seems to be quite advanced in comparison of *R. crustata* and *R. frostii*. The sex-competition within the population is aided by regular spore production and the tubercules production, ensuring the constant presence of both sexes. The survival strategy employed by *R. gougetiana* allows it to survive in extreme environments existing well over 95% of the time in a dormant state (Schuster, 1992a).

2.6 The case with *Riccia fluitans* and *Riccia rhenana*

So far the *Riccia* species follow well known strategies for reproduction, clone establishment and maintenance, regardless of their mating system. The drastically different group of species within the genus are the aquatic ones, and more precisely *R. fluitans* and *R. rhenana*. Both species are known to be sterile, especially in Europe (Damsholt 2002). If any record of fertile individuals exist it is strongly doubted and most often considered as *R. stenophylla* Spruce (Schuster, 1992a; Damsholt, 2002). *Riccia fluitans* and *R. rhenana* are exclusively connected to water, with different tolerance to its absence. Usually they are found floating on the surface of water bodies, or slightly submerged, or growing at the edges as terrestrial forms, but yet close to water. The diploid *R. rhenana* (n=16) is expected to be more tolerant to water with-

draw and capable to survive for longer periods in such conditions, since polyploids are generally defined as more adaptable. Theoretically, the haploid *R. fluitans* (n=8) is expected to be much more dependent on water availability and respectively more sensitive to drought, compared the polyploid *R. rhenana*. The main mechanism for propagation is based on growing by indefinite series of dichotomies (Schuster, 1992a) and detached furcations become practically free individuals. The frequency of propagation via modified branches, discussed by Jovet-Ast (1979), based on my observations, seems to be relatively rare, and I was able to observe it only in a few cases, among the examined materials. The most common situation within a given population in its period of active growth, is a combination of individuals with different size, and respectively number of furcations. The extent of this variation can reach in some cases individuals up to 3+ centimeters long and 30-40+ furcations, and in the same time, individuals just 3-4 millimeters long, comprised by a single furcation. This mechanism successfully maintains clone persistence while they are active. The critical point of their development is the resting period. Water growing clones generally die off during late autumn - early winter (in Central Poland for example, the general dying off starts at the beginning of November), and sink to the bottom (Donaghy, 1915), having only the areas of apical meristem alive. In the late summer period in Europe, the still water bodies are significantly warmer in comparison of the early spring period. A combination of warm water, decreased oxygen level, together with algal overgrowth, marks the begging of resting period for the aquatic *Riccia* species. The terrestrial forms can exist relatively longer, since the weather conditions allows it, during the cooler and rainy periods, or if the thalli are growing shaded among reeds, protected by the direct sun and under high air humidity. They are attached to the substrate with rhizoids and rising water levels submerge them. The submerged enough individuals are protected from freezing and despite the observations in Indiana of surviving meristem of terrestrial thalli, after winter frost (Donaghy, 1915), the survival out of the water should be considered rare in Europe, due to the prolonged periods of frost in many of its parts (direct frost damage and indirect damage via dehydration).

2.7 Discussion

This strong dependency to water inevitably ties both species to water bodies. The seasonal fluctuations of the water levels have profound effect on their development and on the mechanisms of growth, reproduction, seasonal dynamics, and overall survival. In theory the desiccation stress does not reduce the thallus/shoot size, but drastically reduces the water content of the cells, combined with exposure to high temperatures. This is the complete opposite of the *R. fluitans/rhenana* case, where we observe transition from a period of active growth to period of dormancy, fully submerged in water and exposure to cold temperatures and reduced daylight period. This transition is accompanied by decaying of the old parts and significant reduction in photosynthetic tissue. *Riccia fluitans* and *R. rhenana* rely entirely on the apical meristem areas to develop into a new individual. This strategy seems unprecedented and unique to these species, since for a given period of time, the entire population is dormant and the end of dormancy starts by development of new individuals from the remaining meristems. It is an extreme form of survival not aided by any type of specialized structures such as gemmae or tubers. In fact, we have unified developmental state of the entire population, relying on same unified factors for its reemerging. In other groups of bryophytes, when asexual reproduction is in hand, the already established individuals remain capable of continuous production of fragments, gemmae or tubers. Such clones maintain their populations via the regular presence of more than one state of their developmental forms.

Above I have discussed the gradual prevalence of the superior sex over time in long living species, in the context of populations producing spores. The one sex prevalence over time should be expected much sooner in asexually reproducing populations, since no ratio compensation from spores is available. The resting behavior of aquatic clones predefines the clone reestablishment in the period of active growth, and its maintenance. Since the older parts die off during the resting period and only the apical meristem areas remains green and alive, the initial number of individuals of the clone will be equal on the number of the separated free meristems. By this, we

can expect that the most dominant clone is the one with highest furcation rate, since it will produce highest number of meristems, ensuring highest number of reemerging individuals. In relation of sex ratio, populations comprised only by females occur more frequently, than those comprised only by males (Bowker et al., 2000). In the context of *R. fluitans*, males are extremely rare event, and only the reports of Jovet-Ast (1979), Damsholt and Hallingbäck (1986) and Paton (1973) exists, which are discussed as possible misidentification with *R. stenophylla* by Schuster (1992a). During few years of field expeditions in Bulgaria, two female populations have been found, but no males have been observed. In regards to *R. rhenana*, no records of any type of sexuality exists, confirmed by chromosome counts. An experiment on the dioicous species *Marchantia inflexa* Nees & Mont. by McLetchie and Puterbaugh (2000), shows that females outperform males in growth rate and number of meristematic tips, which are directly related to the clonal growth, while males outperform females in the production of asexual offspring. Another conclusion from the same work is the phalanx-like strategy of the females and guerrilla-like strategy of the males. Size difference is also observed in favor to females. Such difference between the size of the sexes is quite well expressed and documented in *R. frostii* and *R. gougetiana* (Jovet-Ast, 1986).

These observations can explain to a good extend the *R. fluitans/rhenana* behavior and the lack of males. Larger females, expectedly produce more furcations and more meristem tips, directly affecting the sex ratio by generating more branches, which furcate during the active season and deposit their meristems in the late autumn. *Riccia* males do not produce specialized asexual structures (such as gemmae), so their only way to compensate the meristemal deposid decrease tendency is via spores. The phalanx and guerrilla strategies (sensu Schmid and Harper (1985)) could explain the female prevalence, but since the behavior of the males is not known, only speculations can be made. Growing and branching, the floating thalli form clumps entangled together, which fits the phalanx-like strategy. Until the period of rapid decay prior dormancy, we can expect them to grow closely together. When the de-

cay starts, part of the thalli dies off and the remaining meristems are released and by this they become free smaller fragments capable of covering larger area. This quality fits to some extend the guerrilla-like strategy. Knowing this, we may suspect that the males and the females in the case of *R. fluitans/rhenana* employ alternations from phalanx-like to guerrilla-like strategies. There remains the question how exactly those plants are transferred from one place to another, lacking spores, or other specialized structures. The commonly accepted theory is that the dispersal is made by migrating birds, from one water body to another (Paton?). The significant reduction of the thalli during autumn and spring, fits the migration time of birds in both directions, and the smaller thallus size favors the chances of fragment dispersal.

2.8 Conclusion

The systematic alternation between favorable and unfavorable conditions have different impact on different species. Since various survival mechanisms are developed, dealing with different types of unfavorable conditions, the behavior of the species in the favorable periods is directly influenced. One point of view can be that the favorable condition is an opportunity window to adapt and survive the unfavorable one. In this regard, the aquatic *R. fluitans/rhenana* exhibit extremely simplified behavior, relying only on one not quite sophisticated strategy. The unified state of all individuals during the period of dormancy, critically exposes the entire population to a single wiping-out event. If such event occurs, being in same state, we can expect that all individuals will be affected. Not having any securing option via spore deposition, propagules, tubercules or anything else, is potentially a hazardous behavior. The only applied mechanism securing survival seems to be the rapid growth and propagation during the active period, relying on the entirely statistical possibility that if a wiping-out event occurs, at least some percent of the individuals will survive and rapidly reproduce. So far, this behavior seems rare among bryophytes, considering diverse structures developed, to deal with unfavorable conditions. Yet,

despite being seemingly primitive, this strategy favors the distribution of aquatic *R. fluitans/rhenana* considering their range of occurrence. Furthermore, the behavior of the *Fluitans* group is poorly known in the tropics and reports of fertile plants remain scarce sterility due to unfavorable conditions cannot be ruled out.

There is a theory of non-native origin, possibly even invasive behavior, of *R. rhenana* in Europe (Preston et al., 2011; Roy et al., 2020). I do not discuss this intentionally, since the true distribution of this species remains poorly known. If the invasive potential of *R. rhenana* later becomes proven, this would contribute to the efficiency of its survival strategy. In this work I also do not discuss the fertility or more precisely its lack, due to environmental conditions. In order for such conclusion to be identified, first the favorable conditions, under which a given species is freely and abundantly reproducing sexually must be defined.

Chapter 3

Distribution of *Riccia fluitans* and *Riccia rhenana* (Marchantiales) in Bulgaria – towards a conflict at a ploidy level

3.1 Abstract

Riccia fluitans and *R. rhenana* are widely distributed aquatic liverworts with challenging species delimitation. The insufficient identification causes uncertainty in the species distribution. In this study, the occurrence data for Bulgaria is summarized, and compared with the records available in GBIF for the territory of Europe. Based on the Bulgarian occurrence and the European occurrence, comparison of the frequencies of both species is made. A prevalence of *R. rhenana* over *R. fluitans* in Bulgaria is here reported, which is the opposite to the data from GBIF. The striking prevalence of *R. fluitans* over *R. rhenana* from GBIF data shows the need for careful species determination and highlights the risks of use of such databases. The field data indicated species-specific trends with respect to habitat origin and microhabitat preferences.

3.2 Introduction

Accurate species identification is crucial for understanding species distribution. In the case of closely related species, occupying similar or identical ecological niches, this step has a profound role. Misidentification can lead to conclusions based on observations of multiple competing species rather than a single species. This issue is compounded when analyzing data from diverse sources without critical evaluation, further distorting our understanding and conclusions.

Riccia fluitans L. and *R. rhenana* Lorb. ex Müll. Frib. (Ricciaceae, Marchantiales) are species of liverworts, sharing similar morphology, and similar ecological niches (Schuster, 1992b; Damsholt, 2002). They are the most common aquatic representatives of genus *Riccia* L. in Europe, but being morphologically almost indistinguishable in their aquatic form, their distribution remains poorly known. The lack of stable morphological species-discriminative traits, and the existence of other morphologically similar members of the section, cause the treatment of this group under the generalized name *"Fluitans Complex"* (Berrie, 1964). The morphological

similarity between *R. fluitans* and *R. rhenana* is due to the general lack of stable traits (Evans, 1922), to a large phenotypic plasticity, and on the other hand, on their proposed genetic relationship. It has been hypothesized that *R. rhenana* is an autodiploid deriving from *R. fluitans* (Berrie, 1964). Since both species inhabit similar ecological niches, and moreover being haploid-polyploid genetically related, ancestor displacing events might be speculated (Alix et al., 2017).

Currently, there is no work dealing with the distribution of the complex in Europe. Also, there is no detailed data, based on chromosome counts for the species distribution in Bulgaria. The species determination based on morphology is questionable, and the only reliable method for delimitation, especially of aquatic forms, is based on the chromosome counts, where *R. fluitans* is a haploid n=8 plant, while *R. rhenana* is a diploid n=16 (Müller, 1941). The aim of this work is to present the current knowledge on the distribution of *R. fluitans* and *R. rhenana* in Bulgaria and to draw the attention to the importance of correct species determination. To do this, the distribution of *R. fluitans* and *R. rhenana* in Bulgaria is summarized, and compared with the reports generated from GBIF (https://www.gbif.org/) for Europe. For the purposes of this work, the GBIF datasets were intentionally not revised, so they fully represent the common awareness on the both species.

3.3 Materials and Methods

For the purposes of this study, two different datasets were made. One dataset, representing the haploid-diploid distribution in Bulgaria (Table 3.1), and a second dataset, representing the distribution of *R. fluitans* and *R. rhenana* in Europe. The dataset with Bulgarian samples, contains 63 entries of published (Natcheva, 2007, 2008; Tzonev, 2006; Iordanoff, 1931; Ellis et al., 2021) and herbarium data. Attempt has been made to re-visit all known locations and collect life material for chromosome counting. The dataset dealing with the general distribution of *R. fluitans* and *R. rhenana* across Europe is based on 13 566 entries, acquired from GBIF. Of them, 13 458 entries represent *R. fluitans* (DOI: 10.15468/dl.pg3cy4), and 108

entries represent *R. rhenana* (DOI: 10.15468/dl.svfyvs). No GBIF data was used for the Bulgarian distribution. The geographic parameters specified for the both GBIF sets are as follows: Continent: Europe; Country area: Albania, Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Montenegro, Netherlands (Kingdom of the), Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, United Kingdom of Great Britain and Northern Ireland. For both sets, only entries with geographic coordinates were selected. The entries from GBIF were taken the way they were provided by the platform. No revision of the identifications of the entries were made.

