Genetic variability within and between populations of two species of Racopilum (Racopilaceae, Bryopsida)

Albrecht de Vries, Bernard O. van Zanten and Henk van Dijk


Genetic variability in three populations of Racopilum spectabile and two populations of R. cuspidigerum was estimated by horizontal polyacrylamide electrophoresis. Eight of 22 tested enzyme systems were scorable; they represented 10 loci. The fraction of polymorphic loci in the most variable populations was 0.64 in R. spectabile and 0.55 in R. cuspidigerum. Estimates of gene diversity were 0.26 for both species, which is a high level of genetic variability compared with phanerogams. The genetic distance was positively correlated with spatial distance. Differences in allozyme frequencies between R. cuspidigerum and R. spectabile were considerably larger than differences within each species. One of the populations of R. cuspidigerum lacked genetic variability, which might be caused by asexual propagation. Maintenance of genetic variation within and origin of differentiation between populations are discussed. Our results reinforce the view that the diplohaploplontic Bryophytina possess an evolutionary potential comparable with that of phanerogams.

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Генетическая вариабельность в трёх популяциях R. spectabile и в двух популяциях R. cuspidigerum была выявлена путём полияридамидного электрофореза. Всем из 22 проверенных ферментных систем были измерены; они представляли 10 локусов. Фракция полиморфных локусов в наиболее вариабельных популяциях составляла 0.64 в R. spectabile, а 0.55 в R. cuspidigerum. Подсчёты разнообразия генов составляли 0.26 в обоих видах, что является высоким уровнем генетической вариабельности в сравнении с явиобразными растениями. Генетическое расстояние было положительно коррелировано с пространственным расстоянием. Различия в аллоферментных частотах между R. cuspidigerum и R. spectabile были значительно больше, чем различия внутри каждого вида. Одна из популяций R. cuspidigerum была лишена генетической вариабельности, что, может быть, вызвано бесполым размножением. Обсуждается сохранение генетической вариации внутри дифференциации между популяциями, а также происхождение данной дифференциации. Наши результаты подтверждают ту точку зрения, что диплохаплоидные мохообразные обладают эволюционным потенциалом, который можно сравнить с потенциалом явнообразных.

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Introduction

Rate of speciation

According to several authors, the evolution of bryophytes is very slow (Herzog 1926, Steere 1946, Stebbins 1950, Anderson 1963, Schuster 1969, Crum 1972, Iwatsuki 1972, Cummins and Wyatt 1981). Mechanisms which are often suggested as contributing to this evolutionary conservatism in bryophytes are a) the presence of a prolonged free-living haploid phase (the gametophyte) in the life-cycle, b) the abandonment of sexuality in many taxa and c) the high incidence of inbreeding in monoicous forms (Longton 1976).

In some recent papers however, the antiquity of the bryophytes (and especially of the Bryopsida) has been questioned. Khanna (1964) concluded that the Bryopsida have a “much greater evolutionary activity” and are a more successful group than the Hepaticopsida. This conclusion was based on the relative number of extant species and varieties, exploitation of the environment, and hybridization, which are all higher in the Bryopsida than in the Hepaticopsida. In some families of the Hepaticopsida and in most families of the Bryopsida genera occur with very polymorphic, intergrading species, which probably indicates recent active speciation (Van Zanten and Pocs 1981). According to these authors, the moss flora of New Guinea is rich in endemic species (probably ca. 40%). Most of these are montane species, an indication that their evolution started after the uplift of the Central Mountain Ranges in New Guinea, at the end of the Tertiary (ca. 10 Myr ago).

Genetic variability

It has become possible in recent decades to describe the genetic variability of populations and species by means of enzyme variants with a known genetic basis (Nevo 1978, Brown 1979). Allozymes, enzyme variants coded by the same locus, can be separated by electrophoresis as a consequence of differences in charge due to amino acid substitutions. Allozyme variation has proved to be very useful for systematic purposes, because differences in allozyme frequencies between populations or related species can be expressed in terms of genetic distance, a measure of the genetic distinctness of different populations or species (Nei 1975). Van Dijk and Van Delden (1981) mention some aspects of population genetics for which variation at enzyme loci has shown its usefulness. They suggest that the genetic distance between populations resulting from divergence over time, is an indication of the extent of gene flow. Moreover, a description of the genetic structure of populations presents knowledge concerning the reproductive system (outbreeding as opposed to inbreeding) and the degree of cloning.

