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# Inter-Simple Sequence Repeat (ISSR) markers support the species status of *Weissia wimmeriana* (Sendtn.) Bruch & Schimp. (Pottiaceae, Bryopsida)

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**Abstract** – Based on ISSR molecular markers, *Weissia wimmeriana* is clearly distinct from *W. controversa*. A clade separating the two species is supported by 100% bootstrap support using Neighbor Joining as criterion. An Analysis of the Molecular Variance (AMOVA) supports the high significance value of the ISSR data ($p \leq 0.001$). The genetic variability within *W. wimmeriana* is extremely low, a typical finding in rare species. The two studied populations of *W. controversa* show a relatively low fixation index ($F_{ST} = 0.189$), which may be the result of a high rate of dispersal or a low evolutionary rate combined with a high effective population size.

**Bryophytes / taxonomy / ISSR markers / *Weissia wimmeriana***

**Resumen** – En base a los marcadores moleculares ISSR, *Weissia wimmeriana* es una especie claramente distinta de *W. controversa*. Usando como criterio el análisis "Neighbor Joining", el clado que separa las dos especies tiene un apoyo "bootstrap" del 100 %. Un análisis de la varianza molecular (AMOVA) apoya el valor de alta significación de los datos ISSR ($p \leq 0.001$). La variabilidad genética en *W. wimmeriana* es extremadamente baja. Esto es típico de especies raras. Las dos poblaciones estudiadas de *W. controversa* muestran un índice de fijación relativamente bajo ($F_{ST} = 0.189$). Esto puede ser el resultado de una alta tasa de dispersión o de una baja tasa evolutiva combinada con un elevado tamaño efectivo de la población.

**Briófitos / taxonomía / marcadores ISSR / *Weissia wimmeriana***

## INTRODUCTION

*Weissia wimmeriana* (Sendtn.) Bruch & Schimp. is a rare moss known from Iceland and Scotland in northwestern Europe, several mountain systems in North and Central Europe, the Pyrenees, Corsica, Sardinia and also from some central and southern regions of Italy. In Asia it has been reported from Turkey, the Caucasus and Kashmir (Smith, 1978; Düll, 1984, 1992; Ganeva & Düll, 1999;

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Pavletic & Martincic, 1999; Cortini Pedrotti, 2001). Its discovery in Sierra Nevada (southern Spain) represents the most southern limit of its range known to date.

It is restricted to higher altitudes or zones with a cold climate. This species is morphologically very similar to the widespread *Weissia controversa* Hedw. The most relevant difference between the two species is the sexuality, paroecious in *W. wimmeriana,* and autoecious in *W. controversa*. Other less conspicuous differences are the longer and more subulate perichaetial leaves in *W. wimmeriana*, while the peristome, which is always present in *W. controversa* (poorly to well developed) is never completely developed in *W. wimmeriana*, and may sometimes even be absent. Therefore, Smith (1978) and Guerra (2002) expressed some doubts, whether these subtle differences warrant the recognition of *W. wimmeriana* at species level, and Blockeel and Smith (1998) reduced it to the rank of a variety of *W. controversa.*

We were able to collect some specimens of the rare *W. wimmeriana* in order to conduct a study at the molecular level. We decided to use ISSR (Inter-Simple Sequence Repeat) markers. A recent study treating the taxonomic status of the closely related *Anacolia webbii* (Mont.) Schimp. and *Anacolia menziesii* (Turner) Parihar demonstrated the utility of this technique in bryophytes working at population level and for closely related species (Werner *et al*., 2003). ISSR is similar to RAPD (random amplified polymorphic DNA; Williams *et al.*, 1990) in that it uses only a single primer in the PCR reaction, but differs in that it does not use arbitrary primers. ISSR-primers are designed to match microsatellite sequences, short repeated sequence motifs that are found many times throughout the genome. If two identical microsatellites are found near one another and in opposite orientation, the ISSR-primer amplifies the region between the two microsatellites. This technique was introduced by Gupta *et al*. (1994) and Zietkiewicz *et al*. (1994) and is widely used by plant breeders, but has only recently been adopted by investigators interested in plant population genetics or taxonomy (Wolfe & Liston, 1998). Its main advantage over RAPD is that longer primer sequences allow more stringent reaction conditions to ensure high specificity (and therefore reproducibility) of the amplification products.

