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Isoenzymatic variability in populations of the genus *plagiochila* in Portugal and Madeira

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ABSTRACT

The *Plagiochila* genus (Dum.) Dum. (*Hepaticae*) in Portugal and Madeira comprises a big amount (60%) of the total of species in Europe. Until now, the knowledge on biodiversity of Portuguese *Plagiochila* was been based on morphological treatment. This study is the first approach to study interspecific as well as intraspecific variability in *Plagiochila* from Portugal and Madeira using isoenzyme analysis.

Twenty populations of *Plagiochila* (*P. bifaria, P. porelloides, P. puntacta, P retrorsa, P. spinulosa, P. stricta*) from several sites in Portugal and Madeira Laurissilva forest were investigated.

The results were obtained by poliacrilamide gel electrophoresis (PAGE). Extracts were prepared from young upper parts of shoots. Four enzyme systems were analyzed: acid phosphatase EC 3.1.3.2 (ACP), peroxidase EC 1.11.1.7 (PER), Glutamate oxaloacetate transaminase EC 2.6.1.1 (GOT) and malic enzyme EC 1.1.1.40 (ME).

Isoenzyme polymorphism was found for all enzyme systems studied,though variability was dower in GOT. The comparative isoenzymes banding patterns of ACP, PER, and Me show interspecific variations and made it possible to identify the species studied. Fairly intraspecific variability was detected, so a difference between populations could not be distinguished.

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RESUMO

Variabilidade isoenzimática nas populações do género *Plagiochila* em Portugal e na Madeira

Em Portugal e na Madeira estão presentes cerca de 60% do total de espécies do género *Plagiochila* (Dum.) Dum. (*Hepaticae*) existentes na Europa. Até ao presente, o conhecimento da biodiversidade deste género em Portugal, baseou-se apenas em estudos morfológicos. Este trabalho representa, assim a primeira abordagem, para o estudo da variabilidade interespecífica e intraespecífica das espécies de *Plagiochila* existentes em Portugal e na Madeira, utilizando a análise isoenzimática.

Foram investigadas vinte populações de *Plagiochila* (*P. bifaria, P. porelloides, P. puntacta, P. retrorsa, P.* aff. *retrorsa, P. spinulosa, P. stricta*) de vários locais de Portugal e da floresta de Laurissilva da Madeira.

Os extractos foram preparados a partir das extremidades superiores mais jovens de cada indivíduo. Os quatro sistemas enzimáticos estudados foram: fosfatases ácidas EC 3.1.3.2 (ACP), peroxidases EC 1.11.1.7 (PER), Glutamato oxaloacetato transaminase EC 2.6.1.1 (GOT) e enzima málica EC 1.1.1.40 (ME), e os resultados foram obtidos por meio de electroforese com gel de poliacrilamida (PAGE).

Em todos os sistemas enzimáticos estudados foram encontrados polimorfismos isoenzimáticos, sendo contudo a variabilidade de padrões inferior no caso da GOT. A comparação dos padrões das bandas isoenzimáticas das enzimas ACP, PER, e Me evidenciam variações interespecíficas que tornam possível a identificação das espécies estudadas. Detectou-se ainda, uma reduzida variabilidade intraespecífica que não permite diferenciar populações de uma mesma espécie.

1. Introduction

Bryophytes are essential components of the biodiversity of terrestrial ecosystems, playing an important role in maintaining the water equilibrium trough an effective retention mechanism of the rain water and fogs in forests (Hodgetts, 1996). The moss and liverwort developed important mechanisms of water control that are a valuable contribution for the water equilibrium in forests. In addition, bryophytes stabilize the soil crust through colonization of bare grounds and rocks.

The Madeira's Laurel Forest (Laurissilva), classified as World Natural Heritage under protection of UNESCO (Sunyer, 2000), has a great biological diversity with a large number of endemic species some of them listed as rare. The Laurel Forest, also know as "water producer" occurs mainly in zones where the atmosphere is quite humid, usually above 85%, and is the main responsible for retaining the water from fogs and vertical precipitation. It is a place, where the diversity of bryophytes is much prominent (Jardim & Fontinha, 2000).