It seems reasonable to assume a relation between allozyme variation and rate of speciation, but the exact evolutionary significance is still unclear in most cases. Selander et al. (1970) and Levin and Crepet (1973) found levels of genetic variability in the horseshoe crab Limulus polyphemus and the clubmoss Lycopodium lucidulum, two extant species with a long history in the fossil record, comparable to more recently evolved species. Therefore, an important question is how the genetic variation is maintained. If there is no adaptive significance i.e., the genetic variation is selectively neutral, enzyme polymorphisms must be a product of chance processes such as mutation, gene flow and genetic drift (Kimura 1968); but when it has a functional meaning, balancing selection may be responsible for the maintenance of variation (Ayala et al. 1974).

Genetic variability and bryophytes

Research investigating levels of genetic variability in bryophytes was begun only recently and has centered in the Hepaticopsida. For the Pellia megaspora-endivi-folia complex (Krzakowa 1981) and Scapania nemorosa (Zehr 1980), enzyme variants were used as taxonomic characters. For Plagiochila asplenioioides (Krzakowa and Szweykowski 1979), Atrichum angustatum (Cummins and Wyatt 1981), Conocephalum conicum (Yamazaki 1981) and Sphagnum pulchrum (Daniels 1982), attempts have been made to quantify the variation. Yamazaki's (1981) study is particularly valuable from a population genetics point of view, because of the relatively great number of enzyme loci (viz. 11) which he studied.

Most of these studies show levels of allozyme variation which are comparable to those measured in phanerogams. This suggests evolutionary potentials in bryophytes as great as that of phanerogams.

This study involved measurement of genetic variability in a number of populations of the tropical Racopilum spectabile and R. cuspidigerum (Racopilaceae, Bryopsida). Allozyme frequencies were determined for 10 enzyme loci. Genetic differences between populations of the two species and differences at the species-level were analysed. Implications for the understanding of the population genetics and population ecology of the Racopilaceae are discussed.

The species

Distribution and morphological variability

The genus Racopilum P. Beauv. belongs to the family Racopilaceae (Hypnobryales, Bryopsida). R. spectabile Reinw. et Hornsch. is common in all parts of the Malesian region and southern part of Oceania, where it occurs in rain forests from nearly sea-level up to ca. 3000 m elevation. It always grows in wet shaded places where it covers earth, rocks, bases of living trees and, rarely, leaves of flowering plants. R. cuspidigerum (Schwaegr.) Aongstr. has a wider range and reaches southern Japan to the north, southeast Australia to the...
south and India to the west. It grows in both wet and rather dry habitats; it is often found at the edges of rain forests, in plantations and other disturbed vegetation where it covers rocks, rotten wood or bark of living trees from low elevation to ca. 2000 m, rarely up to 2500 m. It can tolerate periodical sunshine and dry seasons.

*R. spectabile* is a polymorphic species. The juxtacostal cells may cover more than half of the leaf base or be nearly absent, while all possible intergradations occur. The typical lowland form of *R. spectabile* has very small, narrow dorsal leaves with a relatively long-excurrent nerve. In the montane rain forest the species is weakly heterophyllous to subisophyllous. Intergrading forms are especially abundant in the lower montane rain forest. Whether or not these distinctions are genetically based is unknown, but on the basis of culture experiments it is probable that a considerable part of this morphological variability is environmentally induced.

*R. cuspidigerum* is extremely polymorphic. The characters which vary most are a) size, b) shape of the leaf apex, c) degree of dentation of the leaf margin, d) degree of heterophylly, e) length of the excurrent part of the nerve, f) size of the laminae cells and g) papillosity of the laminae cells. At least some of these characteristics (e.g. degree of heterophylly and papillosity of the lamina cells) are probably genetically based, since they are much more frequent in one part of the geographical range of the species than in others. Because the adaptive significance is not obvious this may indicate genetic drift as a result of a founder effect.