In plants, ISSR markers have mainly been used to study cultivated species, especially in cultivar identification and genomic mapping projects (Wolfe & Liston, 1998), and only recently the technique has been applied to natural populations and relationships among closely related species (Ayres & Strong, 2001; Camacho & Liston, 2001; Deshpande *et al.*, 2001; Wolfe & Randle, 2001).

## MATERIAL AND METHODS

*Plant material* – A total of 27 accessions were used in this study, six of *W. wimmeriana* (one from the Spanish Sierra Nevada and five from the Italian Alps), and 21 of *W. controversa* (14 from the Spanish Sierra Nevada and seven from the Italian Alps). The plants were classified corresponding to their sexual condition (paroecious = *W. wimmeriana*). Details on geographic origin and voucher data are given in Table 1.

*DNA extraction* – Total DNA was extracted by the NaOH extraction method described by Werner *et al*. (2002) with some minor modifications. Basically, the dried plants were ground using mortar and pestle together with acid-

Table 1. Samples included in the ISSR analysis.

| *Species* | *Voucher* | *Origin (mountain system / country)* | *Acronym used in figures* |
|---|---|---|---|
| *Weissia wimmeriana* (Sendtn.) Bruch & Schimp. | MUB 14749 | Sierra Nevada / Spain | wimm Sp1 |
| *Weissia wimmeriana* (Sendtn.) Bruch & Schimp. | MUB 14988 | Alps / Italy | wimm ST1 |
| *Weissia wimmeriana* (Sendtn.) Bruch & Schimp. | MUB 14989 | Alps / Italy | wimm ST2 |
| *Weissia wimmeriana* (Sendtn.) Bruch & Schimp. | MUB 14990 | Alps / Italy | wimm ST3 |
| *Weissia wimmeriana* (Sendtn.) Bruch & Schimp. | MUB 14994 | Alps / Italy | wimm ST4 |
| *Weissia wimmeriana* (Sendtn.) Bruch & Schimp. | MUB 14995 | Alps / Italy | wimm ST5 |
| *Weissia controversa* Hedw. | MUB 14751 | Sierra Nevada / Spain | cont Sp1 |
| *Weissia controversa* Hedw. | MUB 14752 | Sierra Nevada / Spain | cont Sp2 |
| *Weissia controversa* Hedw. | MUB 14856 | Sierra Nevada / Spain | cont Sp3 |
| *Weissia controversa* Hedw. | MUB 14858 | Sierra Nevada / Spain | cont Sp4 |
| *Weissia controversa* Hedw. | MUB 14859 | Sierra Nevada / Spain | cont Sp5 |
| *Weissia controversa* Hedw. | MUB 14862 | Sierra Nevada / Spain | cont Sp6 |
| *Weissia controversa* Hedw. | MUB 14864 | Sierra Nevada / Spain | cont Sp7 |
| *Weissia controversa* Hedw. | MUB 14221 | Sierra Nevada / Spain | cont Sp8 |
| *Weissia controversa* Hedw. | MUB 14223 | Sierra Nevada / Spain | cont Sp9 |
| *Weissia controversa* Hedw. | MUB 14225 | Sierra Nevada / Spain | cont Sp10 |
| *Weissia controversa* Hedw. | MUB 14227 | Sierra Nevada / Spain | cont Sp11 |
| *Weissia controversa* Hedw. | MUB 14228 | Sierra Nevada / Spain | cont Sp12 |
| *Weissia controversa* Hedw. | MUB 14232 | Sierra Nevada / Spain | cont Sp13 |
| *Weissia controversa* Hedw. | MUB 14233 | Sierra Nevada / Spain | cont Sp14 |
| *Weissia controversa* Hedw. | MUB 15014 | Alps / Italy | cont ST1 |
| *Weissia controversa* Hedw. | MUB 15017 | Alps / Italy | cont ST2 |
| *Weissia controversa* Hedw. | MUB 15007 | Alps / Italy | cont ST3 |
| *Weissia controversa* Hedw. | MUB 15005 | Alps / Italy | cont ST4 |
| *Weissia controversa* Hedw. | MUB 15008 | Alps / Italy | cont ST5 |
| *Weissia controversa* Hedw. | MUB 15006 | Alps / Ital | cont ST6 |
| *Weissia controversa* Hedw. | MUB 15010 | Alps / Italy | cont ST7 |

washed sand. In order to release the DNA from the plant tissue, 20 µl of 0.5 M NaOH were added to the resulting powder and after a short centrifugation step, 10 µl of the supernatant were diluted with 90 µl 100 mM Tris/ 1mM EDTA.

