Review

Classification and phylogeny of the cyanobiont Anabaena azollae Strasburger: an answered question?

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The symbiosis Azolla–Anabaena azollae, with a worldwide distribution in pantropical and temperate regions, is one of the most studied, because of its potential application as a biofertilizer, especially in rice fields, but also as an animal food and in phytoremediation. The cyanobiont is a filamentous, heterocystic cyanobacterium that inhabits the foliar cavities of the pteridophyte and the indusium on the megasporocarp (female reproductive structure). The classification and phylogeny of the cyanobiont is very controversial: from its morphology, it has been named Nostoc azollae, Anabaena azollae, Anabaena variabilis status azollae and recently Trichormus azollae, but, from its 16S rRNA gene sequence, it has been assigned to Nostoc and/or Anabaena, and from its phycocyanin gene sequence, it has been assigned as non-Nostoc and non-Anabaena.

The literature also points to a possible co-evolution between the cyanobiont and the Azolla host, since dendrograms and phylogenetic trees of fatty acids, short tandemly repeated repetitive (STRR) analysis and restriction fragment length polymorphism (RFLP) analysis of nif genes and the 16S rRNA gene give a two-cluster association that matches the two-section ranking of the host (Azolla). Another controversy surrounds the possible existence of more than one genus or more than one species strain. The use of freshly isolated or cultured cyanobionts is an additional problem, since their morphology and protein profiles are different. This review gives an overview of how morphological, chemical and genetic analyses influence the classification and phylogeny of the cyanobiont and future research.

Introduction

The small, heterosporic, free-floating, aquatic pteridophyte Azolla, belonging to the family Azollaceae, has a worldwide distribution, mainly in pantropical, subtropical and temperate regions in quiet waters (rivers, dams, creeks, etc.), and is considered an invasive species. During an Azolla bloom, which occurs when the environmental conditions (light, temperature, photoperiod, nutrients, etc.) are optimal, its overgrowth can form dense, thick mats. The multi-branched sporophyte has a main rhizome with alternating lateral ramifications, covered by small, alternate and imbricate deeply bilobed leaves (Fig. 1a). The adventitious roots protrude from the ventral side of the rhizome at ramification branching points. Each leaf has two lobes: an aerial, thick, chlorophyllous dorsal lobe and a thin, hyaline, floating or partially submersed ventral lobe (Lumpkin & Plucknett, 1980; Peters et al., 1982; Shi & Hall, 1988; Wagner, 1997; Carrapício et al., 2000; Lechno-Yossef & Nierzwicki-Bauer, 2002; Bergman et al., 2008; Carrapício, 2010).

Carrapício et al., 2000; Lechno-Yossef & Nierzwicki-Bauer, 2002; Bergman et al., 2008; Carrapício, 2010).

The Azolla pteridophyte forms a symbiosis with a filamentous, heterocyst-forming, nitrogen-fixing cyanobacterium called Anabaena azollae and with members of several bacterial genera. In each dorsal lobe, the cyanobiont occupies a narrow space in the periphery of the extracellular ovoid cavity. The cavity is lined by a mucilaginous network and is limited by external and internal envelopes, leaving the centre empty (Fig. 1b). This unique symbiosis is the only known everlasting plant–cyanobacterium symbiosis, since the cyanobiont is transmitted through Azolla generations without a de novo infection: to new leaves by the vegetative propagation of Azolla and to new plantlets by the sexual reproduction of the pteridophyte. Through vegetative propagation, some cyanobiont filaments at the apical meristem (Fig. 1c) of Azolla are partitioned into the forming cavities (Fig. 1d) and, as the leaves develop, differentiation of heterocysts occurs. In fully developed cavities, vegetative cells and heterocysts form the cyanobiont filaments (Fig. 1e).
negative bacterium, is synchronous with foliar cavity of the cyanobiont with the fern without a cotyledonary leaf, becoming associated with the apical forming filaments of undifferentiated cells that surround occurs (Perkins & Peters, 1993). These akinetes remain (Fig. 1f) and, at the same time, the differentiation of akinetes microsporocarp), some cyanobiont filaments partition to unfavourable for vegetative propagation. During the forma-
tion of sporocarps (the female megasporocarp and the male
chiorphyllous dorsal lobes (*,;). (b) Cross-section of a fully
developed dorsal lobe. The cavity is lined with a layer of epidermal
cells (EC) and the cyanobiont filaments of A. azollae (arrow)
and epidermal trichomes (**) embedded in a mucilaginous matrix
occupying a narrow space near the periphery. (c) Section of the
apical meristem with an inoculum of A. azollae filaments formed by
small vegetative cells (arrow). (d) Partition of cyanobiont filament,
(arrow) from the fern apical meristem to the forming foliar cavity.
(e) Filaments of A. azollae isolated from a mature dorsal lobe foliar
cavity of the fern. The heterocysts (H) are between the vegetative
cells (VC). (f) Cross-section of the megasporocarp of A.
filiculoides showing the location of the cyanobiont (arrow) in a
chamber of the indusium (i).