**Reproduction**

**Sexual reproduction**: All species of *Racopilum* are phyllodioicous, this means that the male plants are very small and grow on the leaves or tomentum of the much larger female plants. The dwarf males consist only of a few caulonema threads with some chloronema, and one or a few male buds on very short stems which bear only a few, very small leaves. In *R. cuspidigerum*, as well as in *R. spectabile*, there occur very rarely free-living male plants of the same size as the female plants. These “normal” male plants have, however, not been observed in Philippine material.

Phyllodioicism promotes (at least theoretically) inbreeding because spores which fall on the mother plant produce male sex organs and spermatozoids which can fertilize the archegonia of the mother plant, at least if they are compatible.

In both species sporophytes are more often absent than present; when produced, however, they are abundant. Of *R. cuspidigerum* about 30% of herbarium specimens bear sporophytes, and of *R. spectabile* ca. 60%. The percentages of non-fruiting populations are probably higher in nature, because fruiting specimens are probably more often collected than specimens without sporophytes.

Asexual reproduction: *R. cuspidigerum* sometimes produces flagellae bearing small, caducous leaves. These may produce (after breaking off) buds, which can develop into adult female plants. The buds are usually produced at the base of the nerve, whether or not via a secondary protonema. Such flagellae have never been observed in *R. spectabile.*

All *Racopilum* species are able to produce new female plants from leaves which are broken off from the adult female plants. As in the caducous leaves, these new plants are usually produced from the base of the nerve, whether or not via a secondary protonema. It is also possible that new female plants are produced from stem fragments by the sprouting of “sleeping” buds which are often present in the leaf axils or just above them.

**Dispersal ability**

The spores of *R. cuspidigerum* and *R. spectabile* are small (10–16 μm) and can easily be transported by air currents over long distances. The spores can probably survive transportation at low and high altitudes because they can withstand desiccation periods of up to ca. 7 months in *R. spectabile* and up to ca. 2 yr in *R. cuspidigerum*. Because the spores of both species remain viable at temperatures of ~30°C, dispersal over moderately long distances by typhoons, where the spores may be sucked into the air to high altitudes, is possible. Because the updrafts during typhoons are usually very strong, there is a good possibility that caducous leaves or stem fragments are transported over moderately long distances from island to island in this way since, like the spores, stem fragments and detached leaves can survive desiccation and freezing. On the whole, however, we think that dispersal of caducous leaves and stem fragments is predominantly of importance for local dispersal and that transportation over longer distances is probably the exception.

**Materials and methods**

We used specimens of five populations from the Philippine islands of Luzon and Mindanao, collected in 1980 by Van Zanten and in 1981 by Van Zanten, Boeken and Boele: *R. spectabile* from Mt. Data, Mt. Banahao and Mt. Apo and *R. cuspidigerum* from Mt. Mayon and Mt. Apo (Fig. 1). From each of the five populations, 7 to 20 specimens were sampled. The distance between samples ranged from a few decimeters to a maximum of three kilometers.

Samples were sent in plastic bags to our laboratory. They were grown in plastic containers covered with glass on a mixture of black earth-sand (1:1, autoclaved) in culture-units (1000–2000 Lux, 12 h light at 21°C, at night 18°C). Rapid growth from side-shoots was obtained by freezing of stems for 5 d at ~5°C. Speci-
mens were harvested as portions weighing 400 mg and were stored at \(-80^\circ\text{C}\) for a maximum of 4 months.

Extracts were prepared by crushing the frozen plants in a mortar with a pestle after addition of 4 mg Polyclar AT (BDH), just before the start of electrophoresis. Mercaptoethanol (400 µl 6% in 10× diluted gelbuffer) was added and the frozen sample was again ground to powder. After centrifugation for 5 min at 5500 rpm, the supernatant was applied in the origins of horizontal 6% polyacrylamide gels, containing electrophoresis buffers diluted 10×. Electrophoresis was carried out for 3 h at 4°C and 15 V/cm. The best results were obtained using the following buffers: tris citrate, pH 7; tris maleate, pH 6 (preparation according to Van Dijk and Van Delden 1981) and Poulik buffer (Poulik 1957).

