Diversity of Batrachospermales (Rhodophyta) in the Iberian Peninsula

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Abstract: Fifty–four streams of the Iberian Peninsula were investigated to document the species richness and phylogeny of Batrachospermum and morphologically similar genera, determine phylogenetic affinities of taxa and infer biogeographic trends. Species were identified using morphology and DNA sequence data for the rbcL gene as follows: Batrachospermum gelatinosum, B. pozoazulense, Paludicola keratophyta, Sheathia arcuata, S. boryana, S. confusa, Torularia atra, Virescentia helminthosa, V. vogesiaca, and Volatus carrionii. Batrachospermum gelatinosum and S. boryana were most frequent (sampled from 16 and 11 streams, respectively). Torularia atra and V. vogesiaca were each collected in a single stream, whereas the other five taxa were present in three to six streams. Virescentia vogesiaca is well known from other parts of Europe but had previously not been reported from the Iberian Peninsula. Biogeographic trends based on DNA sequence data show a flora with most species being well represented in parts of Europe, two taxa (B. gelatinosum and V. carrionii) that are shared between Europe and North America, one between Europe and South America (Paludicola keratophyta) and one cosmopolitan species (T. atra). Previous studies reported twelve taxa in the region using current taxonomy. This study has confirmed seven of these taxa with sequence data and morphology.

Key words: Batrachospermum, freshwater, Iberian Peninsula, Paludicola, phylogeny, rbcL, Sheathia, Torularia, Virescentia, Volatus

INTRODUCTION

Biodiversity of inland freshwater habitats represent a valuable natural resource, which has measurable economic, cultural, aesthetic, scientific and educational value (GOWDY 1997; DUDGEON et al. 2006). A biodiversity hotspot is an area with high species richness and frequently requiring protection (MYERS 1988; MITTERMEIER et al. 1999; MYERS et al. 2000). The Mediterranean Basin region has been identified as a biodiversity hotspot not only for number of endemics, but also for its freshwater biotic richness and, unfortunately, for the environmental threats to its biodiversity (MALCOLM et al. 2006; CUTTELOAD et al. 2008; FIGUEROA et al. 2013). The Iberian Peninsula (Portugal and Spain) has freshwater habitats that are recognized as naturally rich and diverse in their biota (MÉDAIL & DIADERMA 2009; SABATER et al. 2009).

Southern European peninsulas, such as the Iberian, Italian and the Balkans appear to have been Pleistocene refugia (HEWITT 2004). Numerous freshwater animal species have colonized western and northern Europe from the Iberian Peninsula after the Ice Ages (LENK et al. 1999; BEEBEE & ROWE 2000; DURANT et al. 1999; KOTLIK & BERREBI 2001; PAULS et al. 2006), but little is known about the phylogeography of freshwater macroalgae in Europe. Only a few studies have been conducted with the green alga, Aegagropila linnaei Kütz. and red alga, Batrachospermum gelatinosum (L.) DeCandole, both showing a clear imprint of recolonization of northern and central Europe from southern refugia (BOEDEKER et al. 2010; KEIL et al. 2015).

Europe has a long tradition of freshwater algal
studies, including red algae (Kvlin 1912; Skuja 1928; Israelson 1942; Reis 1974; Starmach 1977), yet many regions remain little explored (Eloranta et al. 2011; Ceschi et al. 2013; Chasson et al. 2014). The majority of freshwater red algal studies have focused on northern and central Europe (e.g. Starmach 1977; Sieminska 1992; Athanasiadis 1996; Kucera & Maran 2004; Gutowski et al. 2004; Schauberg et al. 2004; Kostkевич & Laučūtė 2005; Eloranta & Kwandrans 2007; Eloranta 2019), with only a few studies conducted in the Mediterranean Basin (Reis 1974; Sabater et al. 1989; Simic & Rankovic 1998; Cvićan 2002; Ceschi et al. 2012; García–Fernández et al. 2015). A recent checklist of freshwater rhodophytes for the Iberian Peninsula showed this region to have a large number of taxa, many of which are in the Batrachospermales (Chapuis et al. 2014). In addition, a new genus Volatus and a new species of Batrachospermum in the Batrachospermales were described from the region (Chapuis et al. 2017). These studies hint at potentially high diversity of freshwater red algae in this region in comparison to other investigated areas in Europe (Israelson 1942; Reis 1974; Chasson et al. 2014; Eloranta 2019).