*ISSR* – For ISSR, 2 µl of DNA-extract was used in a 25 µl PCR reaction with 0.8 units Taq polymerase (Oncor Appligene), 2 mM $MgCl_2$, 200 µM of each of the dNTPs, 400 µM of ISSR-primer (Table 2) and the buffer supplied by the manufacturer of the enzyme. Two percent BLOTTO (10% skimmed milk powder and 0.2% $NaN_3$) were added to the reaction mix. BLOTTO is reported to attenuate PCR inhibition in the presence of plant compounds (De Boer *et al.*, 1995). The cycling conditions were as follows: 3 min at 94°C followed by 30 cycles of 15 sec at 94°C, 30 sec at 45°C and 1 min at 72°C, with the exception of the primer ISSR2, where the annealing temperature was lowered to 40ºC. These cycles were followed by a final extension step of 7 min at 72°C. After the addition of 5 µl gel

Table 2. ISSR primer sequences and amplification products. The total number of bands per primer, the number of bands that are presented by all samples, and the number of bands that are presented by all samples of the two species are given. There exists no band that is exclusively shown by all samples of *W. controversa,* but 12 bands are found in all samples of *W. wimmeriana* that are not present in *W. controversa*.

| *Name* | *Sequence 5´->3'* | *Number of loci* | *Monomorphic loci* | *Fixed loci* W. wimmeriana | *Fixed loci* W. controversa |
|---|---|---|---|---|---|
| UBC-840 | GAGAGAGAGAGAGAGYT | 22 | 2 | 1 | - |
| ISSR2 | CAACAACAACAACAA | 25 | - | 1 | - |
| ISSR4 | GACAGACAGACAGACA | 11 | 1 | 2 | - |
| OW1 | GAGAGAGAGAGAGAGAA | 11 | - | 1 | - |
| OW2 | GAGAGAGAGAGAGAGAC | 20 | 1 | 4 | - |
| OW3 | GAGAGAGAGAGAGAGAT | 16 | - | - | - |
| OW5 | GAGAGAGAGAGAGAGAYC | 23 | - | 3 | - |

loading buffer (50% glycerol, 0.05% bromophenol blue) 5µl of the amplification reaction were separated on 6% polyacrylamide gels. The DNA bands were visualized by silver staining and the gels scanned with a TWAIN compatible computer scanner with a transillumination module. Reproducible bands were scored as present (1) or absent (0) and the resulting data matrix was entered in the RAPDistance programme (Armstrong *et al.*, 1995). The index of Nei & Li (1979) was used to calculate genetic distances. The resulting distance matrix was used to infer a Neighbor Joining tree. PHYLIP 3.6 (Felsenstein, 1989) was used to estimate the bootstrap support of the different clades (1,000 replicates).

*Estimation of diversity indices and of molecular variance* – The analysis of molecular variance (AMOVA), (Excoffier *et al.*, 1992) was performed using ARLEQUIN (Schneider *et al.*, 2000). To obtain the number of pairwise distances the program simply counts the number of different alleles between two haplotypes. The same program was used to calculate a minimum spanning tree (Rohlf 1973) based on the matrix of pairwise distances. To test the differenciation between groups of populations, the user of the program defines a particular genetic structure that will be tested. The significance of the fixation indices is tested using a non-parametric permutation approach described in Excoffier et al. (1992). The significance of the number of pairwise differences between *W. controversa* and *W. wimmeriana* was tested performing a Student´s *t*-test as implemented in Excel 2000 (Microsoft Corporation).