The enzyme staining methods of Shaw and Prasad (1970) were used, but in the case of dehydrogenase stains, MTT was used instead of NBT.

Eight of 22 tested enzyme systems provided distinct allozymes which were easy to score. Tab. 1 lists these enzymes (with their usual abbreviation) as well as the pH of the electrophoresis buffer which gave the best results.

### Results

#### Enzyme loci and allele frequencies

The specimens of the five populations were screened for variation of 8 enzyme systems, representing at least 10 loci. Tab. 2 shows the allele frequencies of the populations. Mobility of alleles as running distances (in mm) from origins is given in brackets (bromophenol blue = 100). It can be seen that 3 of the loci are non-variable. Of the polymorphic loci, 6-Pgd, Gpi-1 and the Pgm-loci show particularly striking differences in allele frequencies between *R. spectabile* and *R. cuspidigerum*.

#### Extent of genetic variability

The fraction of polymorphic loci (P), mean number of alleles per locus (\( \bar{A} \)) and gene diversity (\( H_1 \)) are expressions of the level of genetic variability. They were determined for each population (Tab. 3).

The fraction of polymorphic loci (P) is the fraction of variable loci. Although the free-living haploid gametophyte is never heterozygous, it is possible to estimate the expected heterozygosity from the allele frequencies. This “gene diversity” (\( H_1 \)) of Nei (in Ferguson 1980) is calculated as follows: for one locus is \( H_1 = 1 - \sum x_i^2 \), where \( x_i \) is the frequency of the i-th allele in the population; for all k loci together the mean gene diversity \( H_1 = \frac{1}{2} \sum H_i/k \) (\( H_1 \) varies from 0 to 1; when \( H_1 = 0 \) the population has no variation and is possibly a product of cloning).

The values in Tab. 3 are based on all sampled specimens (N\(_A\)). When part of the plants of a population shows the same banding pattern (the same alleles) for all studied loci, this could suggest that the plants involved are asexual progeny of one plant: if so, they can be considered as a clone having the same genotype. The number of variable loci is, however, too low, to draw this conclusion with full assurance. Yet, when plants with the same banding pattern are taken together then the number of presumed genotypes in each population is given by N\(_2\) in Tab. 3.

Tab. 3 shows varying values of genetic variability in...
Tab. 2. Allele frequencies of populations of *R. spectabile* and *R. cuspidigerum* (number of specimens per population is given in brackets).

<table>
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<tr>
<th>locus</th>
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<th><em>R. cuspidigerum</em></th>
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<td>F (38)</td>
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a. According to the resulting bands, each specimen probably represented only one single plant.
b. Plants of Mt. Data and Mt. Banahao gave poor bands for Got.
c. ‘O’ denotes a null allele, an allele without staining activity.

The degree of polymorphism in *R. spectabile* and *R. cuspidigerum* is comparable to Hamrick’s (1979) category of “trees and woody shrubs”, which are the most variable of phanerogams. This may be a result of the comparable dispersal potentials of both mosses and trees and woody shrubs that have wind-dispersed pollen populations, for P and A this is perhaps a consequence of varying number of specimens per population. The Mt. Apo population of *R. cuspidigerum* is, however, an exception: it has a very low level of variability, whereas the number of sampled plants is relatively high (N1 = 16). Asexual reproduction possibly plays a prominent role in this population (N2 = 2). Indeed, sporophytes were not found after an intensive search.