(Lumpkin & Plucknett, 1980; Peters et al., 1982; Wagner, 1997; Carrapiço et al., 2000; Lechno-Yossef & Nierzwicki-Bauer, 2002; Carrapiço, 2010). Sexual reproduction of Azolla
only occurs in nature when environmental conditions are
unfavourable for vegetative propagation. During the forma-
tion of sporocarps (the female megasporocarp and the male
microsporocarp), some cyanobiont filament partition to
an indusial chamber in the distal part of the megasporocarp
(Fig. 1f) and, at the same time, the differentiation of akinetes
occurs (Perkins & Peters, 1993). These akinetes remain
dormant until embryogenesis, when the akinetes germinate,
forming filaments of undifferentiated cells that surround
the cotyledonary leaf, becoming associated with the apical
meristem of the new plantlet and promoting the continuity
of the cyanobiont with the fern without a de novo infection
by an external cyanobacterial pool (Peters & Perkins, 1993).

The development of the cyanobiont A. azollae, a Gram-
negative bacterium, is synchronous with foliar cavity
development. At the apical meristem of Azolla, the
cyanobiont filaments have uniform vegetative cells with
numerous carboxysomes, thylakoid membranes running
parallel to the cell membrane, polyglycoside granules
and without nitrogenase activity, due to the absence of
heterocysts. In mature cavities, the cyanobiont filaments
have (i) vegetative cells with fewer carboxysomes than
those in the apical meristem and thylakoid membranes
distributed throughout the entire cytoplasm, forming
whorls; and (ii) heterocysts, in which nitrogen fixation
occurs; these are larger cells, with extra cell-wall layers, a
narrow neck with a plug of cyanophycin and high vesicula-
tion of the thylakoid membranes (Neumüller & Bergman,
1981). In the mature foliar cavity, the cyanobiont filament
density is high, but is circumscribed by the periphery of the
cavity, indicating that cyanobiont growth is probably under
the control of the pteridophyte. However, the mechanism
by which this achieved is not known. The cyanobiont has
chlorophyll a, carotenoids and phycobiliproteins, which
make it capable of photosynthesis, but the photosynthetic
rate is low. The sugar source essential for generation of
the energy necessary for the highly demanding nitrogen
fixation is provided mainly by Azolla. Therefore, restriction
of the sugar flow from the host to the cyanobiont could be a
way of controlling cyanobiont growth – a nutrient control.
In addition, the frequency of heterocysts is much higher
(30–40%) than in free-living cyanobacteria (5–10%),
which makes them more suitable for high nitrogen fixation
rates (Peters & Mayne, 1974; Becking & Donze, 1981;
Neumüller & Bergman, 1981; Peters et al., 1982).

Over the years, the cyanobiont has been named Nostoc
azollae, Anabaena azollae and Trichormus azollae, but,
to date, no definitive classification exists. Morphological,
metabolite and genome and gene sequencing data have
been used in an attempt to address questions such as (i)
the classification of the cyanobiont; (ii) its phylogeny; (iii)
the possible existence of more than one genus or species or a
major (dominant and non-cultivable) and a minor (non-
dominant and cultivable) cyanobiont in the Azolla foliar
cavity; and (iv) co-evolution between the two partners.
Genome sequencing of the cyanobiont isolated from Azolla
filiculoides was a major advance. Analysis of the cyanobiont
genome showed a high percentage of pseudogenes (31.2%)
and large numbers of transposable elements (~600), pointing
to genome erosion (Ran et al., 2010). The mechanisms
that lead to genome erosion in the cyanobiont are not know, but,
since microvesicles containing DNA were found budding
from the cyanobiont, this could be a mechanism of DNA
loss (Zheng et al., 2009). Even so, the sequenced genome has
given an advantage to classification and phylgeny of this
cyanobiont, since it is now possible to compare the whole
genome of the cyanobiont ‘Nostoc azollae’ isolated from
A. filiculoides with the cyanobiont genomes of other Azolla
species and other symbiotic and free-living cyanobacteria.

This review presents the state of the art of the conflicting
classifications and phylgenies of the Azolla cyanobiont
and addresses future research.
Is there co-evolution between the two partners?

The symbiosis between Azolla and the cyanobacterium must have begun many millions of years ago. However, since the limited number of rhizome fossil records make it difficult to date the beginning of the symbiosis, it must be assumed that, even in the oldest Azolla fossils, which date back to the Cretaceous period (Collinson, 2001), the symbiosis probably already occurred. As this is an everlasting symbiosis, co-evolution between the two partners was hypothesized. By using restriction fragment length polymorphism (RFLP) analysis of the nif gene of fresh cyanobionts isolated from four Azolla species belonging to the section Azolla (Table 1), Franche & Cohen-Bazire (1985) were able to detect similarities, but also small differences, in the restriction patterns, probably due to evolutionary divergence, pointing to co-evolution. Now that the possibility of co-evolution between the two partners was hypothesized, the answer must come from analysis of all Azolla cyanobionts. The co-evolution theory implies that the cyanobiont clustering is similar to the Azolla clustering. The seven extant Azolla species are grouped into two sections: (i) Rhizosperma (Azolla nilotica and A. pinnata) and (ii) Azolla (A. filiculoides, A. rubra, A. caroliniana, A. mexicana and A. microphylla) (Reid et al., 2006; Metzgar et al., 2007; Pereira et al., 2011).