## RESULTS

Seven ISSR primers that had given positive results with *Anacolia* (Werner *et al.*, 2003) were surveyed. All primers gave variable banding patterns. Therefore, these primers might be usefull in a wide range of bryophytes and, indeed, they provided useful banding patterns in higher plants too (e.g. Camacho & Liston, 2001; Leroy *et al.*, 2000). All assays were repeated twice and gave highly

reproducible results (only very faint bands were not reproducible in some occasions). A total of 128 loci were identified. Four of these were constant among all specimens, and twelve were found exclusively in all the samples of *W. wimmeriana.* No band was found exclusively in all the specimens of *W. controversa.* Details are listed in Table 2. The number of pairwise differences was very low among the samples of *W. wimmeriana* (values between two and seven, mean 4.6), while a considerably higher number was observed in the case of *W. controversa* (between 4 and 40, mean 25.5). In order to check the statistic significance of the values for the pairwise differences between *W. controversa* and *W. wimmeriana*, a Student's *t*-test was performed. The results show that the the two data sets are significantly different ($p \leq 3.8 \times 10^{-22}$) and that the result is not due to unequal sample sizes. Separating the specimens of *W. controversa* in two groups (Spanish Sierra Nevada versus Italian Alps) resulted between 12 and 40 differences within the samples of Sierra Nevada (mean 23.3) and 4 and 35 within the samples of the Italian Alps. The pairwise differences between samples of *W. wimmeriana* and *W. controversa* ranged from 28 to 47 (mean 36.8).

A minimum spanning tree, calculated on the basis of the pairwise differences, clearly separated *W. wimmeriana* from *W. controversa* (Fig. 1). The analysis of the molecular variance (AMOVA) revealed that 50.4% of the differences were found between the two species and 49.6% were found within the two species (Table 3). This result is highly significant ($p \leq 0.001$). The comparison between the two groups of populations of *W. controversa* showed that 18.9% of the variance components corresponded to two differences between the groups and 81.1% to differences within the groups (Table 4). The differentiation of the two groups is significant at the level of $p \leq 0.001$.

The Neighbor Joining analysis using the index of Nei & Li (1979) confirmed the separation of *W. wimmeriana* from *W. controversa* (Fig 2). The clade of *W. wimmeriana* was supported by all of 1000 bootstrap replicates. The bootstrap support values within species were generally below 50% with some exceptions (Fig. 2).

## DISCUSSION

Our data suggest that *W. wimmeriana* should be recognised at species level. Both Amova and cluster analysis support this point of view with highly significant results. Since the resulting trees are not rooted, it cannot be completely excluded that *W. controversa* might form a paraphyletic taxon. In fact, that would not be surprising as most geographic and demographic scenarios for speciation initially result in paraphyletic taxa when reproductive isolation is the basis for species definition. As time progresses, population extinctions and the fixation of different genetic variants in isolated population systems would give rise to mutually monophyletic taxa (Avise, 2000). Although we cannot reject the possibility that *W. controversa* is paraphyletic, the taxon is morphologically (slightly but consistently) distinct from *W. wimmeriana* and is differentiated genetically. Furthermore, *W. wimmeriana* seems to be restricted to alpine or subarctic/arctic regions, although Guerra (2002) mentions that a specimen of this species has been found in Portugal at lower altitudes.

