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Evolutionary mode, tempo, and phylogenetic association of continuous morphological traits in the aquatic moss genus *Amblystegium*

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Abstract

Evolutionary significance of morphological characters that have traditionally been used for species delineation in the aquatic moss genus *Amblystegium* was tested by partitioning the environmentally and genetically induced morphological variation and focusing on morphological evolution using comparative methods. Cultivation experiments under controlled condition showed that most of the morphological variation in nature resulted from plasticity. Information regarding genetically fixed morphological variation and genetic similarity derived from polymorphic inter-simple sequence repeat markers was combined into an explicit model of morphological evolution. Maximum likelihood estimates of the model parameters indicated that evolution of most characters tended to accelerate in the most recent taxa and was often independent from the phylogeny. Constraining the different characters to be independent from each other most often produced a less likely result than when the characters were free to evolve in a correlated fashion. Thus, the morphological characters that have traditionally been used to circumscribe different *Amblystegium* species lack the independence, diagnostic value for specific lineages, and stability that would be required for distinguishing different species.

Introduction

In systematic biology, it is of critical interest to partition the causes of morphological variation into that due to the environment and that due to genotype in order to search for stable characters (Shaw, 1986). Defining character stability and diagnostic value, however, obviously requires a methodological approach that allows for testing the mode, tempo, and phylogenetic association of morphological evolution. With the increasing availability of phylogenetic information, such an approach has been statistically formalized so that it is now possible to infer historical patterns of evolution from the phylogeny and morphology of the current taxa by using an explicit model of evolution (Martins & Hansen, 1997; Pagel, 1997, 1999; Schluter *et al.*, 1997; Freckleton *et al.*, 2002).

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In this paper, we apply such a model to test the taxonomic significance of morphological characters that have been used for species delineation in the moss genus, *Amblystegium*. In mosses, classification has traditionally been based on a few characters in a context of complete lack of information regarding morphological evolution. As a consequence, the taxonomic relevance of many of these characters has been increasingly challenged by recent data from molecular phylogenies (Buck & Goffinet, 2000). The problem is even more acute in many pleurocarpous aquatic mosses that share almost identical sporophyte features of a possibly terrestrial ancestor (Vitt, 1981), making it necessary to define species boundaries based on gametophytic features that are especially prone to plasticity and convergence (Vitt & Glime, 1984; Hedenäs, 1996).

In the genus, *Amblystegium* in particular, species form a series of intergrades, differing only in continuous characters that defy precise definition such as size and texture of plants, strength of the costa, and length to width ratio

of cells (Crum & Anderson, 1981). Depending on the weight given to the different characters, a number of taxonomic positions, ranging from recognition of several genera, including *Amblystegium s.s.*, *Hygroamblystegium*, *Leptodictyum* and *Orthotheciella* to acceptation of a single genus, *Amblystegium s.l.*, with conflicting species circumscription (see Vanderpoorten *et al.*, 2002a for review), have been adopted. In this context, phylogenetic analysis of DNA sequences helped in resolving a monophyletic *Amblystegium* genus of revised circumscription (Vanderpoorten *et al.*, 2002a). Within the genus, the low level of sequence divergence of several noncoding chloroplast and nuclear regions however seriously limited deeper phylogenetic investigation.

A first goal of this study was to assess whether morphological variation in nature is genetically fixed or reflects environmental variation. Although it has long been acknowledged that a large part of morphological variation in many mosses is due to plasticity (see Hedenäs, 1996 for review), few experimental studies have been conducted to assess the influence of environmental conditions on phenotypic expression of morphological traits (Stenoien *et al.*, 1997; Van der Hoeven *et al.*, 1998; Sastad *et al.*, 1999). In aquatic mosses in particular, information is very limited and restricted to marsh taxa (Zastrow, 1934; Lodge, 1960; Hedenäs, 1996).