Data from fatty acid profiles (Table 2) (Caudales et al., 1995), RFLP (Van Coppenolle et al., 1995) and short tandemly repeated repetitive (STRR) (Zheng et al., 1999) analyses (Table 1) and 16S rRNA gene sequencing (Table 3) (Papaefthimiou et al., 2008b) support the co-evolution theory. There is always a clear distinction between the cyanobionts from the ferns of the two sections Rhizosperma and Azolla, which agrees with the fern taxonomy (Reid et al., 2006, Metzgar et al., 2007; Pereira et al., 2011). The ferns of the two sections were the first to diverge, about 50.7 million years ago (Mya), and the two Azolla species of the section Rhizosperma (A. nilotica and A. pinnata) diverged about 32.5 Mya (Metzgar et al., 2007). Given that these Azolla species have had more time to diverge, their cyanobionts probably evolved differently, allowing a clear distinction between them.

As for the cyanobionts from ferns of the section Azolla, the distinction is more complex, following the as-yet unresolved phylogeny and taxonomy of the five species that are grouped in this section. The estimated time of divergence of the cluster A. filiculoides/A. rubra from the other species was 16.3 Mya (Metzgar et al., 2007). By using fatty acid profiles (Table 2) (Caudales et al., 1995), STRR profiles (Zheng et al., 1999) (Table 1) and sequenced 16S rRNA genes (Table 3) (Papaefthimiou et al., 2008b), the A. filiculoides cyanobiont clusters in a group distinct from the others, matching the fern phylogeny (Reid et al., 2006; Metzgar et al., 2007; Pereira et al., 2011). However, the cyanobionts of A. filiculoides are closer to the cyanobionts of A. mexicana, A. microphylla and A. caroliniana according to the RFLP profiles (Table 1) (Van Coppenolle et al., 1995). In the case of the A. rubra cyanobiont, instead of clustering with the A. filiculoides cyanobiont, the fatty acid profile points to relatedness with cyanobionts of section Rhizosperma (Caudales et al., 1995) or with cyanobionts of A. mexicana, A. microphylla and A. caroliniana using STRR (Zheng et al., 1999).

The cyanobionts from the A. mexicana/A. microphylla and A. caroliniana groups are the most difficult to analyse. As showed by the fatty acid profiles (Table 2) (Caudales et al., 1995), RFLP (Van Coppenolle et al., 1995) and STRR (Zheng et al., 1999) profiles (Table 1) and even the sequenced 16S rRNA gene (Table 3) (Papaefthimiou et al., 2008b), the clustering of these cyanobionts into distinct groups is more complex. This is probably because the cyanobionts have not had sufficient time to evolve. In fact, the hosts A. mexicana/A. microphylla and A. caroliniana diverged about 3.9 Mya, and A. mexicana and A. microphylla diverged 2.3 Mya (Metzgar et al., 2007). Even so, all the data point to evolutionary divergence that makes possible the correlation of a cyanobiont with an Azolla species; however, the clustering problems of the cyanobionts from the section Azolla reflect the phylogenetic and taxonomic difficulties of the host. Instead of using one or several gene sequences, a more reliable approach is to sequence the whole genome of the cyanobiont of each Azolla species and to compare them. In this way, the phylogeny will include all genes, increasing the differences and similarities between them and probably providing a better understanding of the co-evolution.

Is there more than one genus or species or a major/minor cyanobiont inside the Azolla foliar cavity?

The earliest researchers of the Azolla cyanobiont isolated and cultured the cyanobiont. By comparing fresh isolates and cultured cyanobionts from several Azolla species, the presence of more than one genus or species or major/minor cyanobionts inside the Azolla foliar cavity was hypothesized. However, data on the morphology (Table 4), antigen, allozyme and protein profiles (Table 2), STRR and RFLP profiles (Table 1) and sequenced 16S rRNA genes (Table 3) of the presumptive cultivable A. azollae resulted in more conflict and controversy. Analysing the morphology of fresh isolates and cultured cyanobionts, Newton & Herman (1979) indicated that they had distinctive morphologies, but did not state which morphological characters were different. Even so, the isolate of Newton & Herman (1979) was used in later investigations because it was thought to represent the Azolla cyanobiont (Gates et al., 1980; Ladha & Watanabe, 1982; Nierzwicki-Bauer & Haselkorn, 1986; Gebhardt & Nierzwicki-Bauer, 1991; Zheng et al., 1999).