The major difficulty in the implementation of the biological conservation programs is the inadequate knowledge about the taxonomy of several groups (Sim-Sim, 1999; Sim-Sim *et al.*,2000; Fontinha, 2000; Fontinha *et al.*, 2001). Based on several morphological and ecological data already collected, the present study is focused in a biochemical approach for the characterization of the *Plagiochila*

genus. With this study we intend to contribute for a better knowledge of bryological diversity and contribute for the conservation of the Madeira Laurissilva.

The *Plagiochila* genus, with more than 450 species worldwide is the largest genus of liverworts (Heinrichs, 2002; Heinrichs *et al.*, 2002a). This genus is referred for several places in Portugal mainland and is abundant in the Laurel Forest of Madeira when compared with the other parts of Europe (about 60% of the total *Plagiochila* species in Europe were detected in Portugal). The morphological characterization of the *Plagiochila* genus is complex due to the high plasticity of leaf cell patterns, leaf dentation, leaf position and leaf shape of this kind of liverwort. Krzakowa & Szweykowski (1979) were the first authors to study the genetic variability of the genus *Plagiochila* using electrophoresis for isoenzymes. With the present isoenzyme analysis study we expected to contribute for a better characterization of the interspecific as well as intraspecific variability of the genus *Plagiochila* from Portugal mainland and Madeira.

2. Material and methods

2.1. Material

Twenty populations of *Plagiochila* (*P. bifaria* (Sw.) Lindenb., *P. porelloides* (Torrey ex Nees) Lindenb., *P. puntacta* (Taylor) Taylor , *P retrorsa* Gottsche, *P. spinulosa* (Dicks.) Dumort., *P. stricta* Lindenb.) from different sites in Portugal and Laurel Forest of Madeira were investigated. The provenance of each *Plagiochila* sample is shown in Table 1.

The fresh material was carefully cleaned from contaminants. Only the upper young shoots of each individual were collected, weighted, freezed with liquid nitrogen and stored at -80°C.

2.2. Methods

2.2.1. Extraction of total protein

The plant material samples (with about 0,03g), freezed in liquid nitrogen, were grinded in a mortar and pestle until a fine powder was formed.

The extraction was done with 100mM Tris-HCl buffer, pH 8.0 ((tris-hidroxi-metil aminometano-HCl) and 10mM mercaptoethanol (ME).

Table 1

The species and origins of Plagiochila *samples in Portugal and Madeira studied*

Population	Specie	Origin
Mad 1	*P.*aff. *retrorsa*	Madeira, Santana, Caldeirão Verde
Mad 2	*P.*aff. *retrorsa*	Madeira, Santana, Caldeirão Verde
Mad 3	*P. bifaria*	Madeira, Santana, Caldeirão Verde
Mad 4	*P. spinulosa*	Madeira, Santana, Caldeirão Verde
Mad 5	*P. retrorsa*	Madeira, Santana, Caldeirão Verde
Mad 6	*P. retrorsa*	Madeira, Santana, Caldeirão Verde
Mad 9	*P.*aff. *retrorsa*	Madeira, Santana, Caldeirão Verde
Mad 11	*P.*aff. *retrorsa*	Madeira, Santana, Rib° Bonito
Mad 13	*P. stricta*	Madeira, Santana, Rib° Bonito
Mad 16	*P. bifaria*	Madeira, S. Vicente, Folhadal, Levada do Norte
Mad 17	*P. stricta*	Madeira, S. Vicente, Folhadal, Levada do Norte
Mad 19	*P. spinulosa*	Madeira, S. Vicente, Folhadal, Levada do Norte
Mad 20	*P. punctata*	Madeira, S. Vicente, Folhadal, Levada do Norte
Mad 21	*P. punctata*	Madeira, S. Vicente, Montado dos Pessegueiros
Min 1	*P. porelloides*	Minho, Montalegre, Gêres
Mst 1	*P. bifaria*	Estremadura, Sintra, Monserrate
Mst 2	*P. bifaria*	Estremadura, Sintra, Monserrate
Mst 3	*P. bifaria*	Estremadura, Sintra, Monserrate
TM 1	*P. porelloides*	Trás-os-Montes e Alto Douro, Bragança, Montesinho
TM 2	*P. porelloides*	Trás-os-Montes e Alto Douro, Bragança, Montesinho

The resulting homogenate was centrifuged 100min at 17500 *g* at 4°C in an eppendorf centrifuge.