**Genetic distance**

Tab. 4 shows comparative genetic relationships within and between species, which have been analysed by means of the formulae of Nei (1975; also in Ferguson 1980). An estimate of the mean number of codon-differences per locus between two populations or species is the genetic identity I. I is calculated as I = \( \frac{X_i Y_j}{\sqrt{\sum X_i^2 \sum Y_j^2}} \), where \( X_i \) and \( Y_j \) are the frequencies of the i-th allele in the populations X and Y respectively. Depending on the conformity between populations, I ranges from 0 to 1. The genetic distance D is determined by \( D = -\ln I \) (when I = 1, then D = 0; is I = 0, then D = \( \infty \)), with standard error \( S_D = \sqrt{\frac{1-I}{k(k-1)}} \) (k loci; Nei 1971).

Within species the mean genetic distance in *R. spectabile* is 0.139; for *R. cuspidigerum* 0.131 is the only value. Between the two species the genetic distance is markedly higher: D = 0.672.

**Discussion**

**Extent of genetic variability**

Our results suggest that bryophytes are as variable genetically as phanerogams. We even expect a rise of values of the fraction of polymorphic loci and mean number of alleles per locus when the number of studied specimens per population is increased. Both taxa show broadly the same extent of variability; in both species the upper limit of gene diversity is 0.26.
or seeds. Moss spores disperse even more effectively (Van Zanten 1978, Van Zanten and Pócs 1981), because of their small size and probably their better chance of reaching appropriate substrate; spores, unlike pollen, are not dependent on “landing” upon receptive female organs. A result of gene flow between populations is the maintenance of genetic variation and a slowing of genetic differentiation.

The variability of the two Racomitrium species possibly corresponds with that of Conocephalum conicum ($H_1 = 0.174$, $P = 0.64$; Yamazaki 1981). The variability within Plagiochila asplenioides is $H_1 = 0.198$, which is smaller than in R. spectabile and R. cuspidigerum, but this value is based on allele frequencies of only three peroxidase loci (Krzakowa and Szweykowski 1979).

Differences in genetic variability ($P$ and $A$) between populations probably are due to the varying number of specimens collected per population. As mentioned earlier, the Mt. Apo population of R. cuspidigerum is an exception: of the 16 specimens 15 showed the same banding pattern, and only one specimen was found genetically different. This genetic uniformity corresponds with the lack of sporophytes in this population. Predominant asexual reproduction perhaps is consistent with the fact that at least half of these plants were growing in the unnatural environment of a plantation where the population was probably founded rather recently by only a few spores, in contrast to the older R. spectabile population on the mountain.

With regard to mode of reproduction and level of genetic variability the results of Daniels (1982) and of Krzakowa and Szweykowski (1979) are interesting. Although Sphagnum pulchrum (Daniels 1982) and Plagiochila asplenioides (Krzakowa and Szweykowski 1979) reproduce asexually in the studied populations, the amount of genetic variability within each population is considerable. In contrast to the probable recent history of the R. cuspidigerum population of Mt. Apo, Daniels (1982) postulates that the S. pulchrum populations are fragmentary relics of a once more common species. The origin of the variation then arose at a time when S. pulchrum did reproduce sexually.

The high level of variability in our other populations is the more remarkable, considering the potential for inbreeding as a result of the capacity for asexual reproduction and phyllodioicism. It is possible, however, that a mechanism exists that inhibits “son-mother” crosses, based on an unknown genetic incompatibility-system.

Populations of R. spectabile and R. cuspidigerum may be subdivided into several subpopulations of closely related individuals. This hypothesis could not be verified, as the exact position of samples was unknown (although samples of the same height were more often identical in band patterns).

### Variation within populations

The two currently conflicting opinions regarding the maintenance of genetic variation within populations are expressed by Cummins and Wyatt (1981) and Yamazaki (1981). Yamazaki states that it is simplest to assume the neutrality of allozyme polymorphisms and certainly only very few studies demonstrated effects of allozymes upon the fitness of their carriers (e.g., Van Delden et al. 1978). Therefore, it seems reasonable to suppose that chance processes like mutation and gene flow are responsible for the greatest part of allozyme variation. Cummins and Wyatt (1981), on the other hand, suggest that close adaptations to local microhabitats, in which selective advantages vary over short distances, could be responsible for the maintenance of variation.