The second goal of the study was to focus on morphological variation that is genetically fixed among *Amblystegium* species and study morphological evolution. Morphological evolution was investigated in the context of a tree of genetic similarity derived from polymorphic inter-simple sequence repeats (ISSR) to circumvent the low level of gene sequence divergence of noncoding cpDNA regions, internal transcribed spacers of 18S-26S ribosomal DNA (ITS), and issues of paralogy with other nuclear genes (Vanderpoorten *et al.*, 2004). ISSRs may reveal a high level of polymorphism as primers anneal to simple sequence repeats that are abundant throughout the eukaryotic genome and evolve rapidly (Zietkiewicz *et al.*, 1994; Li & Ge, 2001). In addition, ISSRs may produce more reliable and reproducible bands than other fingerprintings techniques such as random amplified polymorphic DNA (RAPDs) because of the higher annealing temperature and longer primer sequences (Qian *et al.*, 2001). ISSR markers have therefore proved to be useful in population genetic studies, especially in detecting clonal diversity (e.g. Esselman *et al.*, 1999), and in resolving taxonomic relationships at or below the species level, when available nucleotide sequences do not exhibit the appropriate level of polymorphism (e.g. Mort *et al.*, 2003; Vanderpoorten *et al.*, 2003a; Werner *et al.*, 2003).

Combining information on genetic similarity and fixed morphological variation then allowed us to test the stability, phylogenetic association, and independence of morphological characters that have been used for species delineation in the genus, employing an explicit model of morphological evolution.

Methods

Population sampling, culture experiments and morphological measurements

Fifteen populations of five putative species from different environments were sampled in order to include a wide range of morphologies of the different species and their intermediates (Table 1). Each sample consisted in 10–50 shoots collected from populations occurring on 1 to several m^2. An attempt to produce completely new shoots from a protonemal stage following the protocol of Shaw (1986) failed, and experiments consequently started from gametophytes. Shoot fragments were grown 3 months in a phytotron at 18 °C in a saturated atmosphere on permanently wet neutral sand with a 16 h photoperiod at 130 μmol m^{-2} s^{-1}.

Phenotypic traits of plants can change dramatically over the course of their development (Coleman *et al.*, 1994). Although it cannot be excluded that the different *Amblystegium* species have, even under identical environmental conditions, different growth rates, harvesting was performed after a fixed period of 3 months in order to have comparable data. At this stage, new branched and mature shoots, 2–3 cm long, were obtained.

Nineteen morphological characters that have traditionally been used for species delineation in the genus (e.g. Crum & Anderson, 1981; Frey *et al.*, 1995; Nebel & Philippi, 2000) were measured before and after cultivation (Table 2). Many other characters, including colour of the basal cell walls, colour and aspect of the rhizoid surface, length and shape of the axillary hairs, and stem anatomy, were identical among the studied populations and thus not included in the analyses.

Measurements were performed on five shoots and five stem leaves per shoot selected at random for each of the 15 populations before and after cultivation.

Statistical analyses of morphological variation

Statistical analyses were conducted to examine how morphological variation was partitioned within and among populations before and after cultivation. The series of 25 measurements for almost each population were normally distributed for all but the bi- or plurimodal characters according to the Kolmogorov–Smirnov test at the 0.05 signification level but exhibited significantly different variances according to the Levene test at the 0.05 signification level, even after log-transformation of the variables. Differences in variance among populations were largely due to differences in variance before and after cultivation, as shown for each character by an *F*-test. However, the balanced ANOVA is robust to heteroscedacity (Zar, 1999) and two-way ANOVA was performed to search for significant differences in morphology depending on the factors 'population', 'treatment', and the interaction between them. Factor

Table 1 Collection details.