Morphological analysis of the cyanobacterium is very important to its description and identification, but the literature describes morphological characters for both cyanobionts isolated from the Azolla cavity and cultured cyanobionts. Morphologically, cyanobionts freshly isolated...
<table>
<thead>
<tr>
<th>Type of cyanobiont</th>
<th>Azolla species (host)</th>
<th>Major findings from genomic analysis</th>
<th>Reference</th>
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<tr>
<td>STRR</td>
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<td>Zheng et al. (1999)</td>
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<td>RFLP</td>
<td></td>
<td></td>
<td>Franche &amp; Cohen-Bazire (1985)</td>
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<tr>
<td>F, C and Tel-Or’s isolate from A. filiculoides</td>
<td>A. caroliniana, A. mexicana, A. microphylla, A. filiculoides</td>
<td>Fresh cyanobiont has a similar nif gene RFLP pattern, indicating co-evolution.</td>
<td>Nierwitski-Bauer &amp; Haselkorn (1986)</td>
</tr>
<tr>
<td>F, C (Newton’s isolate from A. caroliniana)</td>
<td>A. caroliniana</td>
<td>Restriction patterns of the fresh cyanobiont and Newton’s isolate for the genes glnA, psbAI, nifH and rbcL–rbcS are not identical. Cultured cyanobionts and Tel-Or’s isolate are contaminants.</td>
<td>Meeks et al., 1988</td>
</tr>
<tr>
<td>F</td>
<td>A. caroliniana, A. filiculoides, A. mexicana, A. microphylla, A. nilotica, A. pinnata var. pinnata and imbricata</td>
<td>Cyanobionts of A. nilotica, A. mexicana, A. rubra and A. microphylla have a unique genotype. All cyanobionts belong to two divergent evolutionary lines. Geographical differences. Cyanobionts related to Nostoc and not to Anabaena.</td>
<td>Plazinski et al. (1990)</td>
</tr>
<tr>
<td>F, C and Newton’s isolate from A. caroliniana</td>
<td>A. mexicana, A. pinnata</td>
<td>Cultured and fresh cyanobionts have different RFLP patterns for glnA, rbcS and psbA genes. Fresh cyanobionts have a common restriction site for glnA, rbcS and psbA. Cultured cyanobionts from A. pinnata and Newton’s isolate have similar RFLP restriction sites. Cultured cyanobiont is not the major cyanobiont inside the Azolla foliar cavity. Cultured cyanobiont is a contaminant or a minor cyanobiont inside the foliar cavity. Perhaps more than one species of Anabaena or Nostoc inhabits the foliar cavity.</td>
<td>Gebhardt &amp; Nierzwicki-Bauer (1991)</td>
</tr>
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<td>F, C</td>
<td>A. caroliniana, A. microphylla, A. mexicana, A. rubra, A. filiculoides, A. pinnata var. pinnata</td>
<td>Fresh and cultured cyanobionts have different restriction profiles for the 16S rRNA gene. Major and minor cyanobionts inhabit the Azolla cavity. Major cyanobiont is not cultivable. Minor cyanobiont is cultivable.</td>
<td>Sood et al. (2008b)</td>
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</table>
from the host cavity have long, wavy (Papaefthimiou et al., 2008a) or straight (Sood et al., 2008b) filaments, round (Gebhardt & Nierzwicki-Bauer, 1991) or elliptical (Papaefthimiou et al., 2008a) heterocysts, with (Gebhardt & Nierzwicki-Bauer, 1991) or without (Papaefthimiou et al., 2008a) akinetes, no terminal heterocysts, no mucilaginous envelope and no hormogonia (Papaefthimiou et al., 2008a). However, Zheng et al. (2009) refer to the presence of hormogonia. The terminal cells of the filaments are round, although they are difficult to visualize. Presumptive cultivable A. azollae (Newton’s isolate, Tel-Or isolate and other isolates) showed different morphological characteristics, depending on the host (Table 4): (i) the filaments are contorted (Zimmerman et al., 1989), coiled or straight (Sood et al., 2008a); (ii) they show colonial, globular or tubular growth (Zimmerman et al., 1989); (iii) hormogonia are present (Zimmerman et al., 1989; Gebhardt & Nierzwicki-Bauer, 1991); (iv) they display ellipsoid, spherical or cylindrical heterocysts (Zimmerman et al., 1989); (v) oval akinetes (Rajaniemi et al., 2005); and (vi) the A. mexicana cyanobiont (Gebhardt & Nierzwicki-Bauer, 1991) and the presumptive Azolla cyanobiont T. azollae (Rajaniemi et al., 2005) show apoheterocystic akinete development. Even the heterocyst frequency (determined by counting heterocysts with polar nodes and a thick cell wall dividing the vegetative cells) is different: 20–25 % or more inside the foliar cavities and 7–10 % in culture (Pabby et al., 2003). Although morphological characters are very useful for determining whether there is more than one cyanobiont inside the Azolla foliar cavity, their morphology is very challenging. For example, the type of akinete development has not been determined for freshly isolated cyanobionts, but was determined in the presumptive cultivable cyanobiont (apoheterocystic); however, the presumptive cultivable cyanobiont does not represent the true Azolla cyanobiont (see below for an explanation of cyanobiont culturing). Even the presence of hormogonia of the cyanobiont isolated from the Azolla foliar cavity is contradictory. However, those earlier results that showed morphological differences between freshly isolated and cultured cyanobionts

<table>
<thead>
<tr>
<th>Type of cyanobiont</th>
<th>Azolla species (host)</th>
<th>Major findings from cyanobiont chemical compounds</th>
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<tr>
<td><strong>Fatty acids</strong></td>
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<td><strong>Antigens</strong></td>
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<tr>
<td>F, C (Newton’s isolate from A. caroliniana)</td>
<td>A. caroliniana, A. pinnata</td>
<td>Fresh and cultured cyanobiont have different antigens. The cultured cyanobiont does not correspond to the true cyanobiont.</td>
<td>Gates et al. (1980)</td>
</tr>
<tr>
<td><strong>Allozymes</strong></td>
<td></td>
<td>Dendrogram of the allozyme profile indicates that five cultured cyanobionts are Anabaena. Cultured cyanobiont is the minor cyanobiont inside the Azolla foliar cavity. A. azollae is the major cyanobiont, but is not cultivable. More than one genus inhabits the foliar cavity of Azolla. Cultured cyanobiont is Anabaena variabilis var. azollae.</td>
<td>Zimmerman et al. (1989)</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
<td>Proteome of the cyanobiont is equidistant from Nostoc and Anabaena. Protein profile of cultured cyanobiont is different from that of fresh cyanobiont.</td>
<td>Ekman et al. (2008) Sood et al. (2008b)</td>
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### Table 3. Gene and genome sequencing of *Azolla* cyanobionts freshly isolated from the foliar cavity (F) and from culture (C)