Table 3.1: Geographic coordinates, substrate and habitat origin of the analyzed Bulgarian samples. Missing data is indicated with "-"

Species	**Longitude**	**Latitude**	**Substrate**	**Habitat origin**
R. fluitans	23.039843	42.48568	floating	natural
R. fluitans	23.5957182	42.563935	terrestrial	semi-natural
R. fluitans	23.595718	42.5639352	floating	semi-natural
R. fluitans	23.6260434	42.439938	floating	semi-natural
R. fluitans	26.51591	44.03681	-	semi-natural
R. fluitans	23.80273	42.93452	floating	semi-natural
R. fluitans	23.803647	42.933978	floating	semi-natural
R. fluitans	24.059118	42.312024	floating	natural
R. fluitans	25.210693	43.667163	terrestrial	natural
R. fluitans	26.515838	44.0369	floating	semi-natural
R. fluitans	26.515838	44.0369	terrestrial	semi-natural
R. fluitans	26.522205	44.025891	terrestrial	semi-natural
R. fluitans	26.522205	44.025891	terrestrial	semi-natural
R. fluitans	22.831972	42.396861	floating	natural
R. fluitans	23.039843	42.48568	floating	natural
R. fluitans	23.040283	42.48683	floating	natural
R. fluitans	23.040512	42.486773	floating	natural
R. fluitans	23.040512	42.486773	floating	natural
R. fluitans	23.041445	42.486073	floating	natural
R. fluitans	24.056278	43.186444	terrestrial	natural
R. fluitans	25.212023	43.66694	terrestrial	natural
R. fluitans	25.212729	43.666707	terrestrial	semi-natural
R. rhenana	22.831972	42.396861	floating	natural
R. rhenana	23.239854	42.703737	floating	man-made
R. rhenana	23.446447	42.587126	-	natural

Table 3.1: Geographic coordinates, substrate and habitat origin of the analyzed Bulgarian samples. Missing data is indicated with "-". (Continued)

Species	**Longitude**	**Latitude**	**Substrate**	**Habitat**
R. rhenana	23.509557	42.580081	terrestrial	semi-natural
R. rhenana	23.543663	42.441961	terrestrial	man-made
R. rhenana	23.599141	42.562499	floating	semi-natural
R. rhenana	24.059118	42.312024	floating	natural
R. rhenana	24.151631	43.042051	terrestrial	natural
R. rhenana	25.109342	43.659936	terrestrial	man-made
R. rhenana	27.06145	44.11678	-	natural
R. rhenana	27.06636	44.08954	-	natural
R. rhenana	27.066722	44.089941	terrestrial	natural
R. rhenana	27.079491	44.120281	terrestrial	natural
R. rhenana	27.079491	44.120281	terrestrial	natural
R. rhenana	27.080486	44.120849	terrestrial	natural
R. rhenana	27.080771	44.120761	terrestrial	natural
R. rhenana	27.080771	44.120761	terrestrial	natural
R. rhenana	27.080771	44.120761	terrestrial	natural
R. rhenana	27.08103	44.12092	-	natural
R. rhenana	27.725887	42.331815	floating	natural
R. rhenana	23.156738	42.631653	terrestrial	natural
R. rhenana	23.156738	42.631653	terrestrial	natural
R. rhenana	23.156738	42.631653	terrestrial	natural
R. rhenana	23.156738	42.631653	floating	natural
R. rhenana	23.308	42.6593	floating	man-made
R. rhenana	23.509557	42.580081	floating	semi-natural
R. rhenana	25.109415	43.659771	terrestrial	man-made
R. rhenana	25.210693	43.667163	terrestrial	natural
R. rhenana	27.725887	42.331815	floating	natural
R. rhenana	27.7306	42.24766	terrestrial	natural
R. rhenana	23.367868	42.595414	terrestrial	natural
Riccia sp.	23.445474	42.587292	-	-
Riccia sp.	24.0065	42.05165	-	-
Riccia sp.	24.19033	42.00934	-	-
Riccia sp.	24.861605	43.665751	-	-
Riccia sp.	25.07335	43.645619	-	-
Riccia sp.	25.213052	43.66668	-	-
Riccia sp.	25.2132	43.66687	-	-
Riccia sp.	26.51689	44.026583	-	-
Riccia sp.	27.71116	42.2994	-	-
Riccia sp.	27.7306	42.24766	-	-

Two habitat factors for the Bulgarian samples were scored: microhabitat (aquatic vs. terrestrial) and origin of the site. The aquatic habitat means that the given sample was collected from the water (floating), while the terrestrial habitat means that the sample was collected growing on mud. The origin of the habitat was treated in three categories: 1) natural – a natural wetland with no or minor human impact, which did not significantly change hydrology significantly; 2) semi-natural – a water body that is strongly affected by man and is a remnant of an older natural wetland, possibly harboring remnants of an older local population; 3) man-made – entirely artificial water body constructed over non-wetland habitat, whence the populations of *Riccia* are newly established.

For the chromosome counts, plant material was fixed directly in the field in Clarke's solution (3:1 ethyl alcohol/glacial acetic acid) or plants were collected and kept alive in a greenhouse. After a period of adaptation, a modified Gomori's haematoxylin staining method was used (Melander and Wingstrand, 1953). Fresh actively growing thallus tips were placed in 0.01% solution of colchicine for 90 min. After washing in distilled H_2O they were fixed in Clarke's solution for 2 h at room temperature or in a fridge for 24 h. After washing in distilled H_2O the thalli were placed in 1M hydrochloric acid for 40 min at 60 °C followed by washing in distilled H_2O. The samples were incubated in hydrochloric acid /ether in ratio 1:1 for 15 min at 60 °C, washed in distilled H_2O the stained in Gomori's Hematoxylin for 1.45/2 h at 60 °C. Samples were squashed in 45% acetic acid and observed under a light microscope. At least five thalli per populations were checked.

3.4 Results

Based on the chromosome counts (Figure 3.1) we were able to confirm 53 of the 63 known Bulgarian locations. Of them 31 were confirmed to be diploid *R. rhenana* and 21 haploid *R. fluitans*. Eleven entries could not be relocated and samples obtained. These were treated as *"Fluitans complex"*. The distribution of the complex in Bulgaria clearly shows the prevalence of the diploid *R. rhenana* over the haploid

R. fluitans. The European distribution (Figure 3.3) shows the opposite pattern - strong prevalence of *R. fluitans* over *R. rhenana*. In two occasions mixed populationa have been observed.

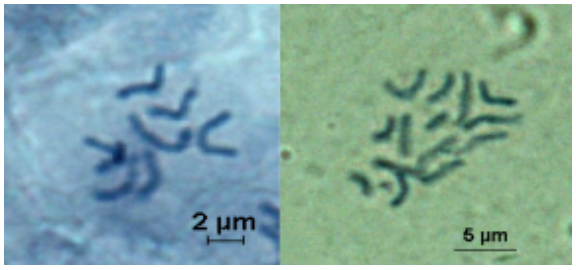

Figure 3.1: Haploid (left) and diploid (right) chromosome sets of *R. fluitans* and *R. rhenana*.

Based on the datasets, two maps have been created, one for the Bulgarian distribution (Figure 3.2) and second one for the European distribution (Figure 3.3).

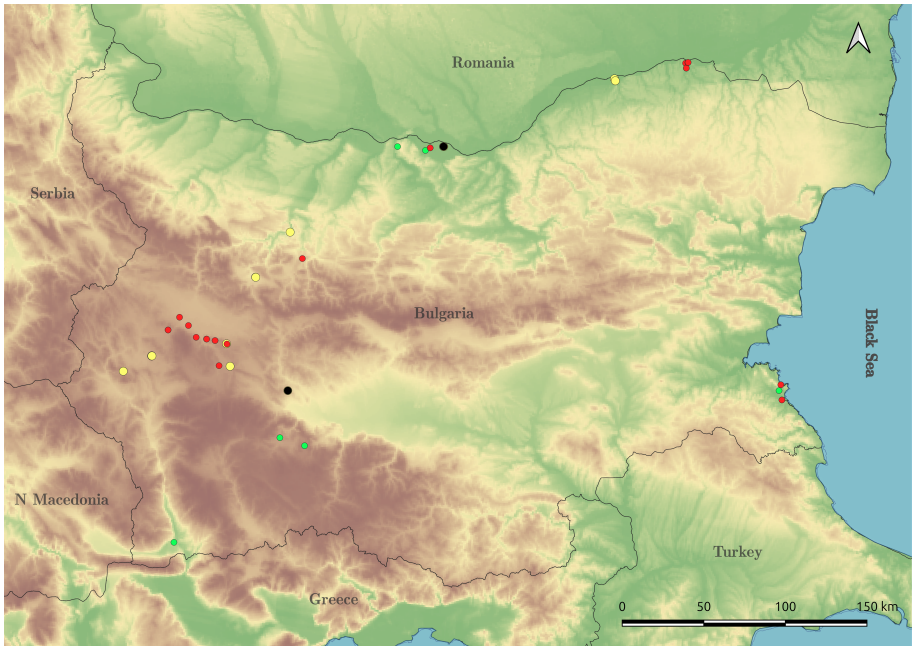

Figure 3.2: Distribution of *R. fluitans* (yellow) and *R. rhenana* (red) in Bulgaria, green – not confirmed cytologically. Black circles indicate the locations where both species co-occur.

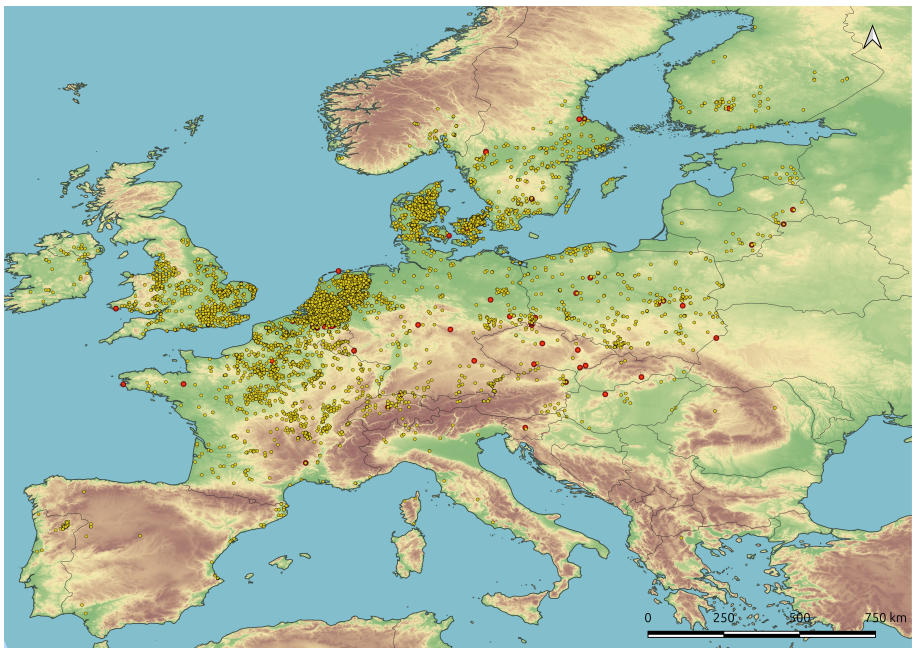

Figure 3.3: Distribution of *R. fluitans* (yellow) and *R. rhenana* (red) in Europe, based on data acquired from GBIF

In Bulgaria, *Riccia fluitans* was found as aquatic in 13 populations, and as terrestrial in eight populations (Figure 3.4). *Riccia rhenana* was collected as terrestrial from 18 populations and from nine populations as aquatic. With respect to habitat origin, the diploid *R. rhenana* was present in all habitat types (Figure 3.5). It was the only species in man-made habitats (four populations), also very common in natural habitats (23 populations), and with low presence (three populations) in semi-natural habitats. whereas the haploid *R. fluitnas* occurred in both semi-natural (11 populations) and natural locations (11 populations) but was not observed in entirely man-made habitats.

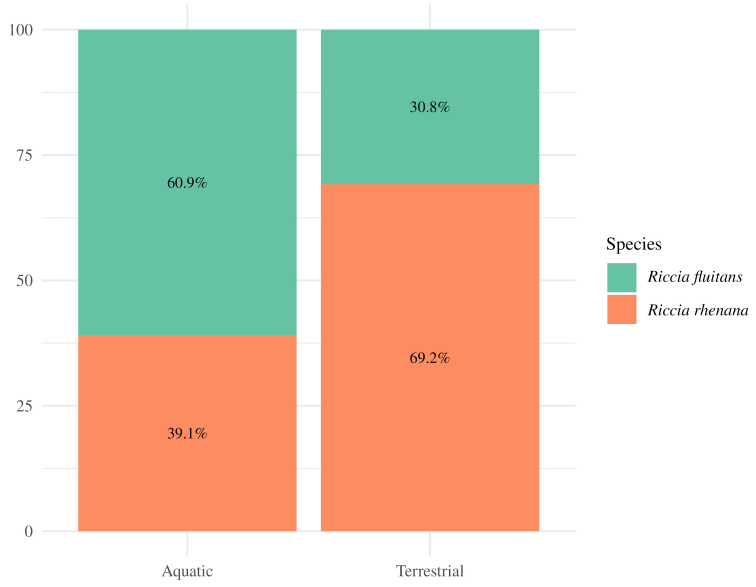

Figure 3.4: Microhabitat occurrence of *R. fluitans* and *R. rhenana*.

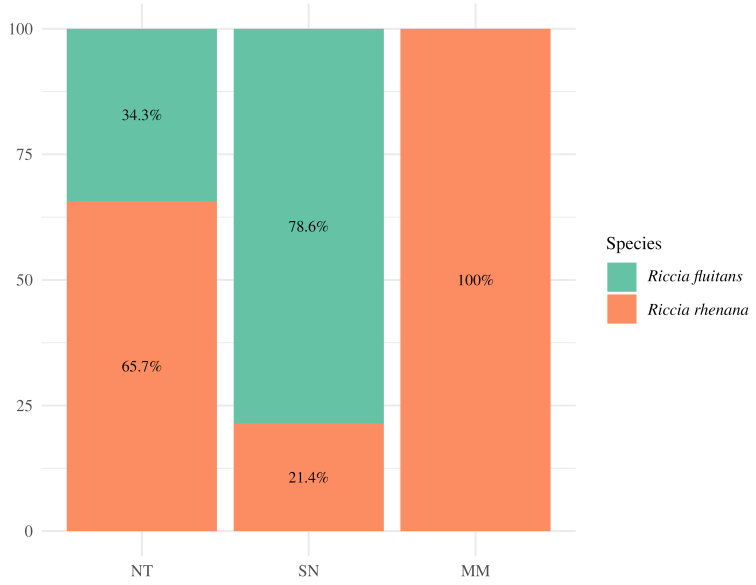

Figure 3.5: Habitat preference of *R. fluitans* and *R. rhenana* in Bulgaria, based on habitat origin. NT - natural, SN - semi-natural, MM - man-made.

3.5 Discussion

Even being a cosmopolitan group of plants, bryophytes are restricted to specific niches (Shaw and Goffinet, 2000). Thus, their distribution is not unlimited, but rather restricted to particular conditions, often found only in very specific types of habitats (Slack, 1990). Given that speciation can also occur through polyploidization (Rieseberg and Willis, 2007), contributing to increased species diversity within the community (Ross, 1972), both species are likely to compete for establishment in the events of coexistence. Despite being potentially genetically related, they should be reproductively isolated and sexual reproduction between the original, and derived chromosome number should not be expected (Newton, 1984), while this isolation would serve for the establishment of two discrete gene pools (Newton, 1990). We have observed a case of coexistence of *R. fluitans* and *R. rhenana* at two locations, and prevalence of the one over the other was not concluded. There might be other cases of coexistence that have remained unnoticed, especially when only aquatic forms were present. This might suggest the possibility of sympatric evolution, for which polyploidization is believed to be the single most important mechanism (Otto and Whitton, 2000).

In general, polyplodization has the potential to alter plant morphology, physiology, phenology, and ecology within just one or few generations (Levin, 1983; Te Beest et al., 2012), providing the newly formed polyploid with features superior to the ancestral progenitor. In this sense, a polyploid can potentially outcompete its progenitor or/and exploit new niches (Leitch and Leitch, 2008; Te Beest et al., 2012). Such advantage can be considered as invasive potential, and the distribution of *R. rhenana* as a invasive species is worth investigating. Newly formed polyploids often exhibit rapid range expansion covering various habitats (Levin, 1983; Hull-Sanders et al., 2009; Treier et al., 2009; Te Beest et al., 2012). For example, the invasive tetraploid *Centaurea stoebe* L., where the invasive populations are dominated by tetraploids (Treier et al., 2009; Henery et al., 2010; Te Beest et al., 2012). The origin of *R. rhenana* remains uncertain, and if it is native to Europe. It

is included in "DAISIE –Inventory of alien invasive species in Europe" (available at https://www.gbif.org/taset/39f36f10-559b-427f-8c86-2d28afff68ca) as "Native to some parts of Europe". Preston et al. (2011) lists it as a "recent introduction" to the British and Irish liverwort flora. In the work of Wyatt and Davison (2013), *R. rhenana* is concluded to be restricted to calcareous seepage fens, at least in Georgia (United States). However, no cytological confirmations of the cited materials seems to be mentioned. In Bulgaria, *R. rhenana* occupies various habitats and such restricted occurrence as the mentioned above, cannot be inferred. Although it was not possible to reestablish a few historical reports and to verify their species identity, the prevalence of *R. rhenana* over *R. fluitans* at a significantly smaller territory as Bulgaria suggests that the abundance of *R. rhenana* is much higher in Europe, than the GBIF data suggests.