Putative species	Label	Locality	Habitat
Amblystegium serpens	N	Belgium, Prov. Namur (Namur)	Weir on the Meuse river, with *Cratoneuron filicinum, Cinclidotus riparius, Cinclidotus danubicus, Fissidens crassipes*
Amblystegium serpens	B	Belgium, Brussels (Uccle)	Base of roadside tree
Amblystegium serpens	A	Belgium, Prov. Namur (Anseremme)	Weir on the Meuse river, with *Amblystegium tenax, Cratoneuron filicinum, Cinclidotus riparius, Cinclidotus danubicus, Fontionalis antipyretica, Fissidens crassipes, Hygrohypnum luridum*
Amblystegium humile	H	Belgium, Brussels (Woluwe)	Alkaline fen, with *Drepanocladus aduncus, Plagiomnium elatum, Calliergonella cuspidata*
Intermediate *Amblystegium humile-tenax*	N	Belgium, Prov. Namur (Anseremme)	Weir on the Meuse river, with *Amblystegium serpens, Cratoneuron filicinum, Cinclidotus riparius, Cinclidotus danubicus, Fissidens crassipes, Fontinalis antipyretica, Hygrohypnum luridum*
Amblystegium fluviatile	L	Belgium, Prov. Liège (Malmédy)	Rocks in the Warche river, with *Hygrohypnum eugyrium, Brachythecium plumosum, Brachythecium rivulare*, *Racomitrium aciculare*
Amblystegium fluviatile	B	Germany, Baden-Würrtemberg	Rocks in the Ravennaschlucht, with *Brachythecium plumosum, Hygrohypnum duriusculum, Scapania undulata, Marsupella emarginata*
Amblystegium tenax	M	Belgium, Prov. Namur (Moha)	Concrete embankment along the Mehaigne river
Amblystegium tenax	N	Belgium, Prov. Namur (Namur)	Weir on the Sambre river, with *Fissidens crassipes*
Intermediate *Amblystegium varium-orthocladon*	G1	USA, Connecticut	On rocks in swampy area along brook
Intermediate *Amblystegium varium-orthocladon*	S	USA, New York	On rocks in moist, shaded gorge along small stream
Amblystegium varium	T2	Belgium, Prov. Namur (Namur)	Weir on the Meuse river, with *Cratoneuron filicinum, Cinclidotus riparius, Cinclidotus danubicus, Fissidens crassipes*
Intermediate *Amblystegium varium-orthocladon*	G2	USA, Connecticut	Wet sandstone outcrops dominated by *Gymnostomum, Atrichum, Conocephalum* and *Pellia*
Amblystegium varium	SR	Belgium, Prov. Liège (Soiron)	On rocks in a spring, with *Platyhypnidium riparioides*
Amblystegium varium	T1	Belgium, Namur (Namur)	Weir on the Meuse river, with *Cratoneuron filicinum, Cinclidotus riparius, Cinclidotus danubicus, Fissidens crassipes*

Vouchers are kept at Duke.

Table 2 Morphological features measured on the investigated specimens.

Leaf shape and size	leaf length (L), width (W, μm), and length to width ratio (R) Leaf apex morphology (AP) (acute/obtuse)* Serrulation of the lamina (S): none (0), slight (1; teeth < 1 μm); sharp (teeth > 1 μm) Percentage leaf length where maximum width reached (MW) Length of the acumen, in percentage of leaf length† (AcL)
Cell dimensions	Laminal (LL, LW, LR), basal (BL, BW, BR), and angular (AL, AW, AR) cell length, width, and length to width ratio
Costa dimensions	Costa width at base (CW) (μm) Costa length, in percentage of leaf length (CL)
Costa shape	Costa straight (0) or hooked at 3/4 of leaf length (CS) (1)

*'Acute' was applied to apices with a single terminal cell whereas 'obtuse' was applied when more than one terminal cells or when the subapical cell reached at least 50% of the length of the apical cell.
†Acumen was defined as the apical leaf portion <10 cells wide.

interaction was consistently significant at the 0.001 level. Hence, the two-way ANOVA was split into two one-way ANOVA's searching for significant differences in morphology depending on the factor 'population' before and after cultivation, respectively.

In order to assess whether the examined populations differed in morphology before and after cultivation in a multivariate context, a principal components analysis (PCA) using the average of the 25 measurements of each morphological feature for each of the 15 populations before and after cultivation was performed. Because of the heterogeneous nature of the variables, the PCA was performed on standardized variables $x_i^0 = (x_i - \bar{x})/\sigma$.

Molecular protocols

Five shoots per population were screened using ISSR markers to search for intra- and interpopulational molecular variation. ISSRs patterns were obtained using the three primers and according to polymerase chain reaction (PCR) and gel conditions described in Vanderpoorten *et al.* (2003a). Polymorphic bands were scored for presence/absence and a phenetic tree of genetic similarity was obtained by neighbour-joining.

Support for the branches of the topology was assessed by NJ bootstrapping using 10 000 random replicates.