Members of the Batrachospermales have gametophytes with either a ‘stick–like’ morphology such as Lemanea or a ‘beaded–appearance’ like the nominate genus, Batrachospermum. Phylogenetic studies of the order have shown Batrachospermum to be paraphyletic and subsequently new genera that superficially resemble this genus have been described, most recently Acarposporophycos, Montagnia, Paludicola, Virescentia and Visia (Necchi et al. 2018, 2019a, b; Vis et al. 2020). Much of the species diversity (~90%) is contained in these macroscopically similar genera. These taxa typically occur in high water quality locations (Necchi et al. 1999; Eloranta 2019). Traditionally, taxonomic identification within the Batrachospermales has been based on the morphological characteristics of the gametophyte thallus. Although morphology remains the primary means of identification, in recent years taxa have been proposed that are more easily identified using DNA sequence data due to lack of a clear morphological distinction. For example, the species of Volatus can only be distinguished with certainty from those of Kumanou by DNA sequence data emphasizing the need for both morphological and DNA sequence data (Chapuis et al. 2017).

The Iberian Peninsula has been underexplored for freshwater red algal diversity especially in the recent past and the combination of both morphology and sequence data are lacking. Therefore, the aims of this study were to collect species of Batrachospermales (Batrachospermum and morphologically similar genera, excluding Lemanea and Paralemanea) from a wide geographic range of the Iberian Peninsula and to use a combination of morphological and DNA sequence data to characterize the taxa. In addition, the flora from this current study was compared with the previously reported flora and biogeographic affinities explored.

**Materials and Methods**

Fifty–four sites were sampled between February and October in the years 2011–2014 as part of a larger study of algal diversity of the Iberian Peninsula (Sánchez Castillo et al. 2009). When possible, water chemistry data (pH, specific conductance and temperature) were measured on site using an YSI 560 multiprobe (Xylem Analytics, UK) and latitude and longitude were recorded using a GPS (Table 1, Table S1, Supplemental Information). These parameters were measured aiming to detect trends of occurrence for these environmental variables for individual species. A total of 55 macroscopic gametophyte samples with Batrachospermum–like morphology were collected from these sites (Fig. 1, Table S1). All algal samples were divided into three portions after removing visible epiphytes. One portion was placed in Sass liquid (Sass 1958) for morphological examination, another was dried in silica desiccant for DNA extraction, and the third portion preserved in Sass liquid was deposited in the University of Granada Herbarium (GDA) as a voucher specimen. Samples were identified according to the morphological characters previously considered as taxonomically important (Necchi 1990; Shihath et al. 1993, 1994; Vis et al. 1995; Kumanow 2002; Salomakij et al. 2014; Eloranta et al. 2011).

Samples for DNA extraction were either ground by hand in liquid nitrogen using a mortar and pestle, or a Tissuelyser Bertin (Bertin Technologies, Bordeaux, France) mod. Precellys®24 with 1–2 tungsten beads. DNA was extracted using the NucleoSpin Plant II Genomic mini kit (MN–Macherey–Nagel, Düren, Germany) following the manufacturer’s protocol or a CTAB extraction protocol (Winnepennickx et al. 1993). A 1,282 bp fragment of the rbcL gene was amplified using the F160 and rbcLR primers (Vis et al. 1998). Amplification was conducted with the following protocol for a total volume of 30 µl (1 µl of DNA, 1X buffer of New England Biolabs buffer, 250 nM dNTP, 0.03% BSA, 2,5mM MgCl2, 7.5 pmoles of each primer and 1U Taq Polymerase (NEB)). For COI–5P (664 bp), samples were amplified using GAZF1 and GAZR1 primers (Saunders 2005). The amplification cycles for the rbcL and COI–5P followed those provided in Salomakij et al. (2014). PCR products were cleaned using Exo–SAP–IT (USB, Cleveland, Ohio, USA) according to the manufacturer’s instructions. Sequencing reactions were performed using the ABI Prism BigDyeTM v3.1 Terminator Cycle Sequencing Ready Reaction kit and the ABI 3100 Genetic Analyzer (Applied Biosystems) at the Ohio University Genomics Facility or were commercially sequenced (Macrogen Inc., Korea). The PCR primers and internal primers (only rbcL) were used for sequencing of both strands (Vis et al. 1998). The sequences generated were edited and assembled using Geneious v7.1.9 software (Biomatters 2014) or Sequencher 5.2.4 (gene Codes Corp., Ann Arbor, Michigan, USA) and submitted to GenBank.