Riccia rhenana has been found in its terrestrial form much more often, than *R. fluitans*. This could partly be a sampling bias, as terrestrial forms are often easier to detect and collect. However, our observations indicate that, the diploid seems to be much more tolerant and freely growing as a terrestrial plant, while the haploid occurs as a terrestrial form in cases where it is forced to do so. The terrestrial haploids might be more short-living, compared to the diploids, and are likely more sensitive to prolonged periods of drying.

Despite the limited data, the habitat origin seems to play a role in the species occurrence (Figure 3.5). The semi-natural habitats are with significant interest since they most likely harbor old species establishments and the haploid *R. fluitans* is found relatively more often, than the diploid. Multiple factors should be considered for the proper understanding of the competing nature of the haploid-diploid occurrence. For example, drastic changes in the water level, could affect negatively a haploid population, since its response to such change should be much slower, and not so effective as the response of the diploid. Another factor is the depth of the given water body. Both the haploid and the diploid rest as fragments at the bottom of the inhabited pond or lake during winter, and rely on the increased light period in

the beginning of the spring to start growing again (Schuster, 1992b). In this period, they are competing with the rest of the inhabitants to grow and reemerge on the surface, where they should establish and maintain their population. Both species are found usually co-occurring with species of *Lemna* L. (most often *L. minor* L. and *L. trisulca* L.), species of *Potamogeton* L., and *Ricciocarpos natans* (L.) Corda. In such competing environment, the haploid seems in a disadvantage position, since all the floating competitors shade the bottom and if the early start is not efficient enough, the population establishment will be negatively affected. Possessing two functional genomes, the diploid is expected to respond faster to the conditions of the environment and to its competitors. By this, we could expect that the population establishment and population maintenance of *R. rhenana* is more effective, favored by the genetic advantage (Te Beest et al., 2012). In the course of our fieldwork, two populations of *R. fluitans* were found to be fertile, comprised only by female individuals (spores have never been observed), while *R. rhenana* has never been observed with reproductive structures. Even not proven to reproduce sexually, the haploid *R. fluitans* invests resources in the production of sexual structures, which indicates that not all resources are focused on the clonal reproduction by fragmentation. Presumably, the diploid should be monoicous, and by this, freely reproducing sexually, which is the case of *R. duplex* Lorb. ex Müll. Frib. (Müller, 1941). Unlike it, *R. rhenana* is always sterile and reproducing exclusively via thallus fragmentation.

3.6 Conclusion

In conclusion, based on the current data, *R. rhenana* is more abundant than *R. fluitans* in Bulgaria and the chromosome number remains the only reliable character for species delimitation. Thus, *R. rhenana* is likely much more common in Europe but routinely misidentified for its haploid relative. Growing in a wider range of habitats and being more often found, fits the polyploid invasive behavior. At this stage, we are not able to discuss further the invasive potential of the diploid, since

no definitive habitat has been identified for the haploid. However, one feature of the preferred habitat of *R. fluitans* seems to be the more stable water levels over the year, while *R. rhenana* seems to be much more tolerant to significant water fluctuations, which might be advantage in result of its polyploid origin. Further studies are needed to elucidate the true distribution, detailed niche preferences, and the competitive relationship of both species.

Chapter 4

Genome size and chromosome counts of some *Ricciella* species

4.1 Abstract

Section *Ricciella* is comprised of species with similar morphological appearance and unresolved genetic relationship. Two pairs of haploid-diploid species are part of this section: *R. fluitans* - *R. rhenana* and *R. canaliculata* - *R. duplex*. The chromosome counts are well known, but no conclusive data on the genome size of the section is available. In this work we provide data on the 1C values of the species *R. fluitans*, *R. canaliculata*, *R. rhenana*, and *R. duplex* s. lat.

4.2 Introduction

Ricciella Broun is a section within genus *Riccia* L. (Order Marchantiales, Class Marchantiopsida). In Europe it is represented by *R. fluitans* L., *R. rhenana* Lorb. ex. K. Müller, *R. canaliculata* Hoffm., *R. duplex* Lorb. ex. K. Müller., and *R. huebeneriana* Lindenb (Schuster, 1966). The species within the section are similar in their appearance and challenging for identification, so a generalized name *"Fluitans Complex"* is often used. Since *R. fluitans* and *R. canaliculata* are haploids (n=8), while *R. rhenana* and *R. duplex* are diploids (n=16) (Müller, 1941), their potential genetic relationship is investigated by Berrie (1964). Colchicine-induced polyploids of *R. fluitans* are shown to be morphologically identical with *R. rhenana*, and induced polyploids of *R. canaliculata* are morphologically identical with *R. duplex*, with exception of the sexuality, where *R. duplex* is monoicous, while the experimental polyploids are dioicous. Based on that work, *R. rhenana* and *R. duplex* are suggested to be autodiploids, and the chromosome counts are used as main species-delimitating factor within the complex.

The genome size of the species across the genus is not well known, and the 1C-value is estimated only for four species, collected in America: *R. campbelliana* M. Howe 1C=0.60pg, *R. crystallina* L. 1C=0.52pg, *R. huebeneriana subsp. sullivantii* (Austin) 1C=0.41pg and *R. trichocarpa* M. Howe 1C=0.51pg (Bainard, 2011). However, no data on the genome size of the *"Fluitans Complex"* seems to be available,

with exception of the mentioned *R. sullivantii*, and the work of Temsch et al. (2010), where the 1C-value is given for *R. fluitans s. l.* and commented as "conspecific with *R. duplex*". The aim of this study was to investigate the genome size of *R. fluitans*, *R. rhenana*, *R. canaliculata*, and *R. duplex*.

4.3 Materials and Metods

Plant material.

A total of seven populations were sampled from Bulgaria, Poland and Sweden (Table 4.1). For the purpose of this study, the samples were cultivated in non-sterile conditions, with daylight regime 12h day/12h night at temperature of 18°C. Chromosome counts and genome size were obtained for each sample. Also, since the species delimitation within the *"Fluitans complex"* is problematic, *trnL-F* and *psbA* chloroplast regions were used for species identification and phylogenetic analysis.

Table 4.1: Plant material collection sites of studied *Riccia* species with herbarium vouchers numbers.

Number	Species	Origin	Geographic coordinates	Collector
TRF61	*R. canaliculata*	Sweden	57.794002° 18.48426°	Peters, K.
SOM 10779-B	*R. canaliculata*	Bulgaria	41.942711° 24.178324°	Gospodinov, G., Natcheva, R.
TRF1	*R. fluitans*	Bulgaria	42.440475° 23.548410°	Gospodinov, G., Natcheva, R.
TRF48	*R. fluitans*	Sweden	55.506138° 13.39021°	Gospodinov, G., Cronberg, N.
TRF49	*R. duplex*	Sweden	55.506138° 13.39021°	Gospodinov, G., Cronberg, N.
SOM 11950-B	*R. rhenana*	Bulgaria	44.120761° 27.080771°	Natcheva, R.
TRF5	*R. rhenana*	Poland	52.475260° 17.19260°	Gospodinov, G.

Chromosome counting.

Fresh, actively growing thalli from each of the populations were used for modified Gomori's haematoxylin staining (Melander and Wingstrand, 1953). They were placed in 0.01% solution of colchicine for 90 min, and washed in distilled H_2O. Fixation was done in Clarke's solution (3:1 ethyl alcohol/glacial acetic acid) for two hours at a room temperature. The thalli were washed again and placed in 1M hydrochloric acid for 40min at 60°C. After another washing, incubation in hydrochloric acid/ether in ration 1:1 for 15 min at 60°C was done and the thalli were washed again and stained in Gomori's haematoxylin for 1.45-2 hours at 60°C. The growing

tips of the thalli were squashed in 45% acetic acid and observed under a light microscope (Gospodinov and Natcheva, 2020). The chromosome counts of at least five individuals for each population were checked. No variation within the populations was observed.

Genome size measurement.

About 25mg of fresh *Riccia* thalli were co-chopped (Galbraith et al., 1983) together with leaf material of the standard organism *Solanum pseudocapsicum* (1.295pg/1C; (Temsch et al., 2010, 2022)) in cold Otto´s buffer I (Otto et al., 1981). The isolate was filtrated with a 30m nylon mesh, supplemented with RNase (0.15 mg/L, Sigma Aldrich, USA) and incubated for 30 minutes at 37°C. Subsequently, Otto´s buffer II (Otto et al., 1981) complemented with propidium iodide (PI; 50mg/L, Applichem, Germany) followed by an incubation between 1 hour up to over-night at the refrigerator. Measurements were performed using a CyFlow flow cytometer (Partec/Sysmex, Germany) equipped with a green laser (532nm, 30mW, Cobolt AB, Sweden). Three runs per sample, in total 10,000 particles, were conducted and the 1C-values were calculated according to the formula (FI = mean fluorescence intensity peak position): 1Cobj = FIobj / FIstd * 1Cstd *2

DNA extraction.

Total DNA was extracted from a single fresh thallus, from each sample, using CTAB method (Murray & Thompson, 1980). The DNA concentration of all samples was equalized in the range of 20-30 ng/μL and stored at -20°C.

PCR amplification and sequencing.

Two chloroplast regions – *trnL-F* and *psbA* were sequenced. Both amplification and sequencing were made with the following primer pairs for *trnL-F* (Taberlet et al., 1991), and *rbcL* (Pedersen and Hedenäs, 2003):

trnL-F

trnC_F (5′-CGAAATCGGTAGACGCTACG-3′)

and

trnF_R (5′-ATTTGAACTGGTGACACGAG-3′).

psbA

trnK2_F (5′-GACGAGTTCCGGGTTCGA-3′)

and

psbA576_R (5′-TGGAATGGGTGCATAAGG-3′)

PCR amplification was performed in a total volume of 20 μL, containing 2 μL of 10× PCR buffer with Mg2+ (Novazym; 25 mmol $MgCl_2$), 1 μL of bovine serum albumin (0.25 mg/ml), 200 μmol of each dNTP (Novazym), 0.4 μmol of each primer, 1 U of Taq DNA polymerase (Novazym) and 1 μL of template DNA (Buczkowska et al. 2018). The PCR reaction was performed in the following cycling steps: 4 min of initial denaturation at 94ºC, followed by 30 cycles of 60 s at 94ºC, 30 s at annealing temperature at 55 ºC, and 60 s at 72ºC, with a final extension step of 7 min of 72ºC. Three microliters from the amplification product was visualized on 1.0% agarose gel, and the remaining volume was purified with Exo-AP (Thermo Scientific), and sequenced in both directions.

Data analysis.

The obtained chromatograms of the DNA sequences were analyzed and edited with MEGA 11.0.13 (Tamura et al. 2021), and consensus sequences were generated with PhyDe v0.9971 (Müller et al. 2010). Alignment was generated using the algorithm of MUSCLE (Edgar 2004). Gaps and missing nucleotides were removed and the final sequences were with total length of 402 nucleotides. Maximum Likelihood tree was generated using MEGA 11.0.13 with bootstrap analysis of 1000 pseudoreplicates and GTR + G model, based on Bayesian Information Criterion (BIC).

4.4 Results

Chromosome counts and genome sizes (Table 4.2) were obtained for four species: *R. fluitans*, *R. rhenana*, *R. canaliculata* and *R. duplex*.

Table 4.2: Chromosome counts (n) and 1-C values of the investigated *Riccia* species

Number	Species	Origin	n	1C-value (pg)
TRF61	*R. canaliculata*	Sweden	n=8	0.4847
SOM 10779-B	*R. canaliculata*	Bulgaria	n=8	0.4871
TRF1	*R. fluitans*	Bulgaria	n=8	0.6661
TRF48	*R. fluitans*	Sweden	n=8	0.6624
TRF49	*R. duplex*	Sweden	n=16	0.8039
SOM 11950-B	*R. rhenana*	Bulgaria	n=16	1.0548
TRF5	*R. rhenana*	Poland	n=16	1.0451

Comparison of the genomes of *R. fluitans* and *R. rhenana*, as well as *R. canaliculata* and *R. duplex*, reveals smaller genomes of the diploid species than the expected additive sum of their parental genomes (Figure 4.1).

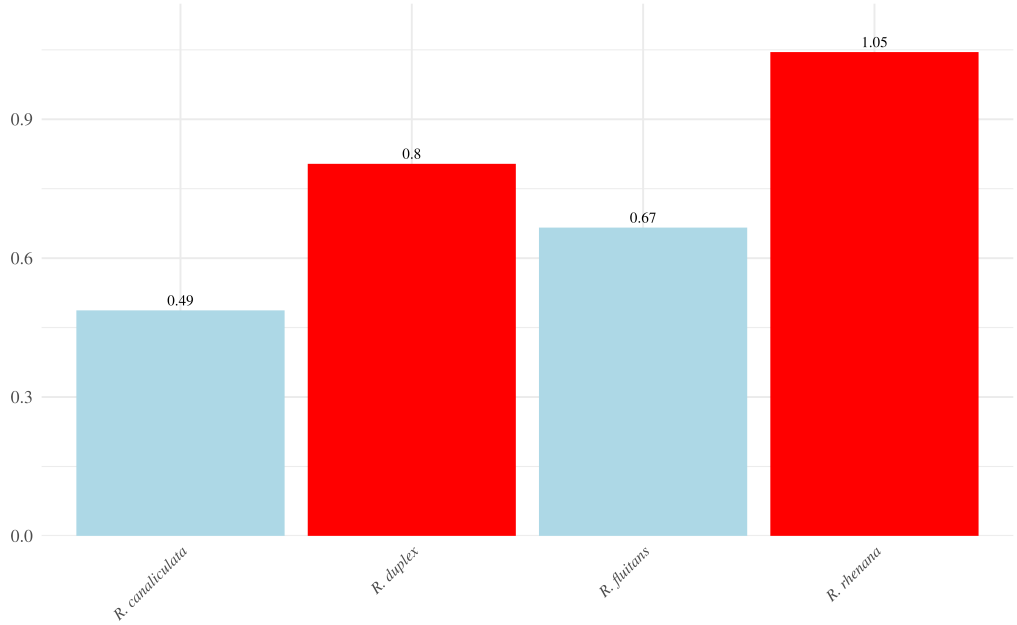

Figure 4.1: Comparison the haploid-diploid *Ricciella* pairs. The genome size (pg) of each species is displayed on the top of its bar.