Statistical analyses of morphological evolution

The NJ tree was used as a rough estimate of the topology and of genetic similarity among lineages to investigate global patterns of morphological evolution. The generalized least-square models implemented by Continuous (Pagel, 1997) were used to investigate the tempo, mode, and phylogenetic component of morphological evolution in the context of the NJ tree allowing for a control of phylogenetic nonindependence in the data. Tempo, mode, and phylogenetic association of trait evolution were assessed through three scaling parameters (Freckleton *et al.*, 2002; Pagel, 2002). The κ parameter tests for punctuational ($\kappa = 0$) vs. gradual mode of trait evolution; the δ parameter detects whether the rate of trait evolution has accelerated ($\delta > 1$) or slowed down from the root to the tips; and the λ parameter reveals whether the tree correctly predicts the character states observed in present taxa (in which case λ takes the value of 1). Each parameter was systematically fit by maximum likelihood and fixed in the calculations. A nested likelihood ratio test (LR) was performed to determine whether the most likely values were significantly higher than 0 and 1. The LR statistics, defined by $LR = -2\ln(H_0/H_1)$, where H_0 represents the simpler model (the parameter is fixed to a constant value of 0 or 1) and H_1 represents the model where the parameter is free to take its most likely value, is asymptotic to a chi-square distribution with 1 d.f. A LR value greater than the chi-square variable indicates that the null hypothesis can be rejected, i.e. that the model allowing the parameter to take its most likely value describes the data significantly better than the model where the parameter equals the fixed constant. LR tests were subsequently performed to determine if a directional random-walk model (B) described the data significantly better than a constant-variance random walk model (A). Model A includes a single parameter α, providing an estimate of the ancestral character value at the root, whereas model B includes α as well as a parameter β measuring the regression of trait values across species against total path length from the root of the tree to the tips.

Likelihood ratio tests were also used to measure the correlation among characters by comparing the fit of a model where character covariance was set to 0 to that of a model where both characters were allowed to co-vary. To try to improve the linearity of the relationship among characters, correlations were calculated among both raw and log-transformed values.

Results

The two-way ANOVA of morphological characters depending on the factors population and treatment consistently revealed significant interactions between the two factors, suggesting that the populations did not exhibit the same morphological trend before and after the experiment. The variance of all but three characters was substantially reduced after cultivation (Table 3). Diminution of variance was either associated with a general trend of the different characters to either decrease (e.g. leaf size characters, proportion of obtuse leaf apices) or increase (e.g. serrulation of the

Table 3 Mean and variance of the 19 investigated morphological features before (0) and after (1) cultivation.

Character	σ_0^2	σ_1^2	*P*-value	m_0	M_1	*P*-value
Leaf length	98 701	36 857	<0.001	993	749	<0.001
Leaf width	17 420	6537	<0.001	425	306	<0.001
Leaf length to width ratio	55.7	50.7	ns	43.4	41.0	ns
Leaf apex morphology	0.16	0.01	<0.001	0.20	0.01	<0.001
Serrulation of the lamina	0.22	0.22	ns	0.45	0.87	<0.001
Maximum width (% leaf length)	31	22.8	<0.01	22.7	17.0	<0.001
Length of the acumen	114.8	116.1	ns	23.7	33.9	<0.001
Laminal cells length	77.4	58.6	<0.01	27	28	ns
Laminal cells width	2.4	1.6	<0.001	9	8	<0.05
Laminal cells ratio	139.1	79.7	<0.001	35.5	31.0	ns
Basal cells length	71.5	24.0	<0.001	30	24	<0.001
Basal cells width	12.6	5.4	<0.001	15	13	<.001
Basal cells ratio	197.3	110.9	<0.001	53.2	56.1	ns
Alar cells length	30.7	16.9	<0.001	20	18	ns
Alar cells width	4.3	2.2	<0.001	12	10	<0.001
Alar cells ratio	359.1	296.0	<0.05	71.7	68.0	ns
Costa width	420.7	172.7	<0.001	55	37	<0.001
Costa length	307.0	513.8	<0.001	76.0	65.9	ns
Costa shape	0.24	0.08	<0.001	61.0	10.0	<0.001

The *P*-value represents the probability that the character mean or variance is identical before and after cultivation.

lamina) after cultivation (Table 3). However, one-way ANOVA of character variation after cultivation still revealed significant differences at the 0.001 signification level among populations for all the investigated characters.

