Pollen-ovule ratios as a method of estimating breeding system in *Trifolium* pasture species

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Abstract

Pollen-ovule ratios were calculated on five species of *Trifolium* from Sardinia, Italy. The five species were known to vary in breeding system, and included the outcrossing species; ball and Persian clover, and the inbreeding species; cluster, woolly and subterranean clover. Pollen-ovule ratios showed that ball clover had the highest rate of outcrossing followed by Persian clover and then subterranean clover. Cluster and woolly clovers were both found to be highly inbreeding as expected. All species were found to be facultative to some degree in their breeding system. Persian clover, although an outcrosser, has a mixed mating system with a significant proportion of inbreeding occurring. Seed weight was shown to be highly correlated to pollen-ovule ratio within a breeding system, and it is suggested that it needs to be accounted for, before accurate levels of outcrossing can be estimated using pollen-ovule ratios. The results have important implications in plant breeding, as the breeding system of a species affects both the method of collection that should be used to conserve its genetic variation most effectively, and how its genetic resources should be used most efficiently in a breeding program.

Key words

Trifolium, breeding system, pollen-ovule ratio, adaptation.

Introduction

Knowledge of the breeding system of a pasture species will help to determine the potential genetic variation of a species and therefore its adaptation when sown in a new environment. This has important implications with the increase in the number of outcrossing pasture species that are currently being developed, particularly the perennial species, as they contain greater levels of genetic variation within a population, which affects both their adaptation when sown and their registration as a cultivar. Breeding system is also important in annual species, as they have to produce viable seed at the end of each growing season to ensure the continuation of the population.

The use of pollen-ovule ratios as a method of estimating breeding system was developed by Cruden (5), such that the higher the ratio of pollen grains to ovules within an individual flower, the higher the rate of outcrossing in a population or species. It is a relatively quick, low cost method of estimating breeding system compared to molecular, and crossing techniques, and therefore would be an easy character to include in new species evaluations. Pollen-ovule ratios have since been used in a number of studies on the evolution of plant breeding systems (7, 10, 12, 16).

Materials and Methods

Pollen-ovule ratios were calculated on five species of *Trifolium* from Sardinia, Italy. The five species were known to vary in breeding system, and included the outcrossing species; ball (*T. nigrescens* Viv.) and Persian clover (*T. resupinatum* L.), and the inbreeding species; cluster (*T. glomeratum* L.), woolly (*T. tomentosum* L.) and subterranean (*T. subterraneum* L.) clover. Pollen-ovule ratios were calculated in four populations per species (three for Persian clover).

The number of ovules and anthers were counted in four plants, from each population of each species. The number of pollen grains was counted in three anthers on each plant. Therefore pollen grains were counted in a total of 12 anthers per population. To count the number of pollen grains the anther head or pollen sac was placed on a slide, a drop of water was placed on top and the pollen sac squashed with a cover slide. The pollen grains were then counted under the microscope using a grid over the cover slide.

Pollen-ovule ratios were calculated as the product of number of pollen grains per anther by the number of anthers per flower, divided by the number of ovules per flower (5).

Cruden and Miller-Ward (6) have suggested that there is a positive relationship between seed size and pollen-ovule ratio, therefore 100 seed weights were determined for each population and correlations between pollen-ovule ratios, pollen grains per flower and mean seed weight were calculated. These correlations were also calculated for the inbreeding species alone.

Table 1. Numbers of ovules, average pollen grains per anther and per flower, pollen-ovule ratios and 100 seed weight per species.

Species	Ovules per flower	Pollen grains per anther	Mean pollen grains per flower	pollen-ovule ratio	Log (p/o) ratio	mean seed weight (mg)
Cluster clover	2.0	30.37	303.72	151.86	2.18	0.38
Ball clover	4.5	595.15	5951.46	1327.48	3.12	0.23
Persian clover	2.0	101.74	1017.46	508.73	2.71	0.40
Subterranean clover	2.0	88.87	888.71	444.35	2.64	5.02
Woolly clover	2.0	31.96	319.61	159.81	2.20	0.42

Results

The number of ovules, pollen grains per anther and per flower, pollen-ovule ratios and mean seed weight are shown in Table 1. Pollen-ovule ratios suggested that ball clover had the highest rate of outcrossing followed by Persian clover and then subterranean clover. Cluster and woolly clover were both suggested to be highly inbreeding as expected. Using a logarithmic scale the results are plotted in relation to Cruden's (5) estimates of breeding system (Figure 1). This shows that there is a large variation between populations within a species, but that most of the populations varied between facultative inbreeding to facultative outcrossing, i.e. they contain some measure of mixed mating. Ball clover was the only exception occurring between facultative outcrossing and obligate outcrossing. Persian clover, although an outcrosser, has a mixed mating system with a significant proportion of inbreeding occurring.