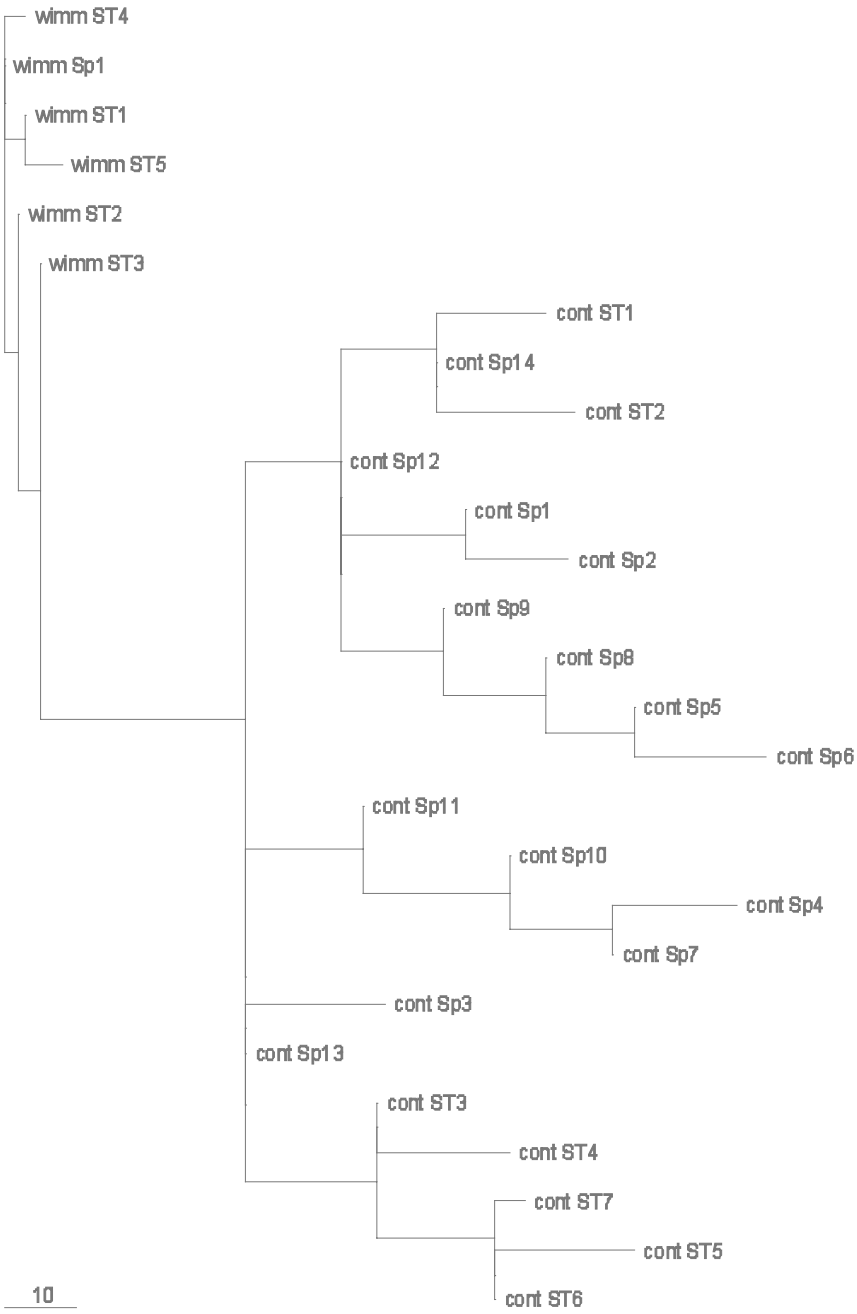


Fig. 1. Minimum spanning tree connecting all studied samples. *Weissia wimmeriana* is clearly separated from *W. controversa*. Five of the seven samples of *W. controversa* from the Alps (ST) are situated on a common clade, but the separation of the two studied populations of this latter species is not absolute. The bar indicates the number of differences. (For abbreviations, see Table 1)

Table 3. Locus by locus AMOVA results based on the ISSR data. The species *W. wimmeriana* and *W. controversa* were compared. Nearly exactly 50% of the observed variance correspond to differences between the two species. The chance to observe random values for Va and $F_{ST}$ greater or equal than the found values is below 0.001.

| *Source of variation* | *Sum of squares* | *Variance components* | *Percentage* |
|---|---|---|---|
| Among species | 111.57 | 10.81 Va | 50.4 |
| Within species | 266.36 | 10.65 Vb | 49.6 |
| Total | 377.93 | 21.46 | 100.0 |
| Fixation Index: | $F_{ST}$ = 0.50368 | | |