In a multivariate context, the first two axes of a PCA performed on the standardized average morphological features of each of the 15 investigated populations before and after cultivation accounted for 40.5 and 26.5% of the total variance, respectively. PCA1 was mostly loaded with leaf length, length of the basal cells and width of the costa and PCA2 with the dimensions of the laminal cells (Fig. 1b). In the first PCA plane, populations before cultivation tended to segregate more or less clearly according to traditional taxonomic concepts whereas populations after cultivation tended to form a morphological continuum with the exception of the populations of *A. serpens* that had the most negative values along the first axis (Fig. 1a).

Examination of morphological variation as well as previous molecular evidence from DNA sequence data (Vanderpoorten *et al.*, 2003b) resulted in an unambiguous molecular and morphological circumscription of *A. serpens*. This, and the fact that ISSR markers displayed little homology among *A. serpens* and the other species, led us to focus on the four remaining aquatic species of the genus.

The three ISSR primers provided a total of 45 scorable polymorphic fragments. All five individuals from the same population exhibited identical band patterns. At the population level, diagnostic markers were found for all but two populations, *varium* nos T1 and T2, which came from two neighbour localities. The tree of genetic similarity among populations obtained by neighbour-joining is presented in Fig. 2.

Table 4 summarizes the mode, tempo, and phylogenetic association of morphological evolution along the branches of the NJ tree. All investigated characters but

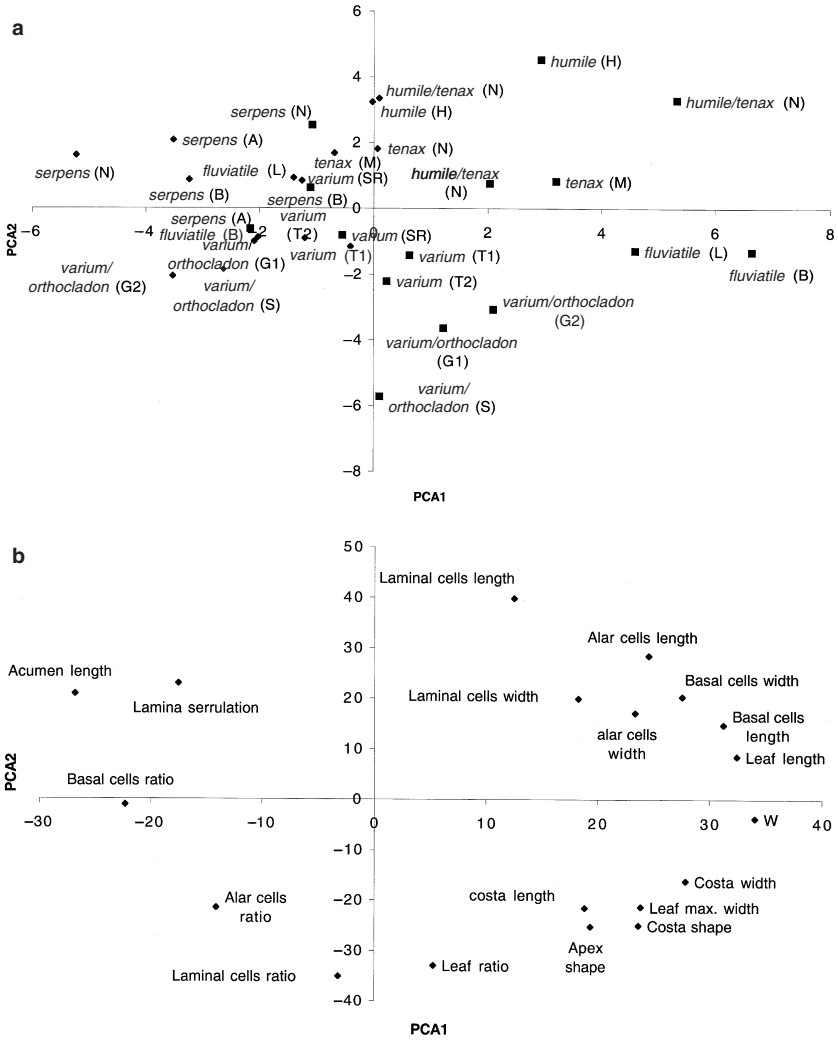

Fig. 1 (a) Scores of the 15 investigated populations before (squares) and after (diamonds) cultivation in the first PCA plane. See Table 1 for the labels. (b) Correlation of the 19 measured morphological variables on the first two axes of the principal components analysis (PCA) on the 15 investigated populations before and after cultivation.