The rbcL sequences were used for phylogenetic reconstruction. Since there were numerous sequences for some of the species, only one or two sequences were used as they only differ by a few base pairs within species. Fourteen sequences from the Iberian Peninsula were combined with 52
The 55 samples collected represent ten species. Based on morphology, the following species were recognized: *Batrachospermum gelatinosum*, *B. pozoozulense* I.S. Chapuis et M.L.Vis, *Paludicola keratophyta* (Bory) M.L.Vis et Necchi, *Sheathia arcuata* (Kylin) Salomaki et M.L.Vis, *S. boryana* (Sirodot) Salomaki et M.L.Vis, *S. confusa* (Bory) Salomaki et M.L.Vis, *Torularia atra* (Hudson) M.J.Wynne, *Virescentia helminthosa* (Bory) Necchi, D.C.Agostinho et M.L.Vis, *V. vogesiaca* (Schultz ex Skuja) Necchi, D.C.Agostinho et M.L.Vis and *Volatus carrionii* I.S. Chapuis et M.L.Vis. Only a single species was collected from each site, except *Torularia atra* and *Sheathia arcuata*, which were both collected at the same site (site 25, Table S1).

Sequence data of the rbcL gene could be obtained from 54 specimens and a BLAST search of GenBank showed sequence similarity to ten taxa identified by morphology in this study (Table S1). Sixteen specimens belonged to *Batrachospermum gelatinosum* and only varied by 0–3 bp. Among the four specimens of *B. pozoozulense*, the sequences were identical, whereas the four specimens of *Paludicola keratophyta* varied by 0–1 bp and the three specimens of *Virescentia helminthosa* also showed little variation (0–1 bp). The species of the genus *Sheathia* showed the

**RESULTS**

The 54 sites sampled had a wide variation in environmental parameters (Table 1, Table S1): pH varied from 4.6 to 8.6, specific conductance ranged from 11.5 to 1400 µS.cm\(^{-1}\) and the temperature varied from 8.4 to 23 °C. However, most taxa occurred in streams that were neutral to alkaline (pH 7.1–8.6), moderately cool to warm waters (14–23 °C) and with elevated ion content (375–847 µS.cm\(^{-1}\)).

![Fig. 1. Map of the Iberian Peninsula showing major rivers, and the 51 sampling localities (site numbers as in Table S1).](image-url)
following variation: *S. boryana* with 0–7 bp among 11 specimens, *S. confusa* with 0–4 bp among five specimens and *S. arcuata* with 0–3 bp among the six specimens. The three *V. carrionii* specimens differed by 1–3 bp. One specimen each of *Virescentia helminthosa*, *V. vogesiaca* and *Torularia atra* were confirmed from the rbcL sequences.

The sixteen COI–5P sequences represented the following taxa: *Batrachospermum gelatinosum* (4), *B. pozoazulense* (1), *Paludicola keratophyta* (6), *Sheathia arcuata* (3), *S. confusa* (1) and *Volatus carrionii* (1) (Table S1). *Batrachospermum gelatinosum*, *Paludicola keratophyta* and *S. confusa* sequences were compared with those from other regions of Europe and were either identical or differed by ≤2 bp. There were no sequences of *Sheathia arcuata* from Europe and the closest match was *S. arcuata* from China, which differed by >7% and probably do not represent the same species. *Batrachospermum pozoazulense* and *Volatus carrionii* were recently described from Spain and these sequences were noted in the protologue.