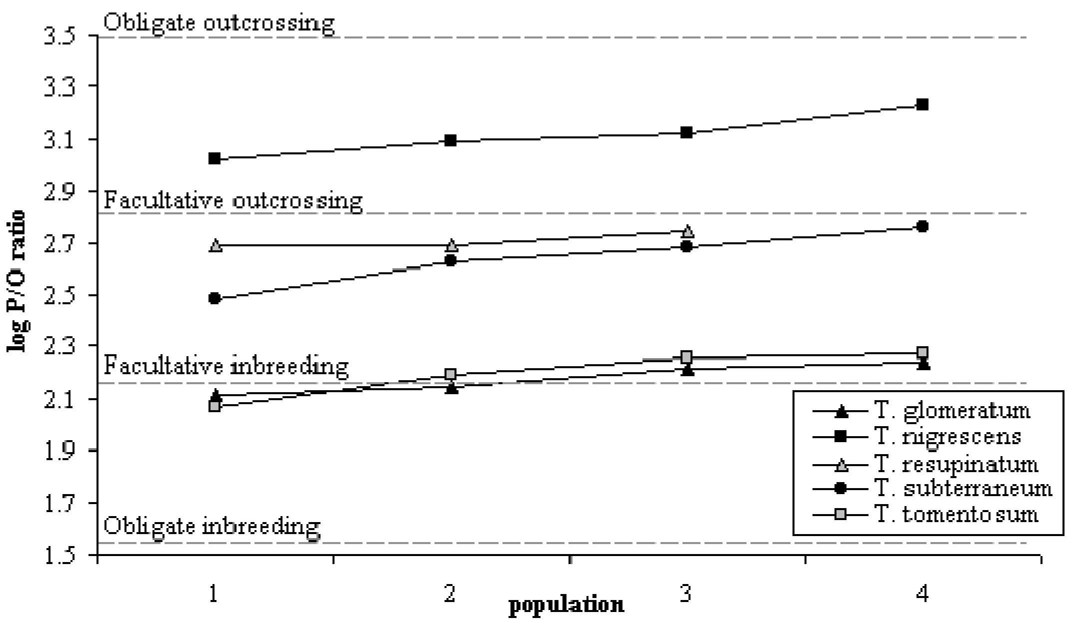

Figure 1. Logarithmic pollen-ovule ratios by species. Dashed lines show Cruden's (5) estimates of breeding system

The results shown for subterranean clover were not as expected, as it has previously been reported as having only a moderate level of outcrossing, 0-22% (8), compared to the mixed mating system shown in Table 1 and Figure 1. It is also the only species of the five included in the study that can be described as being large seeded. All the other species are small seeded with mean seed weights of less than 0.5mg. Therefore correlation coefficients were calculated between pollen-ovule ratios, mean seed weights and pollen grains per flower for all five species, and for the three purported inbreeding species; cluster, woolly and subterranean clover alone. The results are shown in Table 2, where it can be seen that across all five species there is only a very low correlation between seed weight and the other characters. However, when only the inbreeding species are included in the calculation the correlation increases to 0.9 for both pollen-ovule ratio, and pollen grains per flower by seed weight.

Table 2. Correlation of pollen grain characters, pollen grains per flower and pollen-ovule ratio (p/o ratio), in relation to mean seed weight. Correlations are calculated using all species, and the inbreeding species; cluster, woolly and subterranean clover alone.