Specifically, the genome size of *R. rhenana* is 1.57 times that of *R. fluitans*, and *R. duplex* is 1.63 times the size of *R. canaliculata*. This phenomenon is known as genome downsizing (Leitch et al. 2004), supposedly as result of DNA loss after forming polyploids (Soltis et al. 2015).

A similar trend is observed in the polyploid liverwort *Calypogeia sphagnicola f. paludosa*, whose diploid genome (1C = 1.125 pg) is 1.77 times larger than the haploid genome of *C. sphagnicola f. sphagnicola* (1C = 0.634 pg (Buczkowska et al. 2012)) (Figure 4.2).

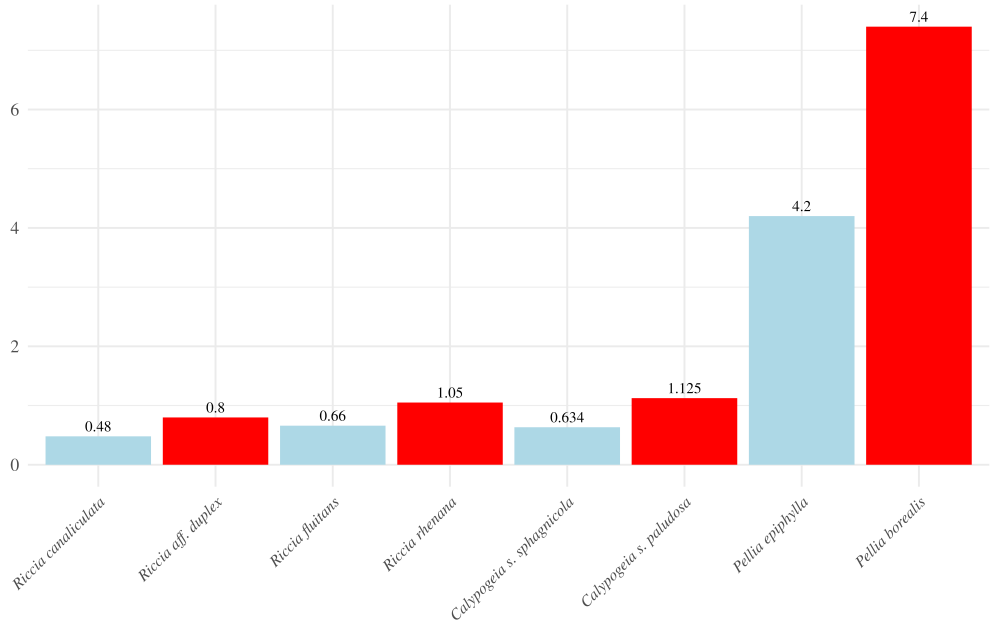

Figure 4.2: Comparison between known haploid-diploid liverwort species pairs. The genome size (pg) of each species is displayed on the top of its bar. With red color are indicated the diploid species.

The observed genome downsizing of the diploids (Table 4.3) ranges from 11.28 to 20.45% of the additional genome with an average reduction of approximately 15.08%.

Table 4.3: DNA loss (%) after diploidization, based on the proposed haploid parent.

Species	***R. duplex***	***R. rhenana***	***C. s. f. paludosa***	***P. borealis***
DNA loss	16.67	20.45	11.28	11.90

A comparison of the known *Riccia* 1C-values is made, with the known 1C values of 89 other species of liverworts (Figure 4.3), illustrating the difference between the *Riccia* species, and the variation range within the genus.

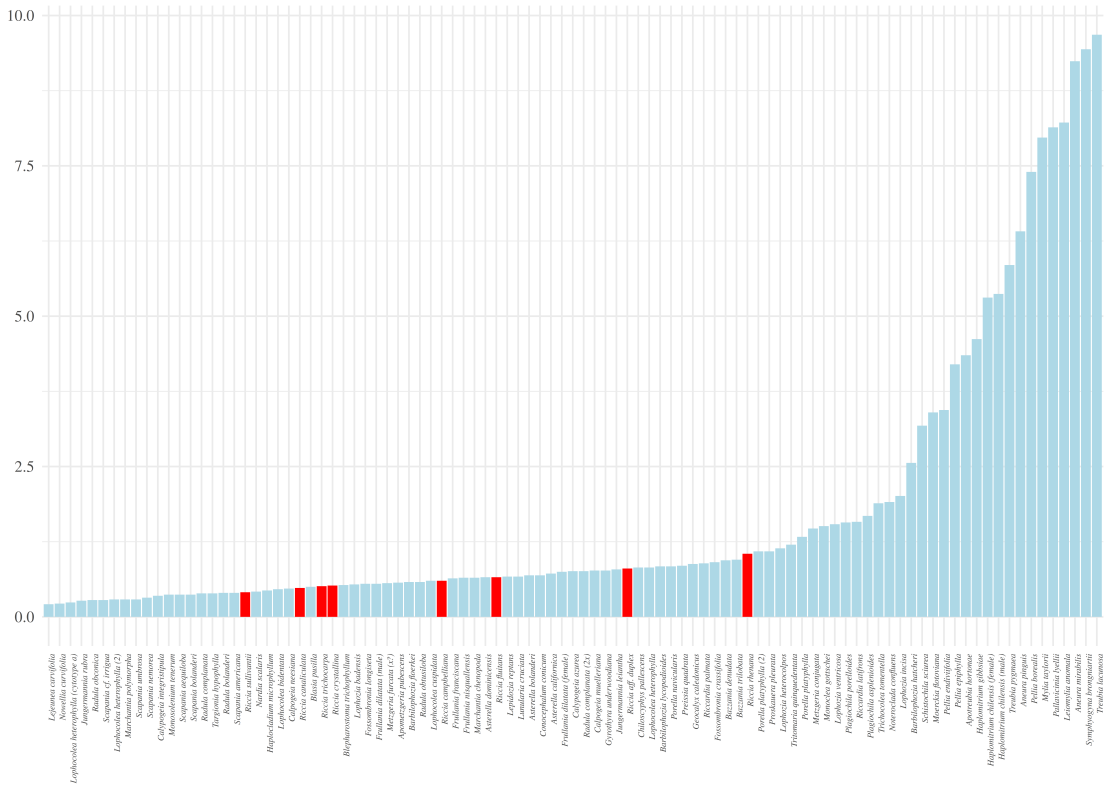

Figure 4.3: Comparison between known 1C-values of *Riccia* with other liverworts.

Barcodes were obtained of the measured samples, and a phylogenetic tree was constructed, with *Ricciocarpos natans* (L.) Corda as an outgroup (Figure 4.4). The investigated species represents two distinct clades, comprised from two haploid-diploid pairs: *R. fluitans* – *R. rhenana* and *R. canaliculata* – *R. duplex*. The investigated taxa *R. fluitans*, *R. rhenana*, *R. canaliculata*, and *R. aff. duplex* form monophyletic clades, supporting the species identification.

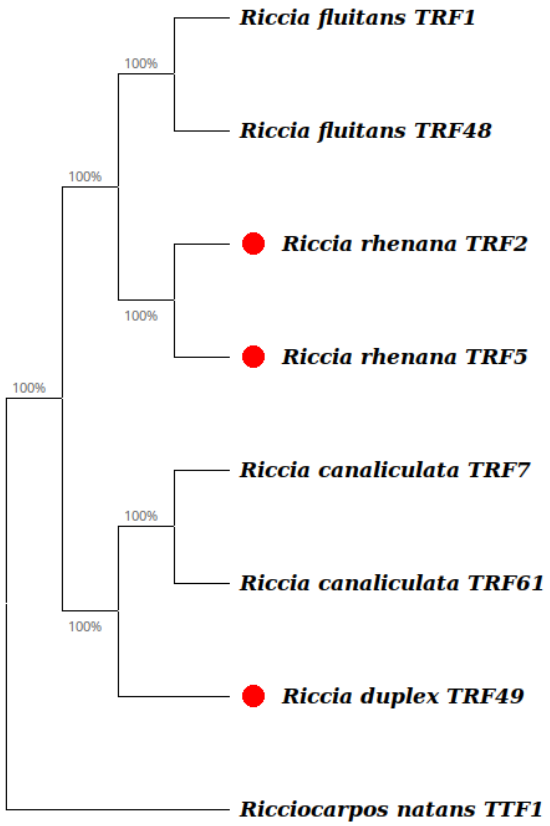

Figure 4.4: ML phylogenetic tree, based on *trnL-F* and *psbA* chloroplast regions, with red dots indicating the diploids.

4.5 Discussion

Since *R. duplex* is prolifically fertile, the parental species are expected to be with similar chromosome architecture, allowing proper chromosome pairing during meiosis (Stebbins, 1947). In contrast, the same suggestion cannot be made for *R. rhenana*, since it has never been documented as a fertile plant in Europe, and the reports for fertile *R. flutans* are extremely rare and questionable (Damsholt, 2002). Despite the only eight known 1C values of *Riccia* species, it seems that their genome size is quite variable, with 1C of 0.41pg for *R. huebeneriana subsp. sullivantii* (Bainard, 2011) to 1C of 1.05pg for *R. rhenana* (here reported).

In relation to the chromosome count across the genus, there is considerable deviation from the base chromosome number, which is n=8 (Jovet-Ast, 1986). In the Fritsch (1991) *"Index to bryophyte chromosome counts"*, more than 30% of the listed *Riccia* species are polyploids, with various chromosome counts, such as n=16, n=24, n=33, n=48. *Riccia lamellosa* Raddi, alone has a chromosome range of n=8, n=16, n=24 and n=33. The nature of such high polyploids remains unknown and of future interest.

The 1C values seems to be with higher species-discriminative value, than the chromosome counts. The chromosome counts can be used only for separation between haplo- and polyploid species, within the complex, while the genome size variation might be used for much more precise identification. Based on the current knowledge on the *Riccia* genome sizes, the 1C values might be useful and fast tool for species delimitation.

Chapter 5

On the polyploidization of *Riccia rhenana*

5.1 Abstract

Polyploidization is a process of rapid chromosome increasment, usually resulting in an offspring having two-three or even more times the chromosome number of their parents. Such drastic genomic event within single generation has profound and significant evolutional role. It can occur in result of different mechanisms in different organisms, but the two main types are via auto- and allopolyploidization. The young autopolyploids are expected to be genetically identical with their parent, since they possesses the same genetic information as their parents, but doubled. The cases of allopoliploidization are a result of hybridization event and by this, the new polyploids are new genetic recombinants, different from their parents. *Riccia rhenana* is a diploid liverwort species, experimentally suggested to be an autopolyploid and by this not a valid taxon, since the autopolyploids are considered as cytotypes of the given haploid species. In this work the genetic complexity of *R. rhenana* is unraveled, and its allopolyploid origin supported, proving the validity of its taxonomic rank.

5.2 Introduction

Among the various mechanisms of plant speciation and diversification, polyploidization is considered to be with significant importance, in both ancient and recent speciation (Stebbins, 1947, 1950; Soltis and Soltis, 1999, 2000). Regardless which of the two main types of polyploidization we have in hand – allo- or autopolyploidization (Kihara and Ono, 1926), the polyploid is genetically favored by factors such as differential niche preference, high fecundity, selfing or asexual mating system (Rodriguez, 1996; Baack, 2005; Rieseberg and Willis, 2007).

In mosses, allopolyploidization is recognized as a main mechanism for hybridization (Wyatt et al., 1988; Wyatt and Odrzykoski, 1998; Ricca and Shaw, 2010; Stenøien et al., 2011; Košnar et al., 2012; Ostendorf et al., 2021). In liverworts, approx. 25% are estimated to be polyploids (Bischler, 1998). Few studies are available at this point, dealing with the polyploidization nature in liverworts, and they all conclude

autopolyploid origins. Species such as *Pellia borealis* Lorb. (Odrzykoski et al., 1996), *Porella baueri* (Schiffn.) C. Jens. (Boisselier-Dubayle et al., 1998a), *Corsinia coriandrina* (Spreng.) Lindb. (Marie-Catherine and Bischler, 1998), a polyploid *Reboulia* (Boisselier-Dubayle et al., 1998b), and *Calypogeia sphagnicola* (Arnell & J. Press.) Warnst. & Loeske (Buczkowska et al., 2012), are all concluded to be allopolyploid hybrids.

In *Riccia*, polyploids are not a rare event, but the nature of their polyploidization has not been yet investigated. More than 30 of the listed *Riccia* species in the Fritsch's Index to bryophyte chromosome counts are polyploids and species such as *R. lamellosa* Raddi and *R. argenteolimbata* Volk & Perold, are listed with multiple ploidy levels as n=8, 16, 20, 24, and 33 (Fritsch, 1991). Moreover, three distinct multilocus genotypes were described within the haploid *R. dictyospora* (n=8) and recognized as sibling species (Dewey, 1989), suggesting hidden genetic differentiation within the haploids.

Riccia rhenana Lorb. ex Müll. Frib., which is the main object of this work, belongs to subgenus *Ricciella*, section *Ricciella* (Müller, 1940). Both morphologically and ecologically it is quite similar to *R. fluitans* L., having aquatic and terrestrial form. In its aquatic form *R. rhenana* is difficult for differentiation from *R. fluitans*. The main species-discriminative trait between the two is the chromosome count, where *R. fluitans* is a haploid plant (n=8), while *R. rhenana* is a diploid (n=16) (Müller, 1940). I have already discussed the colchicine experiment of Berrie (1964) and his conclusion on the taxonomic position of *R. rhenana*, being illegitimate, since the polyploid is autopolyploid cytotype. In the work of Jovet-Ast (1986), *R. rhenana* is also not recognized and discussed. Klingmüller (1958), discusses in detail the morphology of *R. fluitans* and *R. rhenana*, and based on this detailed morphological analysis, he describes *R. media*, which later is synonymized with *R. fluitans* (Bapna, 1961).

The conclusion from the available information is that *R. rhenana* is a diploid plant,

having 16 chromosomes, supposedly originating from somatic doubling in *R. fluitans*. In the case of autopolyploidization, the diploid populations are treated as cytotypes of the parental species, and they are not recognized as separate taxa. Such case is proposed with *R. gougetiana* Dur. et. Mont., where a population in Portugal is reported as a diploid cytotype (Jovet-Ast, 1986). Thus, the aim of this work is to investigate the origin of *R. rhenana*, and more precisely, if it is auto-or allopolyploid hybrid. In this study I attempt to resolve the origin of *R. rhenana*, and based on the obtained results, to support or reject its taxonomic status.

5.3 Materials and Methods

Three different methods were applied for one set of samples: isozyme analysis (***i***), chromosome counting (***ii***), and DNA barcoding (***iii***)

***i*) Isozyme analysis**

Sampling

Samples from *Riccia rhenana* were collected from Bulgaria (four populations) and Poland (two populations) (Table 5.1). Twenty individuals from each population were screened (120 individuals in total).

Table 5.1: Plant material collection sites of the studied *Riccia rhenana* populations. "TRF" indicates personal collection.