The phylogenetic analyses of rbcL sequence data showed the tree topologies of the two reconstruction methods to be similar and only the ML tree is shown with the support values from both analyses (Fig. 2). These analyses confirmed that the specimens from this study were closely related to specimens of the same species from other locations with the exception of *Batrachospermum pozoazulense* and *Volatus carrionii* which are currently only known from the Iberian Peninsula. Six genera, *Batrachospermum*, *Paludicola*, *Sheathia*, *Torularia*, *Virescentia* and *Volatus* were represented in the flora (Fig. 2). In *Batrachospermum* sensu stricto, *Batrachospermum gelatinosum* from Spain was in a clade with sequenced specimens from North America and Europe (France and Italy). The sequences of *Virescentia helminthosa* and *V. vogesiaca* were grouped within this genus and were similar to previously reported sequence data of specimens from France. A sequence of *Paludicola keratophyta* was sister to one from France. The sequence of *Torularia atra* was identical to a specimen from Italy. Within the genus *Sheathia*, the two sequences of *S. confusa* from the Iberian Peninsula were within a well–supported clade with other specimens identified as *S. confusa* collected in Europe (France) and Australasia (New Zealand). Likewise, *S. arcuata* was within a well–supported clade of European *S. arcuata* specimens collected in Bulgaria and France.

*Batrachospermum* was represented with two species occurring in a total of 20 streams. *Batrachospermum gelatinosum* was collected in 16 streams (sites 1–16, Fig. 1) including one on the Balearic Islands (site 2, Fig. 1). The streams in which this species was collected had a broad range in specific conductance (54–1013 $\mu$S.cm$^{-1}$), neutral to alkaline pH (7.1–8.4) and cool to warm water temperature (13–23 °C) (Tables 1, S1). *Batrachospermum pozoazulense* was collected from four streams in the north and east (sites 17–20, Fig. 1). The streams had moderate specific conductance (463–598 $\mu$S.cm$^{-1}$), neutral to alkaline pH (7.2–8.4) and moderately cool water temperature (11–15 °C) (Table 1).

*Sheathia* was represented by three species observed in 23 streams. *Sheathia arcuata* was collected in six streams from the south to north areas of the Iberian Peninsula (sites 25–30, Fig. 1). The streams showed a broad range in specific conductance (375–1237 $\mu$S.cm$^{-1}$), circumneutral to alkaline pH (7.3–8.3) and variable water temperature (8–21 °C) (Table 1). *Sheathia boryana* was collected from 11 streams, primarily located in the south and east regions of the

### Table 1. Mean (range) in stream characteristics for freshwater red algae collected. n = the number of streams with water chemistry data used to calculate the mean stream characteristics from the individual stream data in Table S1.

| Taxon                         | n  | Specific Conductance ($\mu$S.cm$^{-1}$) | pH               | Temperature (°C) |
|-------------------------------|----|---------------------------------------|------------------|------------------|
| *Batrachospermum gelatinosum* | 12 | 394 (54–1013)                          | 7.55 (7.1–8.4)   | 17 (9–23)        |
| *B. pozoazulense*             | 4  | 509 (436–598)                          | 7.60 (7.2–8.4)   | 13 (11–15)       |
| *Paludicola keratophyta*      | 4  | 40 (16–78)                             | 5.3 (4.6–7.5)    | 16 (13–20)       |
| *Sheathia arcuata*            | 6  | 648 (375–1237)                         | 7.55 (7.3–8.3)   | 14 (8–21)        |
| *S. boryana*                  | 11 | 654 (298–1400)                         | 7.81 (7.3–8.6)   | 14 (10–20)       |
| *S. confusa*                  | 3  | 141 (45–277)                           | 6.97 (6.8–7.2)   | 12 (11–14)       |
| *Torularia atra*              | 1  | 750                                   | 7.9              | 13               |
| *Virescentia helminthosa*     | 3  | 179 (68–289)                           | 6.53 (6.1–7.7)   | 19 (16–23)       |
| *V. vogesiaca*                | 1  | 12                                    | 7.4              | 20               |
| *Volatus carrionii*           | 3  | 17 (11–20)                             | 6.70 (6.5–7.1)   | 16 (14–19)       |
north-western streams (sites 49–50) and one close to a hot spring in the east of the Iberian Peninsula (site 48). The streams with *V. helminthosa* had low conductivity (68–289 µS.cm\(^{-1}\)), wide range in pH (6.1–7.7) and moderate water temperature (16–23 °C) (Table 1). *Virescentia vogesiaca* was found in a north-western stream (site 51, Fig. 1) characterized by low light levels due to dense canopy cover and high current velocity as well as very low conductivity (12 µS.cm\(^{-1}\)), pH 7.4 and water temperature 20 °C (Table 1).