We think, as well, that this balancing selection, which maintains variability within a population, plays a significant role in the Bryophyta, but it seems unlikely that selective forces act directly upon allozyme loci. Variation at allozyme loci, however, points to variation at other loci which may have an evolutionary meaning.

Thus, in a heterogeneous environment selective pressures upon non-allozyme loci may be differential with respect to both strength and direction. This differential selection possibly plays some role in R. spectabile and R. cuspidigerum since both species occupy a broad range of habitats. Another form of balancing selection, in which heterozygotes exhibit a higher fitness than homozygotes, could be significant in the diploid sporophyte.

During the diplohaplontic life cycle of mosses there are many points at which selection may act. Among the components of fitness are: success of germination and bud-formation; formation of sex organs; quantity and quality of sperm and eggs; number of spores; resistance of spores against stress.

### Variation between populations

A measure of differentiation between populations is the genetic distance ($D$). From Tab. 4 it is obvious that differentiation occurs within each species. The mean genetic distance in R. spectabile and R. cuspidigerum is 0.139 and 0.131; these values fit in the range of $D$ of 0.00–0.30, which is typical of populations of the same species (Ferguson 1980). The genetic distance between the two populations of R. spectabile of Luzon (0.106) is smaller than the genetic distance between these populations on the one hand and the population of Mt. Apo (Mindanao) on the other hand (mean 0.156). These values correlate positively with the geographical distance since the populations of Luzon, which are 300 km apart, are genetically more closely related to each other than to the population of Mindanao which is 1100 km away.

Genetic differences between populations may arise by chance processes as well as by selection. In the case of selection, adaptive differentiation is the result of natural selection in different habitats. Genetic drift,
however, results in population differentiation merely due to accidental fluctuations in allele frequencies. Drift particularly occurs when successive generations are initiated by small numbers of individuals (bottle neck).

With regard to the Racopilaceae, among other families, genetic drift may play an important role just after the establishment of a new population which has been founded by one or a few individuals: this founder effect has already been discussed for *R. cuspidigerum* of Mt. Apo. Further evidence regarding the role played by (long range) dispersal in the establishment of distribution areas comes from the entirely asexual population of a taxon known as *Powellia breviseta* (a species of the other genus in the Racopilaceae) on Mt. Mayon (Luzon). The lack of sporophytes and the fact that this population shows no genetic variation suggests that this population represents a clone (Van Zanten and De Vries, unpubl.). It has been assumed that the disjunct distribution of many moss species is a reflection of a relictual situation, a consequence of their presumed limited evolutionary potential (Anderson 1963). An alternative explanation is long range dispersal.

Although spore dispersal may be important in genetic drift and subsequent divergence of populations, on the other hand, dispersal between local populations tends to neutralize differences between them, at least when selection plays a minor role. However, the influence of gene flow diminishes with increasing spatial distance as is evidenced by the D-values within *R. spectabile*. It should also be noted that the establishment of a single immigrating settler in an existing population is made very difficult by the presence of large numbers of autochthonous (dia)spores.

**Variation between species and evolution**

The mean genetic distance between our species is 0.672, which is considerably higher than the mean distance within the species (0.139 for *R. spectabile*; 0.131 for *R. cuspidigerum*). The high value for the genetic distance is consistent with the clear morphological differences between the two species (Ferguson 1980, gives the general range of D for species of a genus as 0.10–1.00).

It is obvious that, considering the high level of genetic variability, the Racopilaceae may possess an evolutionary potential comparable with that of phanerogams. This conclusion is supported by the observation of great morphological diversity within *R. spectabile* and *R. cuspidigerum*, although the genetic basis of this diversity has not yet been demonstrated. A preliminary estimate of time of divergence of a number of taxa of the Racopilaceae pointed to a much more recent origin (ca. 5–10 Myr) than was presumed on the basis of bryogeographical considerations (50–80 Myr, De Vries 1981).

Our study has shown that enzyme electrophoresis is an useful tool to elucidate aspects of the population biology of moss species. This field is for the most part unexploited but should in the future unravel many unanswered questions with regard to the peculiar diplohaplontic life style of bryophytes.

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