Number	Origin	Geographic coordinates	Collector
TRF3	Bulgaria	44.120761° 27.080771°	Natcheva, R.
TRF144	Bulgaria	43.042051° 24.151631°	Natcheva, R.
TRF146	Bulgaria	42.703737° 23.239854°	Tanev, A.
TRF150	Bulgaria	42.312024° 24.059118°	Natcheva, R.
TRF5	Poland	52.475260° 17.19260°	Gospodinov, G.
SOM93B	Poland	52.506974° 16.883630°	Gospodinov, G.

***ii*) Chromosome counting**

For all populations a chromosome counting was performed and the diploid number of n=16 for *R. rhenana* was confirmed (following Gospodinov and Natcheva, 2020). Haploid *R. fluitans* n=8 from two populations, one from Bulgaria, and one from Sweden were used as a control (30 individuals in total).

Isozyme electrophoresis protocols

A single thallus was homogenized in 40μL of extraction buffer (Wyatt et al. 1989). Whatmann paper wicks (2 mm $\times$ 11 mm) were soaked with the crude extract and placed into 10% starch-gel (Starch Art). The enzyme systems 6PGD, IDH, MDH, PGM, SDH were analyzed on Sodium-citrate, pH 7.0/Sodium-histidine, pH 7.0 electrode and gel buffer. GOT was analyzed on Sodium-borate, pH 8.0/Tris-citrate, pH 8.66 electrode and gel buffer. The PGI enzyme was investigated on Lithium-borate, pH8.3/Tris-citrate, pH 8.3. All electrode and gel buffers followed the protocols from Cronberg (1995).

iii) DNA Barcoding

Sampling

In order to investigate the relationship of *R. rhenana* with *R. fluitans*, in term of their chloroplast genomes, the regions *rps4* and *trnL-F* were selected. The dataset contains 66 newly generated sequences (Table 5.2), including the out-group (the Bayesian metrics are provided in Supplementary Materials Table 6.2). The dataset dealing with the nuclear *gpd* variants, contains 36 newly generated sequences, including the out-group.

Table 5.2: Analized number of sequences.

Species	***trnL-F***	***rps4***	***gpd***
Riccia fluitans	17	17	17
Riccia rhenana	15	15	18
Riccia perennis	–	–	1
Ricciocarpos natans	1	1	–

Molecular methods

For the both datasets, DNA was isolated following Bączkiewicz et al. (2017). Chloroplast regions *rps4* and *trnL-F* were selected for their complementary evolutionary signals. Region *trnL-F* being fast-evolving non-coding regions are suitable for recent divergences, while *rps4* is a moderately evolving coding region, applied to resolve deeper relationships. Both regions were amplified following Cargill et al. (2016), and

sequenced with the primers described there. For the second dataset, investigating the genetic relationship between the haploid and the diploid, a part of glyceraldehyde 3-phosphate dehydrogenase (*gpd*) was used, with newly developed primers:

RFGPD_F (5′-ATTCGTCGACAAGGATGGTGC-3′)
and
RFGPD_R (5′-CTCGTGCACGTCTCAGGTC-3′).

For all regions the following PCR program was applied: denaturation step of 7 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 sec at 55 °C, and 1 min at 72 °C with final elongation at 72 °C for 10 min, with AllegroTaq polymerase (Novazym). The PCR product was visualized on 1% agarose gel. The fragments were cleaned with Exonuclease I in combination with FastAP™. Each sample was sequenced for both forward and revers primers.

Sequence editing and alignment

Consensus sequences were assembled from the newly generated chromatograms, using PhyDE-1 (Müller, 2005). The new consensus sequences were edited and aligned using MUSCLE (Edgar, 2004) implemented in AliView (Larsson, 2014), and adjusted manually. Incomplete regions at the beginning and the end were excluded. Two main alignments were made, representing the sequences for *trnL-F-rps4* and *gpd*. For the alignment of *gpd* sequences Mixed Sequences Reader (http://msr.cs.nthu.edu.tw/) was used, to separate the two copies (Figure 5.4) of the *gpd* region. For each of the diploids, two sequences were generated and labeled as A and B. For each alignment indels were binary coded, using SeqState (Müller, 2005), with the implemented simple indel coding (sic) method by Simmons and Ochoterena (2000). All gaps generated by the out-group were removed, and the indels were coded only on the ingroup gaps. MrBayes 3.2.7a arm (Ronquist et al., 2012) was used for the phylogenetic reconstruction on a local machine. All regions were first analyzed separately.

Two main datasets were prepared. One dataset investigating the relationship of *R. rhenana* with *R. fluitans*, based on chloroplast markers *rps4* and *trnL-F*. And, a second dataset, investigating the nuclear *gpd* variants in the diploids and the haploids.

Chloroplast dataset

Two datasets were made, each containing concatenated regions. The first dataset included only nucleotide sequence data, while the second dataset combined nucleotide sequence data with indel data. The nucleotide data in both datasets were divided into two partitions, each evaluated with a distinct evolutionary model to account for differing evolutionary dynamics among regions. The fist partition included the entire *trnL-F* region (including the *trnL* gene and *trnL-trnF* intergenic spacer) and the coding part of *rps4*, analyzed using the GTR + I + G model. This model was chosen due to the hight proportion of invariant sites (> 90%) in these conserved chloroplast regions, which reflects strong functional constraints, necessitating the invariant sites (I) parameter to model sites with zero substitution rates. The second partition comprised the non-coding polymorphic part of *rps4*, analyzed with the GTR + G model, as this region is expected to have fewer invariant sites due to its higher variability. These distinct models were applied to accommodate the close phylogenetic relationships among the investigated species, avoiding oversimplification that could arise from a single model. The dataset containing indel data included a third partition, analyzed with an F81-like model to account for the binary nature of indels (presence/absence). Two simultaneous Metropolis-coupled Markov chain Monte Carlo (MCMC) runs, each with one cold and seven heated chains, were performed for 10^7 generations. Posterior probabilities were sampled every 500 generations, with a burn-in of 25% for the summary tree (sumt burnin=25%). All partitions were sampled independently, with model parameters unlined across partitions to allow independent estimation of substitution rates, base frequencies, and rate variation parameters. Tracer v1.7.2 (Rambaut et al., 2018) was used to assess

convergence, ensuring effective sample sizes (ESS) exceeded 200 for all parameters.

Nuclear dataset

For the *gpd* dataset, which investigated haploid-diploid relationships, two partitions were defined: one for the DNA sequence data and one for the indels. The DNA partition, for the *gpd* gene with two copies in diploids, was analyzed with the GTR + I + G model, justified by its moderate proportion of invariant sites (≈ 90%) and higher divergence (3.108%). The indel partition was analyzed with the F81-like model, consistent with the treatment of indels in the other dataset. For the same matrices, maximum likelihood analyses were ran, using RAxML-NG v. 1.2.2-master (Kozlov et al., 2019) on a local machine. Model GTR+G+I was applied for the DNA partitions, while for the indels, BIN+G was applied, with 1000 bootstrap replicates. The matrix without the SIC, and with removed outgroup, was used for reconstruction of a haplotype network (Figure 5.5).

5.4 Results

Isozymes

From all the investigated seven enzyme systems, *R. rhenana* displayed fixed heterozygosity at five: GOT, 6PGD, MDH and SDH. No variation within or between the haploid and polyploid populations was observed.

The * labeled isozymes showed fixed heterozygosity.

Isocitrate dehydrogenase (IDH) was single banded and monomeric for both the haploids and the diploids, and with matching migration speed. One allele (A) coding one protein (A) for the haploid, and homozygous (AA) with one protein (A) for the diploid, matching the haploid. Heterozygosity (AB) with two bands was not observed. The possibility for undetected heterozygosity (AB), due to identical mobilities of the products should be considered for all the homozygous cases.

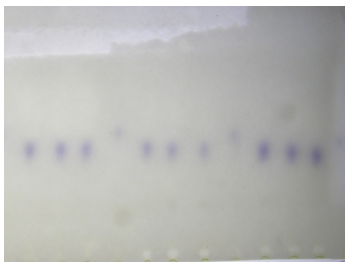

Figure 5.1: IDH represented by three sets of samples. First three - *R. fluitans* and the following two sets of three - *R. rhenana*. The three sets are separated by control of *Ricciocarpos natans*. The system shows no variation or heterozygous loci.

Malate dehydrogenase (MDH)* displayed a complex, multi-banded pattern for the polyploids, and three banded pattern for the haploids. The slowest band of the haploid was not matched by any of the bands of the polyploids. The second and third band, with slightly different migration speed between each other, were matched with two of the multiple polyploid bands. The complex banding of the haploid is more likely due to multiple MDH loci, encoding different isoforms (e.g. chloroplast and cytosolic), including homodimeric (AA, BB) and possibly heterodimeric (AB) forms from heterozygous loci.

Phosphoglucomutase (PGM)* displayed one monomeric enzyme from one locus (A) for the haploid and two similar in size bands, representing heterozygosity at one locus, with two alleles (AB).

Succinate dehydrogenase (SDH)* pattern for the haploid was a single band, suggesting a monomeric protein from one allele. While for the diploid, two bands indicated a most likely monomeric enzyme with two alleles (AB).

6-Phosphogluconate dehydrogenase (6PGD)* (Figure 5.2) exhibited one zone of activity for the haploids and two for the diploids. The eSupplementary Materialsx-hibited two distinct activity zones for the diploids corresponds to different isoforms. One faster-migrating zone, represented as a single band, as a uniform homodimer, and a slower-migrating zone displaying three-banded pattern, indicating heterozygous dimeric enzyme. The two outer bands, of the slower-migrating zone, represent the homodomers AA and BB, from alleles A and B, while the central band exhibit heterodimer AB. The middle larger band is due to equal subunit production, where

the heterodimer forms at twice the rate of each homodimer, yelding appr. 1:2:1 intensity ration.

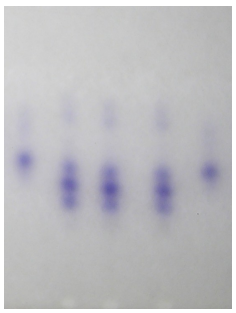

Figure 5.2: 6PGD showing three diploid *R. rhenana* individuals surrounded by two haploid *R. fluitans*.

Phosphoglucose isomerase (PGI) was represented by two homodimeric enzymes from two loci (AA and BB). The diploids were represented by the same banding, and matching speed.

Glutamate oxaloacetate transaminase (GOT)* had only one homodimeric (AA) band, while the diploids had two homodimeric bands (AA and BB) and a smeared heterodimeric (AB) band.

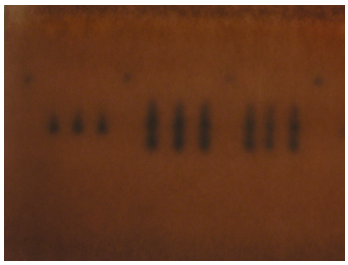

Figure 5.3: GOT by three sets of samples. First three - *R. fluitans* and the following two sets of three - *R. rhenana*. The three sets are separated by control of *Ricciocarpos natans*.

Sequence diversity and phylogeny

The length of the *rps4* region after the editing, resulted in 797 nucleotides, *trnL-F*, resulted in 470 nucleotides, and 353 nucleotides for the *gpd* region. The total length of the concertinaed chloroplast regions resulted in 1267 nucleotides in total. The *gpd* region was represented by two copies in each of the diploid individuals and by one copy for every haploid (Table 5.3).

Table 5.3: Proportional contribution of the partitions. Number of indels, length, *p*-distance, transition/transversion ratio, variable sites and parsimony informative sites.

Region	**bp**	**Indels**	**Indel length**	***p*-dist**	**ti/tv**	**Variable sites**	**p.i.**
gpd	353	2	2	3.108	2.51	9.69	9.12
trnL-F	470	1	1	0.295	0.17	0.59	0.59
rps4	797	0	0	0.355	0.29	0.72	0.72

Double peaks were observed on the chromatograms of the *gpd* region for all the diploid samples (Figure 5.2). The double peaks are with conservative positions in all the diploid samples, and with similar height. For the entire length of the region, seven such positions were observed, represented by one or two double peaks per given site, at conservative position and patter, among all diploid samples.

Summary statistics of the Bayesian analysis e.g. Log-likelihood, Log-prior, Tree Length, Gamma Shape Parameter, Standard Deviation of Split Frequencies, for both trees, are provided in Supplementary Materials Table 6.2.

Bayesian phylogenetic tree (Figure 5.5) was obtained based on the combined chloroplast regions *trnL-F* and *rps4*. The tree is comprised by two major reciprocally monophyletic clades. The haploid *R. fluitans* clade is comprised by a monophyletic subclade and four lineages out of the subgroup. The diploid *R. rhenana* clade is fully monophyletic.

A second bayesian phylogenetic tree (Figure 5.6) was obtained and for the *gpd* region. The tree displays a complex topology, comprised by multiple clades. A large monophyletic clade comprise the haploid and diploid individuals, with *R. perennis* as an outgroup. Within this clade, a large subclade is embedded comprised by two

monophyletic clades - one clade comprised by two sister subclades, encompassing all haploid individuals, and another clade, sister to the haploid one, encompassing two sister clades , one comprised only by "A" copies, and another only by "B" copies from the diploid individuals. One "B" copy (150 B) forms alone suster clade to the haploid and diploid clades.

The obtained haplotype network (Figure 5.7) revealed distinct patterns of genetic variation. A large *R. rhenana* "A" haplotype is connected by five mutation steps to a very large *R. fluitans* haplotype. This *R. fluitans* haplotype is linked by multiple mutations steps to further small *R. fluitans* haplotypes, where they split to different haplotypes: small *R. fluitans*, *R. rhenana* "A", and *R. rhenana* "B". One haplotype of *R. rhenana* "B" is multiple (9) mutation steps.

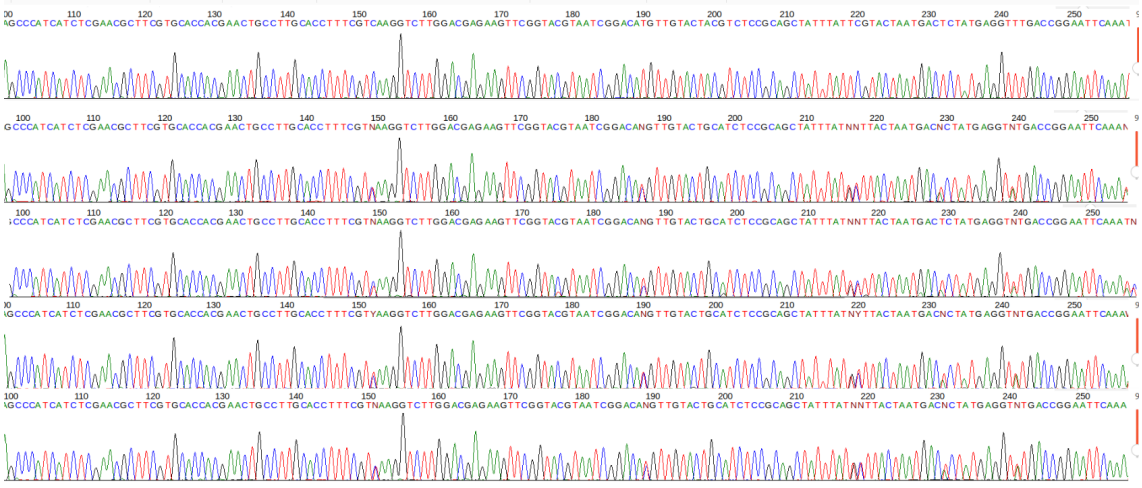

Figure 5.4: Chromatogram comparison showing the conservative heterozygosity positions of the two *gpd* region copies. The first chromatogram represents a haploid *R. fluitans* plant, while the remaining four chromatograms represent diploid *R. rhenana* individuals.