The genera *Paludicola*, *Volatus* and *Torularia* were each represented by a single species. *Paludicola keratophyta* was collected from a total of four streams. *V. helminthosa* was observed in three sites (48–50, Fig. 1) with two north-western streams (sites 49–50) and one close to a hot spring in the east of the Iberian Peninsula (site 48). The streams with *V. helminthosa* had low conductivity (68–289 µS.cm\(^{-1}\)), wide range in pH (6.1–7.7) and moderate water temperature (16–23 °C) (Table 1). *Virescentia vogesiaca* was found in a north-western stream (site 51, Fig. 1) characterized by low light levels due to dense canopy cover and high current velocity as well as very low conductivity (12 µS.cm\(^{-1}\)), pH 7.4 and water temperature 20 °C (Table 1).

Two species of the genus *Virescentia* were collected from a total of four streams. *V. helminthosa* was observed in three sites (48–50, Fig. 1) with two north-western streams (sites 49–50) and one close to a hot spring in the east of the Iberian Peninsula (site 48). The streams with *V. helminthosa* had low conductivity (68–289 µS.cm\(^{-1}\)), wide range in pH (6.1–7.7) and moderate water temperature (16–23 °C) (Table 1). *Virescentia vogesiaca* was found in a north-western stream (site 51, Fig. 1) characterized by low light levels due to dense canopy cover and high current velocity as well as very low conductivity (12 µS.cm\(^{-1}\)), pH 7.4 and water temperature 20 °C (Table 1).
21–24, Fig. 1). The streams had low specific conductance (16–78 $\mu$S.cm$^{-1}$), acidic to circumneutral pH (4.6–7.5) and moderately cool water temperature (13–20 °C) (Table 1). Volatulus carrionii occurred in three streams in the western and northwestern regions of the Iberian Peninsula (sites 52–54, Fig. 1). The streams had very low specific conductance (11–20 $\mu$S.cm$^{-1}$), mildly acidic pH (6.5–6.7) and cool to moderately cool water temperature (14–19 °C) (Table 1). Torularia atra was collected from a single location (site 25, Fig. 1), having high specific conductance (750 $\mu$S.cm$^{-1}$), alkaline pH (7.9) and cool water temperature (13 °C) (Table 1).

**Discussion**

This research extends the previous limited studies of the diversity of freshwater rhodophytes from the Iberian Peninsula. In a checklist of all literature on the region, Chapuis et al. (2014) listed 29 Batrachospernum–like species. Applying the most recent taxonomy, this number would be 12 taxa as follows: Batrachospernum gelatinosum, Kumanoa henriquesiana (Reis) Necchi et M.L.Vis, K. lusitania (Reis) Necchi et M.L.Vis, K. pseudocarpa (Reis) Necchi et M.L.Vis, Sheathia arcuata, S. boryana, S. confusa, S. exigua, Paludicola keratophyta, Virescentia helminthosa, V. gelbenkiana (Reis) Necchi, D.C.Agostinho et M.L.Vis and Torularia atra. The current study has confirmed seven of these taxa with sequence data and morphology, whereas five species previously recorded were not found in this survey: K. henriquesiana, K. lusitania, K. pseudocarpa, S. exigua and V. gelbenkiana. Sequence data will be important in further taxonomic revisions within the order Batrachospermales not only in the European framework, but also in a global context to delineate floras and investigate biogeographic trends.