<table>
<thead>
<tr>
<th>Type of cyanobiont</th>
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</thead>
<tbody>
<tr>
<td>rpoB (RNA polymerase β-subunit)</td>
<td></td>
<td>Clusters to <em>Nostoc/Nodularia</em>. Belongs to genus <em>Nostoc</em> and not <em>Trichormus</em> or <em>Anabaena</em>.</td>
<td>Rajaniemi <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>rbcLX (ribulose-bisphosphate carboxylase large subunit)</td>
<td></td>
<td>Clusters to <em>Nostoc</em>. Belongs to genus <em>Nostoc</em> and not <em>Trichormus</em> or <em>Anabaena</em>.</td>
<td>Rajaniemi <em>et al.</em> (2005)</td>
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<tr>
<td>16S rRNA gene</td>
<td></td>
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<tr>
<td>F</td>
<td>A. filiculoides</td>
<td>Cyanobiont belongs to genus <em>Anabaena</em>.</td>
<td>Svenning <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>F, C (Nostoc from A. filiculoides</td>
<td>A. pinnata subsp. pinnata, A. rubra, A. caroliniana</td>
<td>Fresh cyanobiont and cultured <em>Nostoc</em> from <em>A. filiculoides</em> cluster near <em>Anabaena</em>. <em>T. azollae</em> groups with <em>Nostoc</em>. Fresh cyanobiont is <em>Anabaena</em>.</td>
<td>Papaefthimiou <em>et al.</em> (2008a)</td>
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<td>and T. azollae KOM BAI/1983</td>
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<td>Whole genome</td>
<td>A. filiculoides</td>
<td>Clusters with <em>C. raciborskii/R. brokii</em>.</td>
<td>Ran <em>et al.</em> (2010); Larsson <em>et al.</em> (2011)</td>
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Table 4. Morphological aspects of the *Azolla* cyanobiont freshly isolated from the foliar cavity (F) and from culture (C)

<table>
<thead>
<tr>
<th>Type of cyanobiont</th>
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<th>Major findings from cyanobiont morphology</th>
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</tr>
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<tbody>
<tr>
<td>F, C</td>
<td><em>A. caroliniana</em></td>
<td>Distinctive morphology of fresh and cultured cyanobionts. Cultured strain resembles <em>Anabaena</em>.</td>
<td>Newton &amp; Herman (1979)</td>
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<td></td>
<td><em>A. caroliniana, A. filiculoides, A. pinnata, A. mexicana</em></td>
<td>Cultured cyanobiont of <em>A. mexicana</em> with contorted trichomes and sheath in vegetative cells; colonial or globular growth. Cultured cyanobiont of <em>A. pinnata</em> with fimbriate or tubular growth. Cultured cyanobionts of <em>A. caroliniana</em> and <em>A. filiculoides</em> form loose aggregates. Cultured cyanobionts of <em>A. pinnata</em> and <em>A. caroliniana</em> have hormogonia. Heterocysts ellipsoid, barrel-shaped, spherical, cylindrical. Cultured cyanobionts are <em>Anabaena</em> and <em>Nostoc</em>.</td>
<td>Zimmerman et al. (1989)</td>
</tr>
<tr>
<td>F, C, Newton’s isolate from <em>A. caroliniana</em></td>
<td><em>A mexicana, A. pinnata</em></td>
<td>Fresh cyanobiont has filaments with round heterocysts, akinetes in the filaments or as single cells. Cultured cyanobionts of <em>A. pinnata</em> and Newton’s isolate have a morphology similar to that of fresh cyanobionts, with intercalary heterocysts. Cultured cyanobiont of <em>A. mexicana</em> has motile hormogonia, terminal and intercalary heterocysts, apoheterocytic akinetes. Cultured cyanobiont of <em>A. pinnata</em> is <em>Anabaena</em>. Cultured cyanobiont of <em>A. mexicana</em> is <em>Nostoc</em>.</td>
<td>Gebhardt &amp; Nierzwicki-Bauer (1991)</td>
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<tr>
<td>F, C (Nostoc sp, from <em>A. filiculoides,</em> T. azollae KOM BAI/1983)</td>
<td><em>A. pinnata subsp. pinnata,</em> <em>A. rubra, A. caroliniana</em></td>
<td>Fresh cyanobiont does not have akinetes, terminal heterocysts or mucilaginous envelope, hormogonia not determined, filaments are long, wavy, elliptical heterocysts. Fresh cyanobiont groups in one cluster outside other cyanobacteria.</td>
<td>Papaefthimiou et al. (2008a)</td>
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<tr>
<td>F, C</td>
<td><em>A. caroliniana, A. microphylla,</em> <em>A. mexicana, A. rubra,</em> <em>A. filiculoides, A. pinnata var. pinnata</em></td>
<td>Fresh cyanobionts have straight filaments. Cultured cyanobiont has coiled and straight filaments. Vegetative cells and heterocysts of fresh cyanobiont are larger than those of cultured cyanobiont. Heterocyst frequency of fresh cyanobiont is higher than that of cultured cyanobiont.</td>
<td>Sood et al. (2008b)</td>
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The different RFLP and STRR profiles (Table 1) of fresh and cultured cyanobionts point towards the foliar cavity harbouring a minor and a major cyanobiont (Franche & Cohen-Bazire, 1985; Nierzwicki-Bauer & Haselkorn, 1986; Meeks et al., 1988; Gebhardt & Nierzwicki-Bauer, 1991; Van Coppenolle et al., 1995; Zheng et al., 1999; Sood et al., 2008b). Thus, the major cyanobiont that inhabits the *Azolla* foliar cavity would be non-cultivable, and the minor cyanobiont is cultivable. Although the preceding data point to the existence of major/minor cyanobionts, the denaturing gradient gel electrophoresis profile of the 16S rRNA gene (Table 3) does not support the hypothesis of a major/ minor cyanobiont or more than one genus or species inside the *Azolla* foliar cavity (Papaefthimiou et al., 2008b).