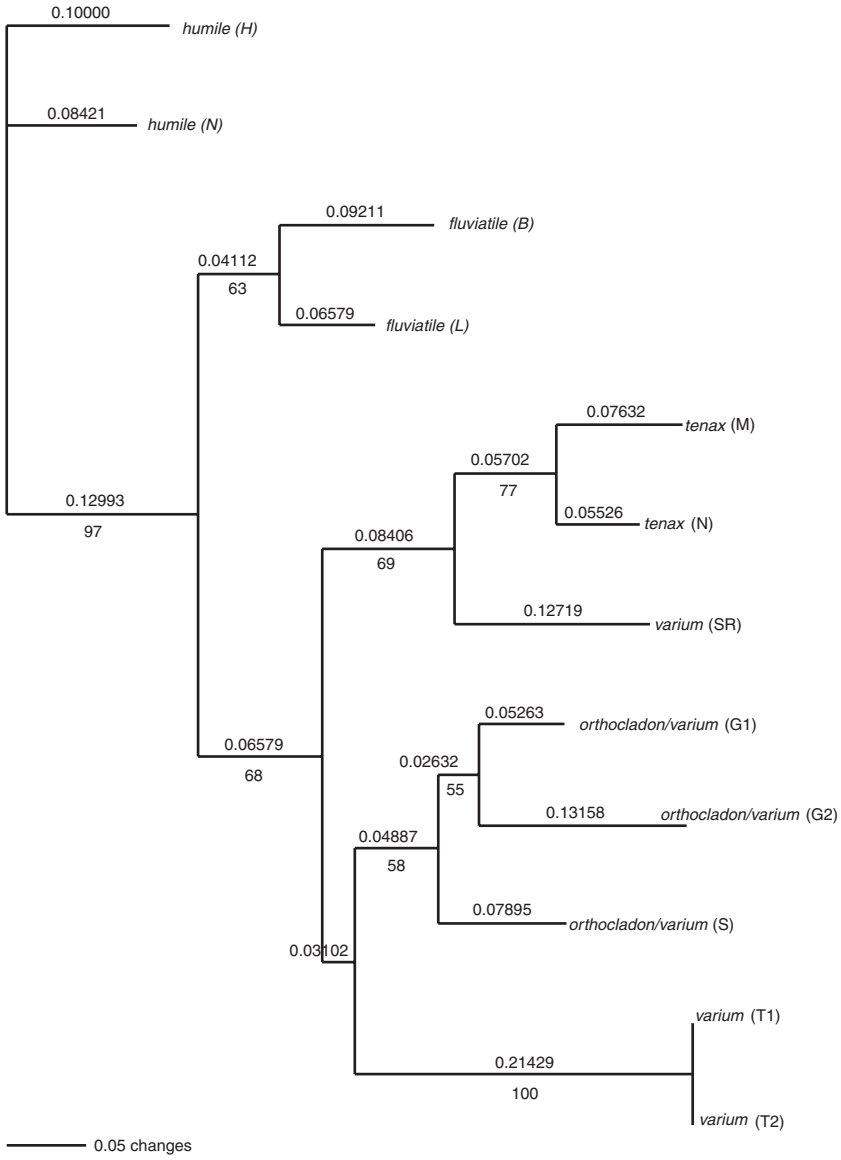

Fig. 2 Neighbour-joining tree resulting from the analysis of the 45 scorable polymorphic bands obtained by the three inter-simple sequence repeats (ISSR) primers. Numbers below the branches are the bootstrap values >50% and those above are the branch lengths used in the models of morphological evolution. See Table 1 for the labels.

the shape of the apex evolved according to a constant-variance random walk model. Hence, these characters did not have any tendency to vary with genetic divergence. The δ parameter was mostly significantly higher than 1, suggesting an acceleration of evolution towards the tips. Six characters, namely the width and width to length ratio of the leaves, the length and the width to length ratio of the basal cells, the width of the angular cells and the shape of the costa, had a λ parameter that did not significantly differ from 0, indicating that these characters evolved independently from the phylogeny.

The matrix of character inter-correlation is presented in Table 5. A number of morphological features were correlated to each other, e.g. leaf length with leaf width and length of laminal and basal cells; leaf width with basal and alar cells width and costal width; costa width with costa shape and length. Only two of the investigated features, namely the shape of the apex and the serrulation of leaf border, seemed independent from the others.