The supernatant was transferred with a Pasteur pipette and utilized directly as an electrophoresis sample.

The extraction, as well as the fallow manipulations and electrophoresis were performed in a cold chamber at 4°C, and the extract was kept always on ice.

2.2.2.Gel electrophoresis under no denaturing conditions -PAGE

A vertical electrophoresis Mini-protean II from BioRad was utilized with glass plates of (10cm X 8cm) and 0.75mm of thickness.

2.2.3. Gel electrophoresis preparation

Gel electrophoresis preparation was performed according to Bollag & Edelstein (1992) and Esquível (1995):

- Stacking gel: 5% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 125mM Tris-HCl buffer, pH 6.8, 0.1% (w/v) ammonium persulfate and 0.05% (v/v) TEMED (N,N'-tetrametilenodiamina);
- Separating gel: 7.5% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 375mM Tris-HCl buffer, pH 8.8, 0.03% (w/v) ammonium persulfate and 0.03% (v/v) TEMED (N,N'-tetrametilenodiamina).

Electrophoresis samples preparation: The extract samples were prepared in sample buffer (0.08 M Tris-HCl pH 6.8, containing 0.1 M mercaptoethanol, 15% (v/v) glycerol e 0.006% (p/v) de *m*-cresol purple). After preparation, the samples were freezed in liquid nitrogen and stored at -80°C.

Electrode buffer and run gel conditions: the electrode buffer had the fellow composition: 25 mM Tris-HCl – 192 mM glycine, pH 8.8.

Just before starting electrophoresis, samples were thawed and centrifuged at 5000*g* for 1min at 4°C. The supernatant with the enzymes was loaded into the gel with a syringe.

The electrophoresis run was carried out at 40mA constant amperage, with a voltage less than 200V. The protein migration was stopped when the dye front (m--cresol purple) was in a short distance from the bottom of the gel.

2.2.4. Enzyme systems analysis

For the isoenzyme analysis the methods are described in Sim-Sim (1995) and Kranner *et al* (2002):

1. Acid phosphatase EC 3.1.3.2 (ACP): 50mM Sodium acetate buffer pH 4.0, was used as the buffer for the enzyme reaction. Acid α naphthyl phosphate was the substrate. "Fast Garnet GBC" was added to enhance the clarity of staining.
2. Peroxidase EC 1.11.1.7 (PER): 50mM Sodium acetate buffer pH 5.0, was used as the buffer for the enzyme reaction. 3-Amino-9-ethylcarbazole and H_2O_2 were the substrates.
3. Glutamate oxaloacetate transaminase EC 2.6.1.1 (GOT): 100mM Tris--HCl buffer pH 8.5, was used as the buffer for the enzyme reaction. L--Aspartic acid and α–Ketoglutaric acid were the substrates.

4. Malic enzyme EC 1.1.1.40 (ME): 100mM Tris-HCl, pH 7.5, was used as the buffer for the enzyme reaction. DL-Malic acid, NADP (nicotinamide adenine dinucleotide phosphate) and the 3-(4,5-dimethyl-2-thiazolyl)--2,5-diphenyl- 2H-tetrazolium bromide (MTT) were the substrates.

3. Results and discussion

As a result of this work, the enzyme systems studied by electrophoresis could be visualized and their bands kept for a future analysis of the interspecific and intraspecific variability of the genus *Plagiochila* from Portugal mainland and Madeira.

For the different *Plagiochila* species analysed, referred in Table 1, the Figures 1, 2, 3 and 4 show the schematic banding patterns for each enzyme system studied, acid phosphatase (ACP), peroxidase (PER), glutamate oxaloacetate transaminase (GOT) and malic enzyme (ME).

The results revealed high enzyme activities for acid phosphatase (ACP) and peroxidase (PER) in all the samples studied. However, the enzymatic activity of glutamate oxaloacetate transaminase (GOT) and malic enzyme (ME) were low and could not be detected in several samples.

For the different enzyme system checked, ACP and PER had the high number of polymorphic bands (bands that are present in an isoenzymatic profile of one population but were absent in the isoenzymatic profile of another population).

Contrasting banding patterns were found between the *Plagiochila* species studied. In global terms, the Figures 1, 2, 3 and 4 revealed at least 7 different banding patterns for acid phosphatase (ACP), 6 banding patterns for peroxidase (PER), 3 banding patterns for glutamate oxaloacetate transaminase (GOT) and 4 banding patterns for malic enzyme (ME).