point to the existence of more than one cyanobiont genus (*Anabaena, Nostoc* or *Trichormus*) or species inside the host cavity.

Other data from metabolite analysis (Table 2) contribute even more to the hypothesis of more than one genus or species inside the foliar cavity. Fresh isolates and cultured cyanobionts of several *Azolla* species show different antigen (Gates et al., 1980; Ladha & Watanabe, 1982) and protein (Sood et al., 2008b) profiles. The allozyme profile could be useful to distinguish the cyanobionts (fresh and cultured), but the only study with these biomarkers was made only with cultured cyanobionts (Zimmerman et al., 1989), so it is not possible to compare these results with the profile of fresh isolates.
In the face of such different data, which apparently support the hypothesis of major/minor cyanobionts, the question is, why those different results have been obtained throughout the decades, since the cultivable cyanobiont must theoretically be identical to the fresh one.

A very crucial point in all the research on the presence of more than one cyanobiont genus or species or a major/minor component is isolation of the cyanobiont. The disinfection of leaves is essential to eliminate any cyanobacteria that may contaminate them and thus to avoid the culture of a non-symbiotic cyanobiont(s). Disinfection of Azolla sporophytes can be done with sodium hypochloride or mercuric chloride and, after disinfection, cyanobionts can be isolated. This procedure was clearly described by Gebhardt & Nierzwicki-Bauer (1991), Pabby et al. (2003) and Sood et al. (2008a, b). Other researchers do not point out which disinfection method was used or whether disinfection was done prior to cyanobiont isolation and cultivation (Newton & Herman, 1979; Gates et al., 1980; Ladha & Watanabe, 1982; Franche & Cohen-Bazire, 1985; Meeks et al., 1988; Nierzwicki-Bauer & Haselkorn, 1986; Zimmerman et al., 1989), although the method of isolating cyanobionts described by Peters & Mayne (1974) was used. This method uses Azolla sporophytes disinfected before cultivation in closed flasks, and not before the isolation of cyanobionts, since it was assumed that the sporophytes are devoid of any contamination. Another key aspect is the difficulty in culturing the cyanobiont (Tang et al., 1990), even if researchers cultured the presumptive cyanobiont. Hindering the multiplication of the true cyanobiont is the genome erosion (gene loss and pseudogenes), affecting genes involved in replication, repair, glycolysis and nutrient uptake, as discovered by genome sequencing, (Ran et al., 2010). For that reason, the cultivated presumptive Azolla cyanobiont might be a cyanobacterium that contaminates the Azolla sporophytes and was not eliminated by disinfection and, for that reason, it is not a true isolate, as previous authors have also stated (Gates et al., 1980; Ladha & Watanabe, 1982; Franche & Cohen-Bazire, 1985; Gebhardt & Nierzwicki-Bauer, 1991). As a result, the presumptive cultivable cyanobiont is probably a resilient contaminant and, since genome sequencing points to genome erosion, the hypothesis of two or more genera/species or major/minor cyanobionts must be rejected, as supported by 16S rRNA gene sequencing (Papaefthimiou et al., 2008b).

**Phylogeny and classification of the Azolla cyanobiont: Anabaena, Nostoc, Trichormus or none of them?**

The classification and phylogeny of the Azolla cyanobiont is a long-term debate within the scientific community that considers this unique symbiosis using morphological (Table 4), metabolite (Table 2), genomic (Table 1) and gene and genome sequence (Table 3) data. Morphological characters are the main way to describe organisms, but, over the years, classification of the cyanobiont based on morphological characters has not been straightforward. The cyanobiont was first described as Nostoc azollae (Strasburger, 1873 cited in Carrapiço, 2010) but, some years later, was renamed as Anabaena azollae (Strasburger, 1884 cited in Carrapiço, 2010) and, since then, this was the name used to describe this cyanobiont. Fjerdingstad (1976) (cited in Lumpkin & Plunkett, 1980), using second-hand morphological data, assigned the cyanobiont as an ecotype of A. variabilis, naming it A. variabilis status azollae. However, later, the re-evaluation of morphological characters carried out by Komárek & Anagnostidis (1989) in order to identify cyano bacteria led to its renaming as Trichormus azollae (Strasburger) Komárek & Anagnostidis. Nevertheless, this name has rarely been used to refer to the Azolla cyanobiont.