Discussion

Morphological variation in *Amblystegium* largely resulted from plasticity. Under controlled conditions, the total variance of almost all the investigated characters substantially decreased. A number of supposedly diagnostic features indeed converged under identical growth conditions. For example, obtuse leaf acumen, characteristic

Table 4 Random walk models of morphological evolution and their associated parameters.

Character	Evolution model	κ	δ	λ
Leaf length	A	1	1	0.98
Leaf width	A	1	3	0
Leaf length to width ratio	A	1	3	0
Leaf apex morphology	B	1	1	0.99
Serrulation of the lamina	A	1	3	0.99
Maximum width (% leaf length)	A	1	3	0.99
Length of the acumen	A	1	1	0.98
Laminal cells length	A	1	3	0.99
Laminal cells width	A	1	3	0.99
Laminal cells ratio	A	1	3	0.99
Basal cells length	A	1	3	0
Basal cells width	A	1	3	0.99
Basal cells ratio	A	1	3	0
Alar cells length	A	1	3	0.99
Alar cells width	A	1	3	0
Alar cells ratio	A	1	3	0.99
Costa width	A	1	3	0.98
Costa length	A	1	3	0.99
Costa shape	A	1	3	0

Parameter values superior than 1 represent maximum likelihood (ML) estimates that are significantly different from 0 to 1 at the 0.05 level. A and B are the standard constant-variance and directional random-walk models, respectively. κ, δ and λ are the scaling parameters for punctuational vs. gradual evolution, accelerated vs. decelerated evolution, and phylogenetic association of morphological evolution.

of *A. fluviatile* and, to a lesser extent, of certain expressions of *A. tenax* (*orthocladon*; see e.g. Crum & Anderson, 1981), became mostly acute under controlled conditions. Similarly, serrulation of the leaf border, sometimes advocated as a means for species distinction (e.g. between *A. tenax* and *A. varium*; Hedenäs, 1992), may be significantly reduced in nature, possibly because of erosion, and was revealed in almost all populations in culture. Curvature of the costa, often considered as a diagnostic feature of *A. varium* (e.g. Nebel & Philippi, 2000), tended to vanish under controlled conditions. All leaf size characters significantly decreased after cultivation. A decrease in leaf size has been commonly observed in a variety of other plant taxa exposed to a high light intensity (e.g. Cornelissen, 1992; Lall *et al.*, 1997; Balaguer *et al.*, 2001; Collado-Vides, 2002). In the present study, reduction in leaf size as a result of light intensity, however, did not affect proportions, as shown by the nonsignificance in changes of the different ratios before and after cultivation.

Despite a substantial reduction in variance for almost all characters, significant differences among populations were still found after cultivation and thus seemed to have a genetic basis. However, the different species were indistinguishable on the basis of the studied characters in a multivariate context except for one species, *A. serpens*, which differed from the others by a series of genetically fixed continuous characters mostly related to plant size. Unambiguous morphological characterization of *A. serpens*, combined with previous molecular circumscription of the species from several gene sequence data (Vanderpoorten *et al.*, 2003b), led us to focus on the remaining aquatic taxa of the genus.

An assessment of genetic variability to test the hypothesis that reduction in fixed morphological variation parallels a lack of genetic variation showed that almost all

Table 5 Character correlation matrix.

	L	W	R	AP	S	MW	AcL	LL	LW	LR	BL	BW	BR	AL	AW	AR	CW	CL	CS
L	1																		
W	82*	1																	
R	74**	–	1																
AP	–	–	–	1															
S	–	–	–	–	1														
MW	–	–	–	–	–	1													
AcL	–	–	–	–	–	–	1												
LL	99***	55*	53*	–	–	59*	–	1											
LW	–	–	–	–	–	–	–	–	1										
LR	69**	–	-	–	–	79***	–	89***	–	1									
BL	79***	–	68***	–	–	–	–	62*	59*	–	1								
BW	66*	67**	–	–	–	-	–	59*	–	–	78**	1							
BR	–	–	62*	–	–	–	–	–	–	–	66*	–	1						
AL	–	–	–	–	–	–	–	65**	60*	–	62*	77**	–	1					
AW	–	67**	–	–	–	–	–	–	–	–	76**	77**	–	–	1				
AR	–	–	–	–	–	–	–	60*	–	–	–	–	–	83***	–	1			
CW	–	75**	–	–	–	–	60*	–	–	–	–	–	–	–	–	–	1		
CL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	56*	1	
CS	–	–	–	–	–	73*	–	–	–	–	–	–	–	–	–	–	52*	–	1