Two species of Batrachospernum were reported in this study, B. gelatinosum and B. pozoazulense. The former is widely distributed in both North America and Europe but differs by only a few base pairs in the rbcL gene from all these locations (House et al. 2010; Keil et al. 2015). Despite the wide geographic distribution of B. gelatinosum in the Northern Hemisphere (Sheath & Sherwood 2002; House et al. 2010; Eloranta et al. 2011; Keil et al. 2015), there are few data on the physical and chemical characteristics of the streams for which there are molecular data for specimens. Three recent studies of streams in France and Italy report B. gelatinosum from a wide range of stream parameters: specific conductance (110–825 $\mu$S.cm$^{-1}$), pH (6.5–8.4), and temperature (9–26 °C), similar to the current and previous studies (Ceschi et al. 2013; Chasson et al. 2014; Abdelahad et al. 2015; Sheath & Vis 2015) also confirm the broader environmental tolerance of B. gelatinosum in North America. B. pozoazulense is a newly described species from four streams with narrow environmental ranges (Chapuis et al. 2017). Since this species can only be distinguished from B. gelatinosum with molecular data, in the future it may become known from more locations and may be shown to have a wider environmental tolerance like B. gelatinosum.

Two species of Virescentia were identified (V. helminthosa and V. vogesiaca), the latter is reported for the first time in the Iberian Peninsula (Chapuis et al. 2014). The environmental parameters recorded for the site of V. helminthosa in the Iberian Peninsula were within those reported by Sheath et al. (1994) for V. virideamericana in North America and by Agostinho & Necchi (2014) for V. viridebrasilensiens in South America. In terms of the environmental characteristics of the Iberian streams containing Paludicola keratophyta, most are acidic, brown water and low specific conductance, especially those in the north–east. Reports of P. keratophyta from other European countries have also noted similar environmental conditions (Reis 1974; Chasson et al. 2014; Eloranta 2019).

The species Sheathia boryana, S. confusa and S. arcuata are widespread in Europe (Vis et al. 2010; Eloranta et al. 2011; Chasson et al. 2014; Salomaki et al. 2014; Abdelahad et al. 2015). A study in Italy reported S. boryana from streams with similar parameters to those of the Iberian streams: specific conductance (487–597 $\mu$S.cm$^{-1}$), pH (7.2–7.3), and temperature (15 °C) (Abdelahad et al. 2015). The Iberian streams with S. confusa share common ecology with the populations reported in France (Chasson et al. 2014). In contrast, the environmental conditions in the Iberian Peninsula streams with S. arcuata extend the range in environmental conditions for temperature, pH and conductivity from those previously reported for France and Italy (Chasson et al. 2014; Abdelahad et al. 2015). Torularia atra is a cosmopolitan species and the morphological characteristics of the specimen in this study match previous descriptions (Sheath et al. 1993; Kumano 2002; Eloranta et al. 2011; Rossignolo & Necchi 2016). Torularia atra (as Batrachospernum atrum) was reported four times previously in the Iberian Peninsula (Reis 1974, Aboal et al. 1995; Moreno Alcaraz et al. 2013; Rossignolo & Necchi 2016). The environmental conditions reported in those surveys were similar to the ones described in this study: specific conductance (577–870 $\mu$S.cm$^{-1}$), pH (6.4–8.1), and temperature (14–20 °C).

Our morphologically identified and molecularly confirmed species data showed some species to be widespread in the world, whereas others have narrower geographic ranges. Five of the taxa identified appear to inhabit streams only in Europe including Batrachospernum pozoazulense, Sheathia arcuata, S. boryana, Virescentia helminthosa and V. vogesiaca. Torularia atra is distributed worldwide and Batrachospernum gelatinosum is common on two continents (Europe and North America). Three species had collections from numerous locations in Europe but
also a single location on another continent, *Paludicola keratophyta* (South America), *S. confusa* (Australasia), *Volatus carrionii* (North America). These widespread species and species with disjunct distributions suggest that these taxa can be dispersed long distances even though a resistant stage is unknown. Dispersal by wind or animals such as flying adult stages of insects or birds (*Proctor* 1959; *Maguire* 1963; *Caceres* & *Soluk* 2002; *Vis* 2016) is a possibility, although still speculative due to the scarcity of experimental studies on dispersal of freshwater algae by waterfowl (*Vis* 2016). In addition, historical and ecological biogeography remain unexplored fields for stream macroalgae, making it difficult to provide other plausible explanation to the presently known distribution of members of the Batrachospermales.

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