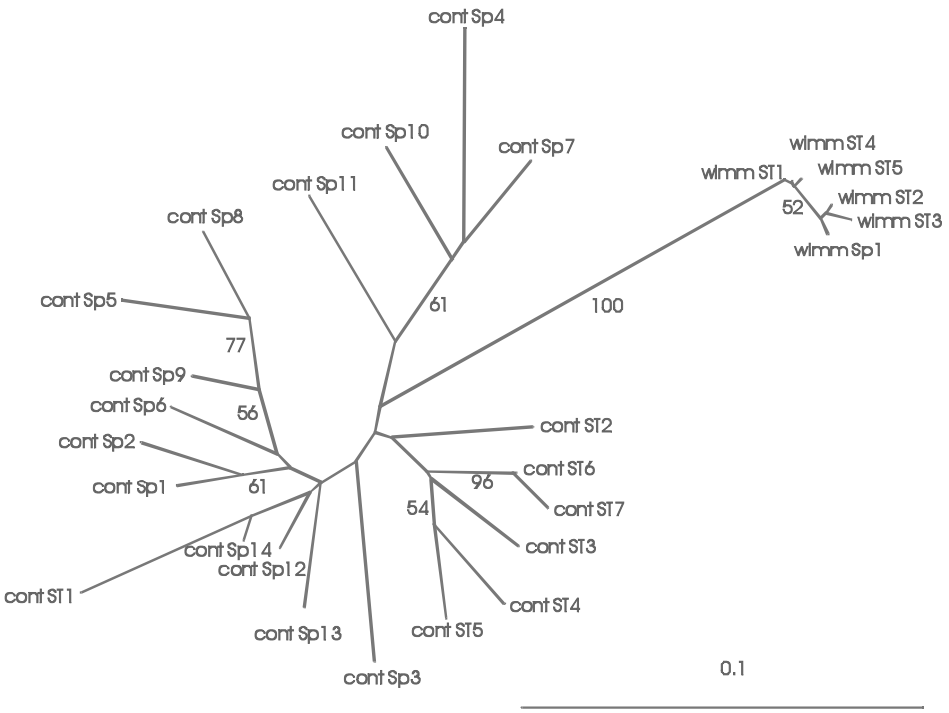


Fig. 2. Unrooted NJ tree based on the distance of Nei and Li (1979). Bootstrap support percentages of higher than 50% are given (1000 replicates). The clade of *W. wimmeriana* receives 100% support. The bar indicates the genetic distance. (For abbreviations, see Table 1)

The differentiation of the two groups of *Weissia controversa* from Spain and Italy, although highly significant, is not very high with $F_{ST}$ = 0.189. Stenøien & Såstad (1999) and Såstad *et al*. (2000) found similar $F_{ST}$ values for *Sphagnum angustifolium* (Russ.) C. Jens. and *Sphagnum majus* (Russ.) C. Jens. based on isozyme analyses. Similar values are generally found in species with a high level of dispersal capacity. An alternative explanation, favoured by these authors

Table 4. Locus by locus AMOVA results based on the ISSR data. The two populations of *W. controversa* (Sierra Nevada versus Italian Alps) were compared. Nearly 20% of the observed variance correspond to differences between the two populations. The chance to observe random values for Va and $F_{ST}$ greater or equal than the found values is below 0.001.

| *Source of variation* | *Sum of squares* | *Variance components* | *Percentage* |
|---|---|---|---|
| Among populations | 36.50 | 2.68 Va | 18.9 |
| Within populations | 218.36 | 11.49 Vb | 81.1 |
| Total | 254.86 | 14.17 | 100.0 |
| Fixation Index: | $F_{ST}$ = 0.189 | | |

consists in low evolutionary rates and large effective population sizes. At present it is not possible to draw a final conclusion about the two alternative explanations as there are no data available that would make it possible to estimate the rate of evolutionary change or the actual dispersal capacity in the case of *Weissia* species.

*Weissia wimmeriana* shows an extremely low level of genetic diversity. The number of differences between the different specimens is even lower than that observed in the equally rare *Anacolia menziesii* and *A. webbii*. Using the same primers the number of pairwise differences between Spanish and North African specimens of *A. webbii* ranged from 8 to 20, and between specimens of *A. menziesii* of European origin between 6 and 21 (out of a total of 147 observed bands) (Werner, unpublished data). Similar low levels of genetic diversity are generally found in endangered species, especially when they are restricted to relict habitats (e.g. Bauert *et al.*, 1998; Jiménez *et al.*, 2002; Maki & Horie, 1999; Palacios & González-Candelas, 1997).

The data do not permit us to draw a clear conclusion about the origin of *Weissia wimmeriana*. Since this species lives in habitats that allow growth only during a few months of the year, the paroecious condition may reduce the time necessary for the development of sexual organs. It is possible that the origin of *W. wimmeriana* lies in isolated populations of *W. controversa* (autoecious) living in arctic-alpine climate conditions.

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