Figure 1

Electrophoresis analysis - schematic banding pattern for acid phosphatase EC 3.1.3.2 (ACP) from Plagiochila *populations (see Table 1)*

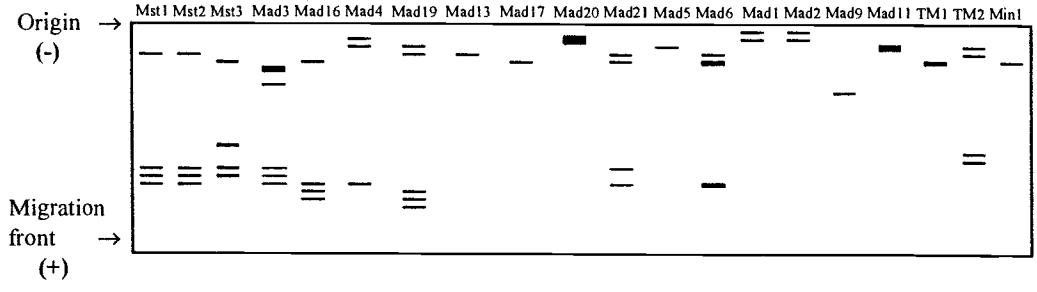


Figure 2

Electrophoresis analysis - schematic banding pattern for peroxidase EC 1.11.1.7 (PER) from Plagiochila *populations (see Table 1)*

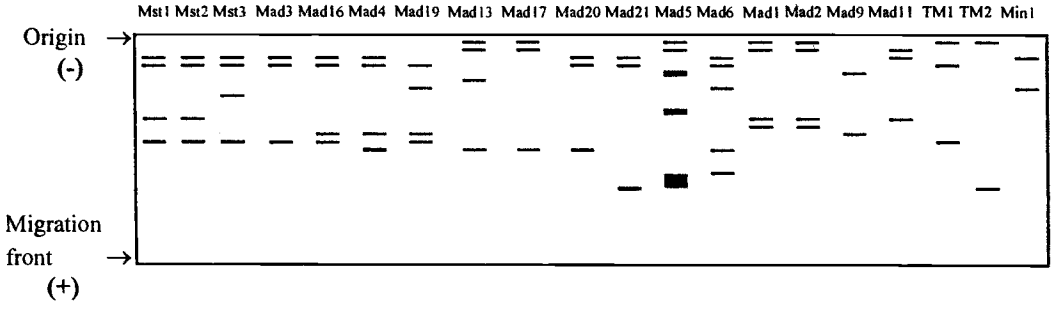
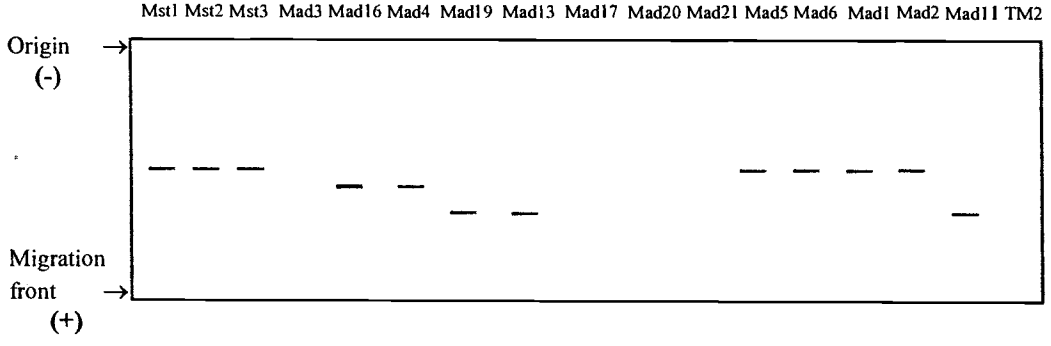


Figure 3

Electrophoresis analysis - schematic banding pattern for glutamate oxaloacetate transaminase EC 2.6.1.1(GOT) from Plagiochila *populations (see Table 1)*