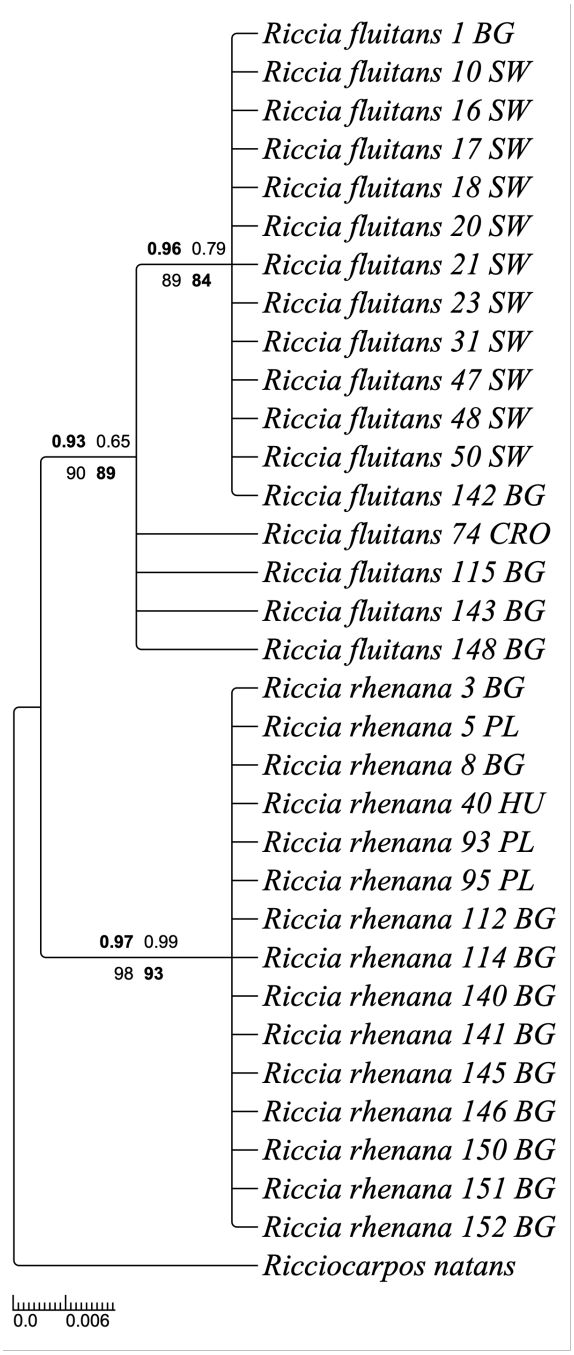

Figure 5.5: Phylogenetic tree based on the combined *trnL-F-rps4* chloroplast markers. The values above the branches represent the support from MrBayes. The values under the branches, represent the support from the maximum likelihood analysis. The values in bold, indicate the results from the matrices with the included indels. The numbers after the taxon names correspond to a private numbering, included in the additional data, provided for the samples in the supplementary materials.

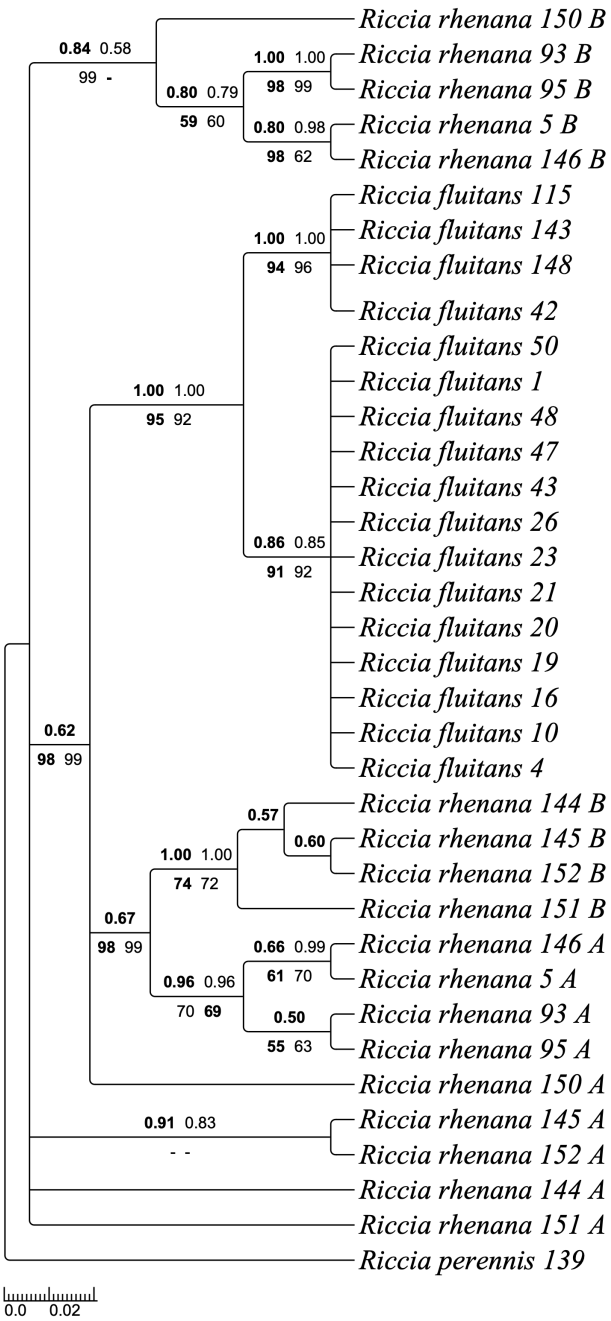

Figure 5.6: Phylogenetic tree based on the *gpd* region. Diploids are represented by two sequences of the region. Major sequences are labeled with "A", minor sequences with "B". Values above the branches represent the support from MrBayes. Values under the branches - support from the ML analysis. Values in bold, indicate results from the matrices with included indels. Support values under 50 are indicated with dash "-". Numbers after the taxon names correspond to a private numbering, included in the additional data, provided for the samples in the supplementary materials.

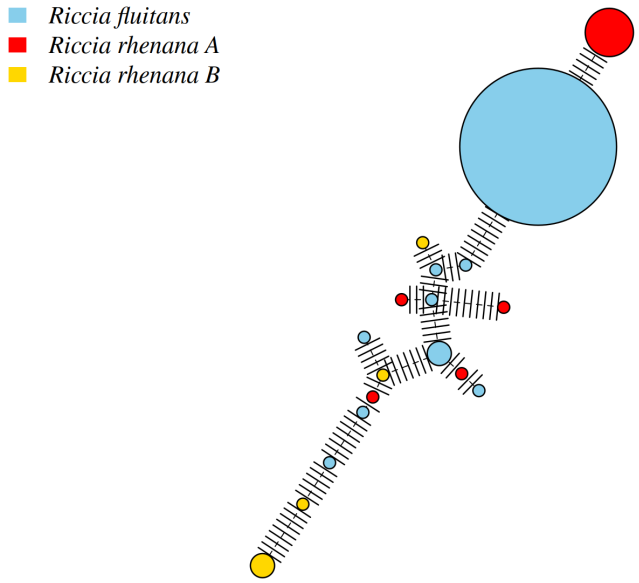

Figure 5.7: Haplotype network based on the *gpd* region. Distinct haplotypes can be observed within the haploid *R. fluitnas*, and even within the copies of the gene of the diploid *R. rhenana*.

5.5 Discussion

Isozyme analysis effectively distinguished diploid *R. rhenana* from haploid *R. fluitans* and provided evidence for the allopolyploid origin of the diploid through consistent additive banding patterns from parental species. An allopolyploid species possesses two sets of chromosomes inherited from distinct parental species (Lloyd and Bomblies, 2016), and in bryophytes, homeologous loci remain largely un-recombined, preserving parental gene variants. The complex isozyme banding in diploid *R. rhenana* results from subgenome-specific homeologous copies of enzyme-encoding genes, which differ slightly in sequence, causing amino acid changes that affect electrophoretic mobility. The isozyme systems employed - GOT, 6PGD, MDH, and SDH - yielded reliable and reproducible patterns, enabling clear separation of the two species. No variation was observed within or between haploid *R. fluitans* populations, suggesting clonal uniformity, while diploid *R. rhenana* populations exhibited uniform isozyme patterns across all experimental replicates, consistent with fixed subgenome contributions in an allopolyploid genome.

The absence of variation is consistent with the predominantly, if not exclusively, asexual reproduction of both species Schuster (1992a); Damsholt (2002). Asexual reproduction limits genetic recombination, resulting in clonal populations with minimal genetic diversity (Cronberg, 2002). Additionally, both species occupy similar habitats and are subject to comparable environmental pressure, which likely contribute to consistent gene expression profiles and uniform isozyme patterns. The primary difference observed, as expected, relates to the number of active genes, with the diploid *R. rhenana* showing fixed heterozygosity indicative of its allopoloid origin. It is important to note that the isozyme analysis in this work was not designed to detect haplotype variations within the investigated species, and the technique's sensitivity may limit its ability to identify subtle genetic differences. Nevertheless, the consistent isozyme patterns observed across GOT, 6PGD, MDH, and SDH systems strongly support their utility for distinguishing *R. rhenana* from *R. fluitans* and confirming the allopolyploid nature of the diploid.

The phylogenetic tree (Figure 5.5) is comprised by two main well-supported clades. One clade comprised only by the haploid *R. fluitans* and a second clade comprised only by the diploid *R. rhenana*. One possible explanation of this topology is that *R. fluitans* is most likely the paternal donor and we observe a distinct chloroplast genome, inherited by another species, which served as a maternal donor. The diploid clade is entirely monophyletic, indicating one universal maternal donor, while the haploid clade, shows polyphyletic topology, indicating variation within the haploid samples, and most likely cryptic speciation within *R. fluitans*. Even considering the variation within the haploid, the diploid samples remain monophyletic.

As described above, the portion of the *gpd* gene was represented by two homeologous copies in every diploid sample of *R. rhenana* (labeled A and B). Since it is proposed to be a single-copy nuclear gene (Wall, 2002; Szövényi et al., 2006), in a case of autopolyploidization, two nearly identical copies would be expected. In all *R. rhenana* samples, two distinct copies were present, without any exception, suggesting allopolyploidization event. The *gpd* tree (Figure 5.6) is much more complex, than the chloroplast data, which is to be expected, with variable sites 9.69% for length of 353 nucleotides. Unlike the chloroplast reconstruction, the *gpd* tree suggests a complex genetic pool within the diploid, forming a large monophyletic ingroup clade, containing all haploids and diploids, with outgroup *R. perennis*. Based on the current labeling, generated by the Mixed Sequence Reader, the A (major) and B (minor) copy have complex phylogenetic relationships. The current labeling of the tree, suggest that minor B copies of the gene are monophyletic, belonging to two separate clades, while the major A copies are paraphyletic. Even if we assume that the generated labeling is wrong, and the copies would have different labels assigned, arrangement suggesting two, monophyletic copies from one individual, cannot be assumed. In every case, one individual combines two copies, belonging to distinct clades. This pattern strongly supports the hypothesis of one or more hybridization events, resulting in different hybrids, originating from the combination of divergent ancestral lineages. As suggested from the tree, the copies of the *gpd* gene in some

hybrids, seems to be predating those in *R. fluitans*, based on their branch lengths, indicating deeper divergence. Furthermore, the haplotype network (Figure 5.7) also supports the hypothesis of distinct haplotypes within the haploids, and more than one haplotype within *R. fluitans*, as inferred from the phylogenetic tree. *Riccia fluitans* shows high intraspecific variation, with multiple haplotypes, some of which give rise to *R. rhenana* "B" and additional *R. rhenana* "A" haplotypes. The haplotype network suggests a complex evolutionary history, potentially involving shared ancestry.

The currently proposed parent *R. fluitans* in theory is the best candidate, but this would be the case, if we assume that the polyploidization occurred only in Europe. Even so, the presence of *R. stenophylla* in Europe has been discussed by Damsholt (2002) and Schuster (1992a), adding even more complication to the *R. fluitans* complex of species.

5.6 Conclusion

The five isozyme systems exhibiting fixed heterozygosity, provide a strong evidence of the allopolyploid origin of *R. rhenana*. The chloroplast dataset, revealed two distinct clades, indicating allopolyploidization, by suggesting a distinct maternal lineage for *R. rhenana*, and excluding *R. fluitans* as a maternal donor. The phylogenetic reconstruction based on the *gpd* region, strongly support the hybrid origin, showing that *R. rhenana* possesses two copies of the *gpd* gene, originating from divergent ancestral lineages. A haplotype network, showing different haplotypes implies again for genetic divergence between the two copies.

Collectively, the fixed heterozygosity of isozymes, the chloroplast clades, the *gpd* phylogeny and the haplotype network confirm *Riccia rhenana* as an allopolyploid species of hybrid origin, and by this, as a distinct and a legitimate taxon.

Chapter 6

Preliminary taxonomic investigation on sect. *Ricciella*

6.1 Abstract

The genetic relationships within the subgenus *Ricciella* and the section *Ricciella* is investigated here. The preliminary results do not support the monophyly of subgenus *Ricciella* or its constituent section *Ricciella*, indicating a need for extensive genetic and taxonomic revision. Two potential sectional grouping are identified: one comprising monophyletic clades of *R. fluitans* and *R. rhenana*, and another including *R. canalicuata*, *R. duplex*, and *R. perennis*. Additionally, genetic diversity within *R. fluitans* suggests cryptic speciation, highlighting potential unrecognized taxonomic unit within this species.

6.2 Introduction

As shown above in the *Literature review* section, the taxonomic arrangement of subgenus *Ricciella* is dynamic and constantly developing. All the mentioned taxonomic concepts and proposed structures of the genus and the subgenus, are based on the classical morphological interpretations from different authors. The only work discussing the taxonomic arrangement of the entire genus based on genetic analysis, is Cargill et al. (2016) "A preliminary molecular phylogeny of the genus *Riccia* L.(*Ricciaceae*) in Australia". In this work the entire genus is investigated with combination of molecular methods and classical morphological evaluations, including spore morphology.

Despite its wide range of investigated taxa, the work is focused on Australian samples (with a few exceptions), making it not entirely valid to the European taxonomic treatments. In this study sebgenus *Ricciella* is represented by *R. crystalina*, *R. cavernosa*, *R. frostii*, *R. luticola*, *R. multifida*, *R. huebeneriana* with its two subspecies (*huebeneriana* and *sullivanti*), and *R. fluitans*. The species *R. canaliculata*, *R. duplex* and *R. perennis* are not investigated genetically, which makes the work not exhaustive enough, in the context of subgen. *Ricciella*.