–, *, **, and *** represents *P*-values >0.05, <0.05, 0.01, and 0.001, respectively.
See Table 2 for character abbreviations.

the studied populations differed genetically from each other. Within the populations, although ISSRs have been successfully used in other plant taxa for studying intra-populational patterns of diversity (e.g. Qian *et al.*, 2001), the five individuals screened in each population exhibited identical fingerprintings. This either suggests that the marker did not exhibit the appropriate level of polymorphism for the studied taxa or that the populations of *Amblystegium*, which were, as has commonly been observed in these taxa (e.g. Crum & Anderson, 1981), sterile, were clonal. More variable genetic markers such as microsatellites, which have recently been designed in mosses (Van der Velde *et al.*, 2001), may ultimately help in resolving this issue.

Using the tree of genetic similarity among populations to study morphological evolution indicated that the morphological features that have been used for species delineation may not be appropriate for taxonomy. First, six of the 19 investigated characters exhibited a λ parameter that was not significantly different from 0, suggesting that these characters evolved independently from the phylogeny. Character states such as the curvature of the costa or leaf width, which were previously used for species circumscription, thus could not be predicted from the phylogeny and may consequently have no taxonomic relevance in *Amblystegium*. The lack of relationships between phylogeny and morphology has sometimes been interpreted in terms of difference in molecular and morphological evolutionary rates. DNA sequences are generally thought to evolve in a regular fashion whereas morphological characters are thought to evolve following a different pattern, with long periods of stasis interspersed with periods of rapid change (Newton *et al.*, 2000). In *Amblystegium*, however, the significant difference of the κ parameter from 0, rejecting the hypothesis of punctuational evolution, suggested that morphological evolution in *Amblystegium* has been gradual.

Secondly, maximum likelihood estimates of the model parameters suggested that evolution of most characters tended to accelerate towards the tips. The results thus favoured a scenario where morphology of current *Amblystegium* taxa has evolved at a rapid pace as opposed to a scenario where rapid morphological evolution would have occurred during the adaptive radiation of ancestor species followed by a slow-down of evolutionary rates in the most recent taxa. As a consequence, character states are likely to change rapidly in the current species and thus lack the stability that would be required for taxon circumscription. The ambiguity in ancestral character state reconstruction within the Amblystegiaceae was also interpreted as an indication of relatively fast rates of morphological evolution within the family to such an extent that the family itself, as well as many intrafamilial taxa including the genus *Amblystegium*, could not be circumscribed by any morphological synapomorphy (Vanderpoorten *et al.*, 2002b).

Thirdly, the significant correlations among characters, many of which were mostly related to plant size, suggests the existence of evolutionary constraints so that the series of characters that have been used for species delineation are not independent. For example, leaf length and length of the laminal cells were highly correlated and thus seem linked in *Amblystegium*. Such an association in character traits may have differently evolved in the different moss lineages as a number of short-celled genera (e.g. *Anomodon, Thuidium*) can include species with variously long leaves. Hedenäs (1996) also found high correlations among continuous characters that have been used to circumscribe species in another complex of sibling species within the Amblystegiaceae based on measurements made on a clonal population exposed to contrasting growth conditions. In both cases, it seems that the characters that were used to circumscribe the different species were all more or less related to plant size.

In conclusion, most of the morphological variation encountered in aquatic *Amblystegium* species results from plasticity. Within the remaining morphological variation that is genetically fixed, accelerated evolution rates, coupled with little phylogenetic influence on the evolution of a number of characters, suggests that the same transition of character states are likely to occur along any branch of the phylogeny. Constraining the different characters to be independent from each other most often produced a less likely result than when the characters were free to evolve in a correlated fashion. Thus, the morphological characters that have traditionally been used to circumscribe different *Amblystegium* species lack the independence, the diagnostic value for specific lineages, and the stability that would be required for distinguishing different species. The genetic relationships found in the present study may, however, constitute a basis for future morphological investigation that may ultimately reveal diagnostic characters for the different genetic entities.

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