The presence of hormogonia, the shape of terminal cells of the filament and the akinete developmental pattern were not observed. A survey made by Papaefthimiou et al. (2008a) pointed to the non-existence of hormogonia-like cells of the Azolla cyanobiont, but Zheng et al. (2009) stated the presence of hormogonia. The shape of the heterocysts is another disputed area. For some authors, those cells are elliptical (Papaefthimiou et al., 2008a), whereas for others they are round (Gebhardt & Nierzwicki-Bauer, 1991). As for the akinetes, Papaefthimiou et al. (2008a) did not observe akinetes in cyanobionts freshly isolated from Azolla species and, therefore, their developmental pattern is difficult to ascertain as apoheterocystic or para heterocystic – a very useful morphological character to describe the cyanobiont.

Despite all the uncertainties about morphological characters, the cluster analysis made by Papaefthimiou et al. (2008a) using four morphological characters (width and length of filaments, presence of akinete-like cells and terminal cells) groups the Azolla cyanobionts in a separate clade, outside the free-living and symbiotic genera Nostoc and Anabaena. Thus, this may support the conclusion that the Azolla cyanobiont could represent a new genus of the family Nostocaceae. Nevertheless, to establish their morphological uniqueness, observation of the morphology of the cyanobionts of all Azolla species is indispensable, especially akinete development – if it was apoheterocystic, it would support their relationship to Nostoc or Trichormus and, if it was para heterocystic, it belongs to the genus Anabaena.

The chemical profile of a family, genus or species can be useful in order to analyse chemical differences and to adopt a chemosystematic approach. The compounds used are mostly alkanes, sterols, hydrocarbons, alkaloids and phenols. Information about the cyanobiont chemical profile is scarce, but the volatile profile of A. filiculoides showed differences between the cultured and non-cultured fern. Some of the compounds, such as the alkanes, benzaldehyde, acetophenone or nor-terpenoids (β-ionone and trans-β-ionone-epoxide), could have originated from the cyanobiont (Pereira et al., 2009), since these also exist in free-living cyanobacteria of Nostoc (Caudales & Wells, 1992; Dembitsky et al., 1999) and Anabaena (Caudales & Wells, 1992). Therefore, volatile compounds could be useful for the
chemotaxonomy of *A. azollae*. Analysis of fatty acid profiles (Table 2) indicates that the *Azolla* cyanobionts were equally distant from free-living *Nostoc* and *Anabaena* (Caudales et al., 1992), which might indicate that the cyanobionts form a distinct taxonomic genus in the family Nostocaceae. Comparison of the *Azolla* cyanobiont proteome (Table 2) with those of other cyanobacteria leads to high protein profile similarities with the filamentous and heterocystic *Nostoc* and *Anabaena* (Ekman et al., 2008), both belonging to the family Nostocaceae. However, a phylogenomic analysis with 404 proteins of 58 cyanobacteria, including the fully sequenced *N. azollae*, clustered this cyanobiont within the group of *Cylindrospermopsis raciborskii/Raphidiopsis brokii* (Table 3) (Larsson et al., 2011), also belonging to the family Nostocaceae.

What about the molecular data? RFLP profiling (Table 1) showed that the cyanobionts were more closely related to *Nostoc* than to *Anabaena* (Plazinski et al., 1990). Analysis of the phycocyanin (PC-IGS) gene indicates that the *Azolla* cyanobiont was not closely related to the genera *Nostoc* or *Anabaena* (Baker et al., 2003). Therefore, since Komárek & Anagnostidis (1989) renamed *A. azollae* based on morphology as *T. azollae*, Baker et al. (2003) proposed the hypothesis that the *A. filiculoides* cyanobiont could belong to the genus *Trichormus*. Phylogenetic analysis of the 16S rRNA gene of *A. azollae* freshly isolated from *A. filiculoides* points to their relationship to *Anabaena* or *Nostoc* (Baker et al., 2003), but the sequence of the entire 16S rRNA gene indicates an association with the *Anabaena* clade (Svenning et al., 2005), supporting the classification as *A. azollae* and not as *N. azollae* or *T. azollae*. Another study with the 16S rRNA gene showed that *A. azollae* belongs to the *Anabaena* clade (Papaefthimiou et al. 2008b). However, in 2010, the whole genome sequence of the *A. filiculoides* cyanobiont was published (Ran et al., 2010), which was clustered in the *C. raciborskii/R. brokii* clade (Table 3) (Larsson et al., 2011). Because there is a lot of misinformation and different phylogenies are apparent, the best way of inferring phylogeny and relatedness is currently by sequencing (i) several genes or (ii) the whole genome of each cyanobiont that inhabits the foliar cavity of each *Azolla* species. Although comparison of gene sequences provides the relatedness with other cyanobacteria and the possible genus where the *Azolla* cyanobiont may fit, it does not provide a deeper insight into the taxonomic implications or even the possible taxonomic rank of the cyanobiont.