Based on the obtained results there, the following conclusions are made (provided

bellow literally), concerning subgenus *Ricciella*:

a) Subgenus *Ricciella* (Braun) Boulay is polyphyletic and nested within subgenus *Riccia*, which is now rendered paraphyletic.

b) On the basis of the above data, we reduce subgenus *Ricciella* to synonymy of *Riccia* subgenus *Riccia*.

c) *Riccia multifida* also shares a similar morphology with the Australian species *R. duplex* var. *megaspora* Na-Thalang.

d) Three other semi-aquatic taxa from the USA, namely, *R. fluitans* (which is also the type species for the subgenus *Ricciella*), *R. huebenariana* and *R. huebenariana* var. *sullivanti* (Austin) R.M.Schust., also share similar gametophore characters, yet are unrelated. These shared morphologies of semi-aquatic species are an example of convergent evolution, where they have developed a set of traits that are adapted to a wet habitat, but do not indicate a shared ancestry.

Despite the conclusion made there for synonymisation of *Ricciella* subgenus with subgenus *Riccia*, the work of Hodgetts et al. (2020) "An annotated checklist of bryophytes of Europe, Macaronesia and Cyprus" cites subgenus *Ricciella* as a valid taxon, with two sections *Ricciella* and *Spongoes*.

The aim of this study is to provide a preliminary data on the architecture of section *Ricciella* and the obtained results to serve as a foundation for further, more detailed and comprehensive analysis of the entire genus *Riccia*.

6.3 Materials and Methods

Sampling

DNA was isolated from *R. fluitans*, *R. rhenana*, *R. stricta*, *R. perennis*, *R. canaliculata*, *R. duplex* s. lat, and *R. huebeneriana* (Table 6.1), following Bączkiewicz et al. (2017). The chloroplast regions *rps4*, and *trnL-F* and *rbcL* were amplified following Cargill et al. (2016), and were sequenced with the primers described there.

Table 6.1. List of samples used for phylogenetic reconstructions. Newly generated sequences for this study are marked with "*"; "-" indicates the marker was not used; accession numbers are provided for sequences from NCBI.

Species	**Isolate**	**Origin**	***trnL-F***	***rps4***	***rbcL***
R. abdita	B623	Australia (AU)	-	-	KX468589
R. albolimbata	X384	Australia (AU)	-	-	ON564419
R. billardierei	B632	Australia (AU)	-	-	KX468597
R. boumanii	X143	China (CN)	-	-	ON564418
R. canaliculata	7	Bulgaria (BG)	*	*	*
R. canaliculata	45	Hungary (HU)	-	-	*
R. canaliculata	51	Sweden (SW)	-	-	*
R. canaliculata	61	Sweden (SW)	*	*	*
R. canaliculata	159	Poland (PL)	-	-	*
R. cavernosa	B682	Australia (AU)	-	-	KX468625
R. ciliifera	-	Switzerland (CH)	-	-	DQ286022
R. cf. crassivenia	DC1267	Australia (AU)	-	-	KX468577
R. crinita	B655	Australia (AU)	-	-	KX468612
R. crystallina	B630	Australia (AU)	-	-	KX468596
R. aff. duplex	49	Sweden (SW)	*	*	*
R. fluitans	1	Bulgaria (BG)	*	*	*
R. fluitans	-	Scotland (UK)	*	*	DQ286023
R. fluitans	94094	-	*	*	DQ645963
R. fluitans	X419	China (CN)	-	*	OL771231
R. fluitans	48	Sweden (SW)	*	*	*
R. fluitans	50	Sweden (SW)	*	*	*
R. fluitans	74	Croatia (HR)	*	*	*
R. gangetica	B649	Australia (AU)	-	-	KX468607
R. gougetiana	60623	Spain (ES)	-	-	KT793582
R. huebeneriana	X181	China (CN)	OL771235	OL771241	OL771229
R. huebeneriana	44	Hungary (HU)	*	*	*
R. huebeneriana	57	Sweden (SW)	*	*	*
R. ssp. *sullivantii*	-	Australia (AU)	AY507554	AY507463	-
R. inflexa	B685	Australia (AU)	KX468760	KX468645	KX468628
R. lamellosa	B409	Australia (AU)	-	-	KX468574
R. lamellosa	B410	Australia (AU)	-	-	KX468575
R. lamellosa	B648	Australia (AU)	-	-	KX468606
R. multifida	B624	Australia (AU)	KX468706	KX468657	KX468590
R. multifida	B647	Australia (AU)	-	-	KX468605
R. cf. multifida	B684	Australia (AU)	-	-	KX468627
R. perennis	139	Bulgaria (BG)	*	*	*
R. pullulans	B646	Australia (AU)	-	-	KX468604
R. rhenana	5	Poland (PL)	*	*	*
R. rhenana	93	Poland (PL)	*	*	*
R. rhenana	95	Poland (PL)	*	*	*

Table 6.1. List of samples used for phylogenetic reconstructions. (Continued)

Species	**Isolate**	**Origin**	***trnL-F***	***rps4***	***rbcL***
R. rhenana	112	Bulgaria (BG)	*	*	*
R. rhenana	141	Bulgaria (BG)	-	-	*
R. rhenana	145	Bulgaria (BG)	-	-	*
R. rhenana	146	Bulgaria (BG)	-	-	*
R. rhenana	152	Bulgaria (BG)	-	-	*
R. rhenana	168	Bulgaria (BG)	-	-	*
R. rhenana	X51	China (CN)	OQ547819	OQ547823	OQ547815
R. rhenana	X123	China (CN)	OQ547820	OQ547824	OQ547816
R. sorocarpa	B683	Australia (AU)	-	-	KX468626
R. cf. subbifurca	B667	Australia (AU)	-	-	KX468615
R. stricta	128	Africa	*	*	*
R. subcrinita	X408	China (CN)	-	-	OL771230
R. subbifurca	B680	Australia (AU)	-	-	KX468624
R. verrucosa	B614	Australia (AU)	-	-	KX468580
R. junghuhniana	X170	Indonesia (ID)	-	-	OL771227
Ricciocarpos natans	TTF1	Bulgaria (BG)	*	*	*

For all regions the following PCR program was applied: denaturation step of 7 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 sec at 55 °C, and 1 min at 72 °C with final elongation at 72 °C for 10 min, with AllegroTaq polymerase. The PCR product was visualized on 1% agarose gel. The fragments were cleaned with Exonuclease I in combination with FastAP™. Each sample was sequenced for both forward and revers primers.

Sequence editing and alignment

Consensus sequences were generated from newly produced chromatograms using PhyDE-1 (Müller, 2005). The sequences were edited and aligned with MUSCLE (Edgar, 2004) in AliView (Larsson, 2014), with manual adjustments as needed. Incomplete regions at the sequence ends were excluded. Two alignments were created, yielding three matrices: one for the *rbcL* region, one for the *trnL-rps4* regions, and a combined alignment. The combined alignment produced two matrices—one with genetic data only and another incorporating genetic data plus simple indel coding (SIC). Indels were binary-coded using SeqState (Müller, 2005) with the SIC method

(Simmons and Ochoterena, 2000). Gaps introduced by the outgroup were removed, and indel coding was applied only to ingroup gaps. The non-SIC dataset was analyzed separately.

Phylogenetic reconstruction was performed using MrBayes 3.2.7a on a local machine. A GTR + I + G model was applied to the sequence data matrix, while an F81-like model was used for the indel matrix. Each region was analyzed independently. Two simultaneous Metropolis-coupled Markov chain Monte Carlo (MCMC) runs, each with one cold and three heated chains, were conducted for 30,000,000 generations. Posterior probabilities were sampled every 500 generations, with a burn-in of 75,000 samples for the summary tree. Model parameters (base frequencies, substitution rates, and gamma shape) were unlinked across partitions for independent estimation, though the proportion of invariable sites was optionally linked. Convergence was assessed using Tracer v1.7.2 (Rambaut et al., 2018), confirming effective sample sizes (ESS) exceeded 200 for all parameters.

6.4 Results

The combined DNA matrix of *trnL-F* and *rps4* regions, resulted in a total of 1 199 nucleotides. Forty three indels were coded, making the final length of the combined DNA/SIC matrix of 1 242 characters. Both matrices generated identical trees, with just a slight difference between a few of the support values. The *rbcL* matrix resulted in 1 266 nucleotides.

In result, two phylogenetic trees were generated: one tree based on *trnL-rps4* regions showing the *Ricciella* section only (Figure 6.1), and a second tree based on the *rbcL* region, summarizing the position of the section and the subgenus *Ricciella* withing the genus *Riccia* (Figure 6.2). Bayesian metrics are provided in Supplementary Materials Table 6.3

The *trnL-F-rps4* phylogenetic tree (Figure 6.1) features a major monophyletic clade encompassing all *Ricciella* species. Within this clade, two main sister clades are identified:

One clade includes all *R. fluitans* samples, together with the European *R. rhenana* samples, and *R. stricta* as a sister taxon. The *R. fluitans* samples form a monophyletic clade, with *R. fluitans* 74 HR sister to a tightly related subclade of *R. fluitans* 1 BG, 48 SW, 50 SW, showing minimal genetic divergence. In contrast, *R. rhenana* is paraphyletic, and splits into two clades: one with 5 PL, 93 PL, 95 PL, 112 BG (European clade), and another with X51 CN, X123 CN (Chinese clade), separated by *R. stricta*.

The second clade includes *R. canaliculata* (7 BG, 61 SW), *R. perennis*, *R. duplex*, *R. huebeneriana* (44 HU, 57 SW, AUS, X181 CN), and *R. multifida*. Within this clade, *R. canaliculata* and *R. huebeneriana* (44 HU/57 SW; sullivantii AUS/X181 CN) form monophyletic subclades, while *R. perennis*, *R. duplex*, and *R. multifida* are singletons. No polyphyletic taxa are observed in this clade.

The *rbcL* tree (Figure 6.2) summarize the section and subgenus branching pattern within the genus. The tree lustrates a complex phylogenetic hierarchy, revealing polyphyletic origin of section *Ricciella* and subgenus *Ricciella* within the genus.

Three main sister clades form the base topology of the tree. A small clade comprised by the species *R. crystalina* and *R. cavernosa* (sect. *Cavernosae*). Above it a clade of complex topology uniting species of different sections - *Sorocarpae, Riccia, Ciliiferae, Lamellosae, Limbata*, Ricciella*. And a top clade with polyphyletic structure comprised by the sections *Ricciella*, *Macrospora*, Limbata*, Albosquamatae*.

With "" symbol are indicated taxonomic subgroups as provided in Na-Thalang (1980).*

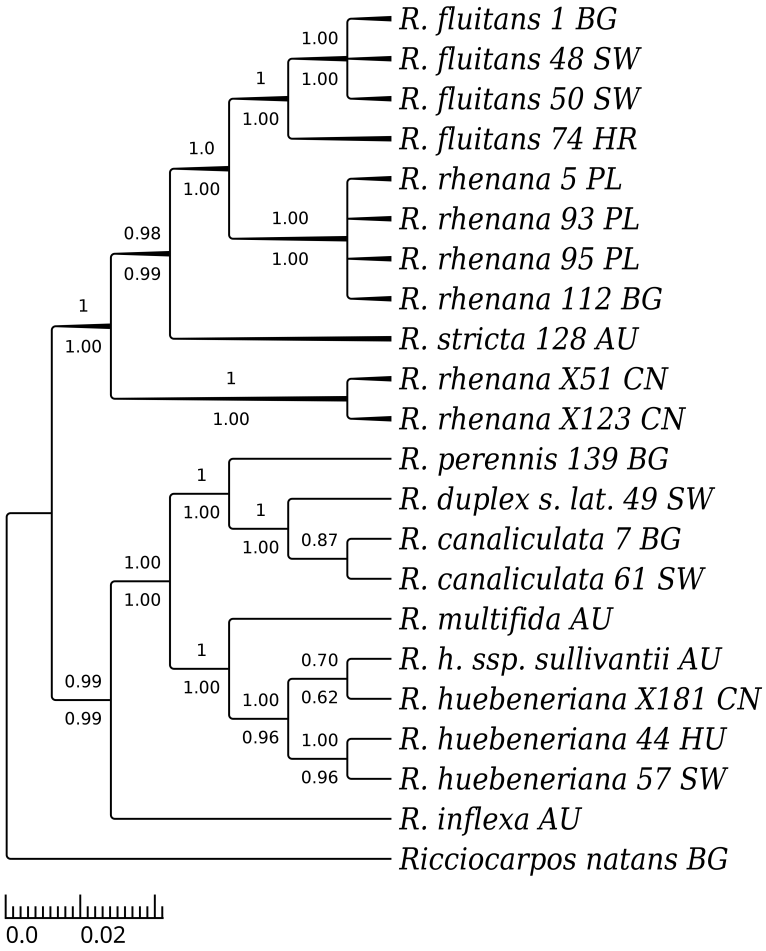

Figure 6.1: Bayesian phylogenetic tree based on the chloroplast regions *trnL-F* and *rps4*. Values above the branches indicates SIC support; under the branches, without

.