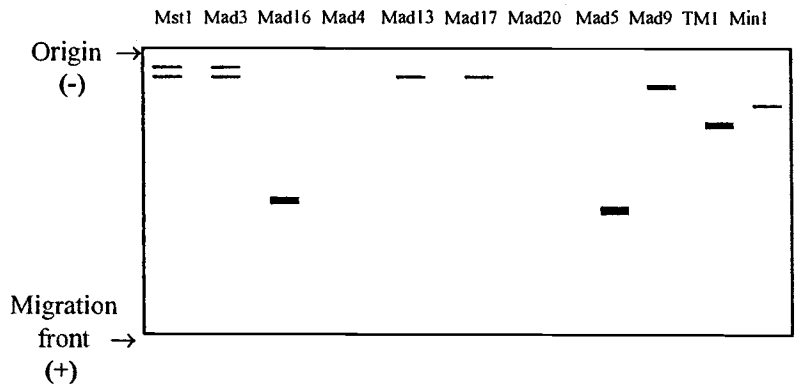
The three banding profiles of glutamate oxaloacetate transaminase revealed to be monomorphics (Fig.3), exhibiting activity only in one band. Those profiles did not give enough information about the genetic variability of the studied populations and hence were not used for interpretation.

The acid phosphatase banding profiles from populations of *P. bifaria* and *P. spinulosa,* (Mst 1 and Mad 19 populations) seems to have many similarities (Fig. 1). For example, the enzyme system showed distinctly two zones of activity with different mobility. One zone situated near the electrophoresis origin (bands with low mobility and called cathodal bands) and the other, with three bands, situated near the migration front (bands with high mobility and called anodal bands).

Similarly, the peroxidase profiles for these species show several analogies (Fig. 2), four bands were observed in two distinct zones of mobility. The results indicate that there is low interspecificity between *P. bifaria* and P. *spinulosa* species.

Figure 4

Electrophoresis analysis - schematic banding pattern for malic enzyme EC 1.1.1.40 (ME) from Plagiochila *populations (see Table 1)*



Plagiochila stricta (Mad 13 and Mad 17 populations) revealed similar isoenzymatic profiles for ACP, PER and ME and distinct from the other *Plagiochila* populations, this fact confirmed that is a distinct specie (Figs. 1, 2 and 4). These results are in accordance to the morphological characters observed and essential oil composition detected in these populations by Sim-Sim *et al.* (2003).

Concerning the populations of *P. retrorsa* (Mad 5 and Mad 6) and *P.* aff. *retrorsa* (Mad 1, Mad 2, Mad 9 and Mad 11), it was observed a high level of heterogeneity in the enzymatic systems, acid phosphatase and peroxidases (Figs. 1 and 2). However, the peroxidase patterns of banding (Fig. 2) from the two populations of *P. retrorsa*, revealed a fifth band unique to this two profiles. The fifth band was not found on the populations of *P.* aff. *retrorsa.* The possibility to distinguish the populations of *P. retrorsa* and *P.* aff. *retrorsa* by isoenzymatic assays, was particularly important. To determine the taxonomic status of *P.* aff. retrorsa, it is necessary to utilize molecular biology techniques. For example, the analyses of nrDNA ITS1 and ITS2 sequences were shown to be a good type of molecular marker in phylogenetic *Plagiochila* studies (Heinrichs, 2002; Heinrichs *et al.*, 2002 b).

Due to the heterogeneity on the isoenzymatic banding pattens of the *P. punctata* populations, we suggest that more populations should be analyzed in the future.

Interspecific variation was observed for acid phosphatase, peroxidase and malic enzyme in the *P. porelloides* populations analysed (Figs. 1, 2 and 4). The banding

patterns of this specie showed also significant differences when compared with the other *Plagiochila* populations. Because these populàtions were collected in distinct sites of Portugal mainland, the polymorphism accumulation in this specie could be due to geographical differentiation and habitats diversity.

With this isoenzymatic study we may conclude that *P. porelloides* populations had the most distinct isoenzymatic banding pattern of all *Plagiochila* populations. In opposite, the two species, *P.bifaria* and *P.spinulosa,* had close isoenzymes profiles for the majority of the enzymatic system studied.

The results obtained from the populations of *P. retrorsa, P.* aff. *retrorsa* and *P. stricta* indicate a clear difference between these populations. We hope, with the future research in this field, to contribute to clarify the taxonomic status of these populations.

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