All species	pollen grains per flower	log (p/o ratio)	Inbreeding species	pollen grains per flower	log (p/o ratio)
mean seed weight (mg)	-0.2210	0.0780	mean seed weight (mg)	0.912	0.9020
log (p/o ratio)	0.8600		log (p/o ratio)	0.984	

Discussion

Cruden (5) developed the method of pollen-ovule ratios as an estimate of breeding system using 80 different species from a range of genera. From these results he was able to provide a table containing the pollen-ovule ratio levels for six different breeding systems; cleistogamous, obligate inbreeder, facultative inbreeder, facultative outcrosser and obligate outcrosser. However, he has been criticised for publishing a table with definitive pollen-ovule ratios for each breeding system when there are a number of other factors that are likely to affect the amount of pollen produced by a flower. These include whether a pollen vector is required for pollination, whether pollen or nectar is the main reward and when the flowers are taken from the plant (12). It is well documented that wind pollinated species produce more pollen than insect pollinated species (for example; 9, 12). Preston (12) therefore conducted a similar experiment using 66 species from the Cruciferae. The species were chosen because they have relatively uncomplicated breeding systems, and their breeding system was previously known. His results also showed that pollen ovule ratios were a relevant measure of breeding system, but that the other factors listed above amongst others needed to be understood and accounted for.

The clover species included in this study all have similar breeding morphology, as they all produce 10 anthers, and bees are the main pollinators where required. The main difference between the species that needs to be accounted for is the large seed size of subterranean clover, compared to the other species. Gallardo *et al.* (7) used pollen-ovule ratios as a measure of estimating the breeding system in *Astragalus* subgenus *Epiglottis*. These species included were all annuals, and were found to vary from inbreeding to outcrossing. However, they found that there was only a low correlation between autofertility results and pollen-ovule ratios, and suggest that this is a result of factors such as seed size which are correlated to pollen-ovule ratio, but only when the breeding system is constant. The results in Table 2 show that there is a high correlation between pollen-ovule ratio when only the inbreeding species are included in the analysis, compared to when all the species. In light of previous work on the rate of outcrossing in subterranean clover (8) which suggests that the rate is lower than the mixed mating system shown in this study, it is suggested that seed size is having an effect on the pollen-ovule ratio, and that it would need to be accounted for before the rate of outcrossing could be determined from the graph presented.

For the other species the rate of outcrossing estimated from the pollen-ovule ratio is thought to be more accurate. Taylor and Gillett(15) following crossing studies on species of the section *Vesicaria* found that woolly clover was inbreeding, and Persian clover was usually outcrossing. However they found one accession of Persian clover which contained a mixture of inbreeding and outcrossing plants. Pagnotta *et al.* (11) found that although woolly clover has been suggested as being highly inbreeding, rare outcrossing events do occur. A similar system has been suggested for cluster clover (1). Ball clover is the only species to have been reported to be self-incompatible and outcrossing (4). Further work is necessary using molecular techniques to provide a definitive measure of rate of outcrossing in each population, which can then be related to the ratios obtained in this study. As all the other species have similar seed sizes and flower morphology, it is felt that the pollen-ovule ratios obtained here are an accurate estimate of their rate of outcrossing.

Variation between species can not be explained by differences in pollination methods, breeding morphology or seed size. Ecogeographical data were collected at the site of collection and data such as rainfall or soil pH may be important in explaining variation in pollen-ovule ratios within a species. Ecogeographical data as a source of within species variation in pollen-ovule ratios will be explored in a later paper, as it will have important implications in understanding changes in the rate of outcrossing in colonising species.

These results have important implications in plant breeding. The breeding system of a species affects both the method of collection that should be used to conserve its genetic variation most effectively, and how its genetic resources should be used most efficiently in a breeding program (3). Using the method of pollen-ovule ratios, none of the species included in this study were found to be 100% inbreeding. Therefore crossing events between cultivars or lines will always be possible. Adaptation to a new environment is thought to be quicker in an outcrossing species, and with current research on new perennial species, adaptation may be an important characteristic. However, even a small amount of outcrossing in an inbreeding species can rapidly increase the genetic variation of a population, and therefore its adaptation. Subterranean clover is a good example of this as limited amounts of outcrossing have lead to numerous new strains appearing in old sown pastures (13, 14). The naturalised clovers in Australia have also shown the same large genetic variation despite being inbreeding, and probably initially suffering from genetic bottlenecks (1,2).

Conclusions

Pollen-ovule ratios are a quick, low cost measure of determining the rate of outcrossing in a population or species. However, care must be taken in interpreting the results as factors such as pollen vector required for pollination, whether pollen or nectar is the main reward and when the flowers are taken from the plant are likely to be affecting the amount of pollen that is produced by a flower. In this study a high correlation was found between seed size and pollen-ovule ratio in the inbreeding species, suggesting that this may also be affecting the results. Further research is required to quantify the effect of seed size.

However, the results obtained for the other species, are felt to be an accurate estimate of the rate of outcrossing, and provide information that will be important in both plant collection missions, and plant breeding programs.

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