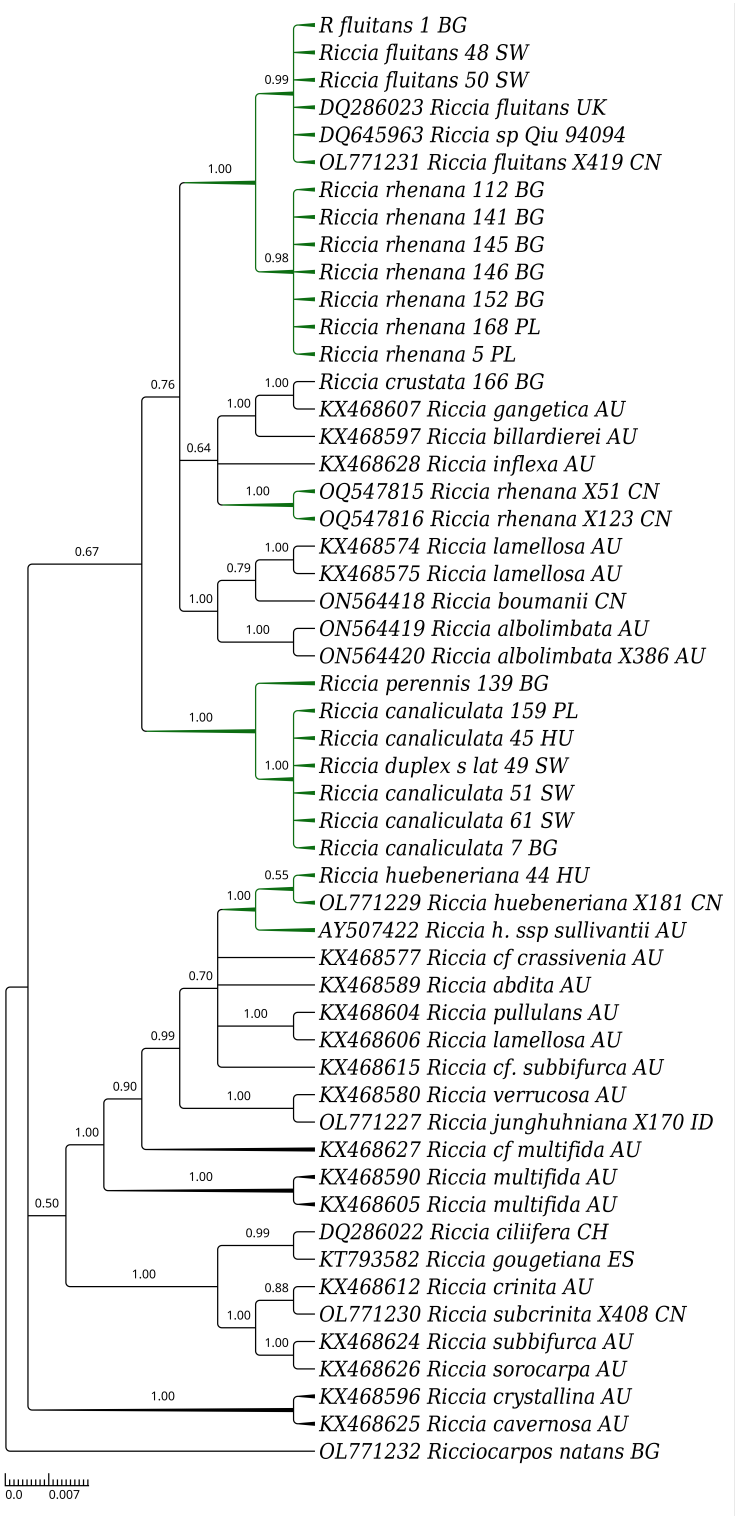

Figure 6.2: Bayesian phylogenetic tree based on the chloroplast region *rbcL*. In green are indicated species belonging to sect *Ricciella*; bolded are species from subgenus *Ricciella*

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6.5 Discussion

The phylogenetic reconstruction, based on the combined *trnL-F* and *rps4* datasets (Figure 6.1), reveals a polyphyletic branching pattern for *R. rhenana*. Samples from China (X51 and X123), as reported in Xiang et al. (2023), form a distinct clade separate from the European *R. rhenana* and *R. fluitans* samples.

Two potential explanations account for this tree topology. First, the European *R. rhenana* may represent a hybrid derived from a different parental combination than the Asian *R. rhenana*. Alternatively, the Asian samples may have been misidentified. A key diagnostic trait for *R. rhenana* is its chromosome number, which was not reported in Xiang et al. (2023). The marked divergence of the Asian *R. rhenana* samples from the *R. fluitans* clade is further highlighted in the *rbcL*-based tree (Figure 6.2). In this tree, the Asian *R. rhenana* clade is separated from the European clade, by multiple nested subclades.

The *Ricciella* section is distributed across the genus and does not form a monophyletic clade, nor does the *Ricciella* subgenus, confirming the work of Cargill et al. (2016). The large number of species and wide geographic range requires thorough sampling for a complete and representative species evaluation. The current data on the *Riccia* genetic structure, reveals a vast and complex speciation. The morphological simplicity of the genus conceals its species diversity and obscures potential processes of allopatric and sympatric speciation. Sympatric speciation may occur in the closely related *R. fluitans* and *R. rhenana*, which can co-occur, remain reproductively isolated, and continue to diverge. An allopatric example might be suggested again with *R. fluitans* and the tropical *R. stenophylla*. In both of the speculated examples, morphologically unified aquatic species are in hand, divided by fundamental biological factors such as their reproductive systems, suggesting the complex evolutionary history of this group.

6.6 Conclusion

The tree topologies suggest two well defined taxonomic groups, which can be considered as a new section candidates - *Fluitans* group, comprised by the species *R.*

fluitans, R. rhenana and *R. stricta*. *Canaliculata* group, comprised by the species *R. canaliculata, R. duplex* and *R. perennis*. The taxonomic position of subgenus *Ricciella* is here not supported. The section *Ricciella sensu* Schuster (1992b) and Jovet-Ast (1986, 1991, 2000) is also not supported.

Main Conclusions from the PhD thesis

The study concludes the following:

- *Riccia fluitans* and *R. rhenana* exhibit survival strategies enhancing reproductive success.
- *Riccia rhenana* is underrecognized in taxonomic studies.
- *Riccia fluitans* shows multiple genotypes, suggesting cryptic speciation.
- *Riccia rhenana* is an allopolyploid hybrid.
- Neither subgenus nor section *Ricciella* is phylogenetically monophyletic, thus unsupported.

Główne wnioski z rozprawy doktorskiej

Badania prowadzą do następujących wniosków:

- *Riccia fluitans* i *R. rhenana* wykazują strategie przetrwania zwiększające sukces reprodukcyjny.
- *Riccia rhenana* jest niedostatecznie rozpoznana w badaniach taksonomicznych.
- *Riccia fluitans* wykazuje wiele genotypów, co sugeruje ukrytą specjację.
- *Riccia rhenana* jest mieszańcem allopoliploidalnym.
- Ani podrodzaj, ani sekcja *Ricciella* nie są filogenetycznie monofiletyczne, a zatem nie są poparte dowodami.

Glossary

after Damsholt (2002)

***Air-chambers*:** large intercellular cavities, opening to the surface of the thallus, usually via a more or less specialized opening (pore).

***Alloploidy*:** crossing between two different species, resulting in an interspecific hybrid, usually with the chromosomes unable to pair at meiosis, either through missing meiosis with development of diploid spores, germinating into diploid gametophytes, or with occurrence of autoploidy (doubling of chromosomes, e.g. via apospory or by gemmae being diploid after mitosis has started, but for some reason lacking a new wall between the two components), also resulting in diploid gametophytes, spermatozoids and egg cells, after fertilization of gametes from two such new cytotypes, able to produce a sporophyte by mitosis, to pair at meiosis and to reproduce by recombined spores (*allopolyploidy*, *Porella baueri*).

***Antheridium*:** the male reproductive organ; a spherical or ellipsoidal body, containing spermatozoids, on a longer or shorter stalk.

***Apical cell*:** the cell giving rise to most of the mature body.

***Apospori*:** development of a gametophyte directly from a sporophyte, without the formation of spores.

***Archegonium*:** the female reproductive organ, consisting of a long neck and a venter containing the egg cell.

***Autodiploid*:** autopolyploidy, leading to a new species by a doubling of the chromosome number within individual organism of a species (e.g. *Chiloscyphus*

pallescens).

***Branch*:** an extension of the main axis; lateral or ventral, leafy or non-leafy.

***Calcareous*:** a substrate containing large amounts of lime.

***Capsule*:** the spore-containing part of the sporophyte.

***Deciduous*:** falling of.

***Dichotomy (true)*:** a longitudinal division of the apical cell into two new apical cells. Reporeted from Anthocerophyta.

***Dioicous*:** antheridia and archegonia develop on separate plants (dioecious).

***Divergent*:** spreading widely from each other.

***Dorsal*:** the face of the stem lacking rhizoids; the upper surface of a thallus, away from the substrate.

***Ephemeral*:** short-lived.

***Epidermis*:** the outer layer of cells of a thallus.

***Female*:** a plant with archegonia or part of a plant with archegonia.

***Fertile*:** shoot bearing archegonia or antheridia, or both.

***Fertilization*:** the fusion of the egg cell and a motile spermatozoid.

***Furcate*:** forked, with two almost equal branches or lobes.

***Gamete*:** haploid reproductive cell; gametes fuse to form a diploid zygote.

***Gametophyte*:** the usually haploid generation with sexual organs.

***Gemma*:** a small bud-like body capable of reproducing the plant.

***Habitat*:** place with a particular kind of environment in which an organism lives.

***Haploid*:** refers to cell with one set of chromosomes, borne by a single gamete (n), e.g. the whole plant of the gametophytic generation.

***Heterothallic*:** male plants much smaller than female plants.

***Lateral*:** attached to the side.

***Male*:** a plant with antheridia or a part of a plant with antheridia.

***Meiosis*:** the two successive nuclear divisions in which the chromosome number is reduced from diploid (2n) to haploid (n) and segregation of the genes occurs; in bryophytes, spores are the result of meiosis.

***Meristem*:** a localized area of growth by cell division.

***Mitosis*:** a process during which the chromosome divide longitudinally and the daughter chromosomes then separate to form two genetically identical daughter nuclei; usually followed by cytokinesis (division of the cytoplasma, etc.).

***Monoicous*:** antheridia and archegonia occur on the same plant (monoecious).

***Pore*:** a small opening in the dorsal epidermis of a thallus, surrounded by specialized cells (e.g. simple pore, barrel-shaped pore).

***Propagule*:** small caducous branchlets for vegetative propagation, usually used as a general term for gemmae, caducous leaves, branchlets, caducous perianths, etc.

***Protonema*:** branched or unbranched filament, discoid cell-surface or a solid mass of cells developed from a spore from which the plant arises. Today including rhizoids by some scientists.

***Rhizoid*:** unicellular protonemal filament, springing from the stem or thallus (sometimes branched at apex, e.g. *Radula*), anchoring the plant to the substrate, smooth or with internal pegs. Also serving in transporting water and nutrition to the plant.

***Scale*:** a thin, flat semi-transparent appendage.

***Seta*:** the delicate stalk of the sporophyte, connecting the foot and the capsule.

***Sibling species*:** species that are difficult or impossible to distinguish using morphological characters.

***Spermatozoid*:** the motle male gamete, with two obliquely inserted flagella at one end and proximal triplet extensions of the basal bodies. Unique for eukaryotes, uniting mosses and liverworts.

***Spore*:** an asexual reproductive unit, developed in the capsule. Able to germinate and develop into a gametophyte.

***Sporophyte*:** the asexual generation or the part bearing spores.

***Thallus*:** a dorsiventrally flattened plant body usually not differentated into stem and leaves.

***Tuber*:** a spherical or ellipsoidal or reniform body, usually developed on the ventral surface of a thallus; caducous or persistent, considered as a mean of vegetative reproduction.

***Ventral*:** the surface of the thallus turned against the substrate.

***Ventral scale*:** thin or small appendage at the ventral side of the thallus.

***Xerophyte*:** a plant adapted to a dry habitat, usually with a high saturation deficit.

***Zygote*:** the diploid cell (2n), resulting from the fusion of male and female gametes.

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Fine-scale species delimitation – genetic approach for closely related species separation 115/13/UAM/0024 ID-UB. 2023.

Double the genes to conquer the niches: Isoenzyme investigation of Riccia rhenana origin and distribution 070/13/UAM/0020 ID – UB. 2022.

Separation within unity. Population genetics of Ricciocarpos natans: EST-SSR markers development. 054/13/SNP/0019 ID – UB. 2022

Cryptic species in Bulgarian flora - molecular species delimitation in the *Anura pinguis* complex. KP-06-H21 from 19.12.2018.

Examined materials

In my research, I analyzed over 350 dried plant specimens sourced from natural habitats, private collections, and various European herbaria, including:

- **Adam Mickiewicz University, Poznan, Poland** – Herbarium Code: POZ
- **Botanischer Garten und Botanisches Museum, Freie Universität Berlin, Germany** – Herbarium Code: B
- **Faculty of Biology, University of Warsaw, Poland** – Herbarium Code: WA
- **Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Bulgaria** – Herbarium Code: SOM
- **Lund University, Sweden** – Herbarium Code: LD
- **Museu Nacional de História Natural e da Ciência, Portugal** – Herbarium Code: LISU
- **Norwegian University of Science and Technology, Norway** – Herbarium Code: TRH
- **University of Silesia in Katowice, Poland** – Herbarium Code: SOSN
- **University of Tartu, Estonia** – Herbarium Code: TU

Additionally, a living collection was established using both sterile (in-vitro) and non-sterile cultures to ensure the availability of fresh material for chromosome counting, genome size measurements, and DNA barcoding. This collection comprised approximately 70 clones from various populations of the species of interest.

Supplementary Materials

Table 6.2: Summary Statistics for Phylogenetic Model Parameters in Chapter 5. Provided are the mean, standard deviation (stdev), and efective sample size values (ESS) for: Log-Likelihood (LnLike), Log-Prior (LnPrior), Tree Length (TL), Gamm Shape Parameter (Alpha), Proportion of Invariant Sites (Pinvar), Standard Deviation of Split Frequencies (SDSF), Potential Scale Reduction Factor (PSRF), Effective Sample Size (ESS).

Region	**LnLike** mean stdev ESS	**LnPrior** mean stdev ESS	**TL** mean stdev ESS	**Alpha** mean stdev ESS	**Pinvar** mean stdev ESS	**SDSF** (avg)	**SDSF** (max)	**PSRF** (avg)	**PSRF** (max)
gpd	-1204.23 7.27 42020	-165.51 8.82 63401	0.483 0.0634 62436	0.9044 0.5382 43654	0.3666 0.1381 36622	0.001705	0.00495	1.000	1.000
gpd SIC	-1229.32 7.28 28371	161.56 8.98 53418	0.512 0.0677 51688	0.9037 0.5582 30485	0.3682 0.1401 24106	0.003183	0.014943	1.000	1.001
trnL-rps4	-2123.31 7.502 41176	244.68 9.80 44605	0.109 0.0179 43471	{1} 1.0063 0.966 62011 {2} 2.4291 1.299 97921	0.405 0.2217 37701	0.001676	0.0055	1.000	1.000
trnL-rps4 SIC	-2126.97 7.35 42742	246.33 9.45 54228	0.107 0.0168 52436	{1} 1.0794 0.987 65837 {2} 2.4202 1.295 95659	0.3749 0.2177 46012	0.000682	0.002137	1.000	1.000

Table 6.3: Summary Statistics for Phylogenetic Model Parameters in Chapter 6. Provided are the mean, standard deviation (stdev), and efective sample size values (ESS) for: Log-Likelihood (LnLike), Log-Prior (LnPrior), Tree Length (TL), Gamm Shape Parameter (Alpha), Proportion of Invariant Sites (Pinvar), Standard Deviation of Split Frequencies (SDSF), Potential Scale Reduction Factor (PSRF), Effective Sample Size (ESS).

Region	**LnLike** mean stdev ESS	**LnPrior** mean stdev ESS	**TL** mean stdev ESS	**Alpha** mean stdev ESS	**Pinvar** mean stdev ESS	**SDSF** (avg)	**SDSF** (max)	**PSRF** (avg)	**PSRF** (max)
trnL-rps4	-3300.8 5.69 33238	-104.66 3.11 36002	0.324 0.0245 36002	0.9418 0.06226 25421	0.9418 0.1417 22073	0.002420	0.010417	1.00	1.001
trnL-rps4 SIC	-3492.06 5.59 81486	103.7 5.59 98873	0.332 0.0251 102143	0.942 0.6268 43918	0.3816 0.1422 33991	0.000978	0.004604	1.00	1.00
rbcL	-3949.68 8.91 18162	382.50 10.76 34102	0.308 0.0272 33699	0.994 0.558 12718	0.7193 0.0605 10735	0.002724	0.014801	1.00	1.002