Another problem that exists with the phylogeny is the several studies with the presumptive cultivable *Azolla* cyanobiont assigned as *T. azollae* KOM BA/1983. Cultivation of the cyanobiont is problematic, as discovered after the analysis of the genome sequenced and discussed above. Therefore, the presumptive cultivable cyanobiont is probably not their true cyanobiont. However, in the NCBI database, there are sequences for the 16S rRNA gene, *rbcLX* and *rpoB* belonging to *T. azollae*, but the phylogenetic relationship is attributed to *Nostoc* (Rajaniemi et al., 2005; Papaefthimiou et al. 2008a).

Besides the still unresolved problem of the cyanobiont phylogeny, another dilemma is their taxonomic rank according to approved international codes. Generally, naming of cyanobacteria follows the *International Botanical Code of Nomenclature*, although it has been proposed that they could be classified according to the *International Code of Nomenclature of Prokaryotes*. Regardless of the code used to classify a cyanobacterium, the name must be published and validated (Oren, 2004; 2011). Komárek & Anagnostidis (1989) updated the classification criteria for the family Nostocaceae and classified the *Azolla* cyanobiont as *Trichormus azollae* (Strasburger) Komárek & Anagnostidis. As far as we know, this is the only valid name for this cyanobiont. All previous names attributed to the cyanobiont by Strasburger (1873, 1884) or Fjerdingstad (1976) are not valid, since they were not validated. Nonetheless, it is not known whether the cyanobiont used by Komárek & Anagnostidis (1989) was freshly isolated from the foliar cavity or a presumptive cultivable cyanobiont which, as stated above, is not reliable. However, nowadays, morphology alone is not used for taxonomic purposes; a molecular approach should be included, normally through the sequence of the 16S rRNA gene (Komárek, 2005, 2006, 2010b). Hence, for phylogeny and classification, it is not possible to use a cultivable cyanobiont; only freshly isolated cyanobionts are acceptable. With fresh isolates, morphological characters must be documented (especially the presence of hormogonia and the akinete development pattern, among other characters) and compared with the 12 genera of the family Nostocaceae (Komárk, 2010a) and with the phylogeny of, at least, the 16S rRNA gene in order to name and validate them. Instead of using the 16S rRNA gene, it is possible to sequence the whole genome of each cyanobiont of each *Azolla* species to ascertain the presence of ecotypes, as stated by Papaefthimiou et al. (2008b). Since all *A. azollae* inhabit the *Azolla* foliar cavity under the same conditions, it can be argued that they are ecotypes, but they can be ecotypes of *A. variabilis*, as stated by Fjerdingstad (1976). In addition, if morphological variation is not observable and not related to the *Azolla* species, since there is gene variation, they can be classified as genotypes. However, more investigation is again needed, especially in the morphological observation of the cyanobionts of each *Azolla* species using the available morphological characters (colonies, vegetative filaments, hormogonia, mucilaginous sheaths, akinete development pattern and others) that, in combination with gene sequence analysis, could give an improved taxonomic analysis, as in the case of soil strains of *Nostoc* (Hrouzek et al., 2005).

**Final comments and future research**

The morphological, metabolite, genomic and gene and genome sequence differences between freshly isolated and cultured cyanobionts led researchers to conclude that more than one genus or species or a major/minor cyanobiont inhabit the *Azolla* foliar cavity. Due to the difficulty in culturing the *Azolla* cyanobiont explained by analysis of the
whole genome, the presumptive cultivable \textit{A. azollae} was probably a resilient cyanobacterium that contaminates the sporophyte and is not removed by disinfection. Therefore, several ecotypes exist inside the foliar cavity.

Phylogenetic analysis using only one type of data can lead to misinterpretation both in the evolutionary conclusions and in the relationship between the cyanobionts isolated from \textit{Azolla}. The data (morphological, metabolite, genomic and gene and genome sequence) point to the relatedness of the \textit{Azolla} cyanobionts to \textit{Anabaena}, \textit{Nostoc} or \textit{Trichormus}, but the sequenced whole genome of the cyanobiont isolated from \textit{A. filiculoides} indicates its relatedness to \textit{C. raciborskii}. \textit{R. brokii}. The data also point to co-evolution between the cyanobiont and the pteridophyte.

The questions of the existence of several genera or species and/or major/minor cyanobionts and co-evolution have been addressed, but could be improved by sequencing several genes or sequencing the cyanobiont genome from several \textit{Azolla} species and the integration of other data such as morphological characters in a polyphasic approach. This approach was made only by Sood \textit{et al.} (2008a), using STRR and protein profiles. The morphological characters that should be applied to \textit{Azolla} cyanobionts should be similar to those used to identify cyanobacteria in a dichotomous key, such as the colonies, vegetative filaments, hormogonia, mucilaginous sheaths and akinete development pattern, allowing their match with an already existing species or assignment to a novel species. In addition, there is still the question of the validation of the cyanobiont name. The classification of the cyanobiont should follow the rules of international nomenclatural codes (botanical or bacteriological) and should be made by integrating morphological characters and metabolite, genomic and gene and genome sequence profiles, making a polyphasic approach. The attributed name should be validated according to the requirements of accepted international committees.

Acknowledgements

European Social Funding (FSE) under the Human Potential Operational Program (POPH) of the National Strategic Reference Board (QREN) supports the fellowship SRFH/BPD/44459/2008 to A.L.P. The figure was prepared by A.L.P.

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