

Sexual Symmetry in Natural Populations of the Patagonian Cypress (*Austrocedrus chilensis*)

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Abstract

Sexual symmetry, defined as equal allelic frequencies among reproduction effective gametes of both sexes, was analysed by means of 10 isozyme loci in three natural populations of *Austrocedrus chilensis* (dioecious and wind pollinated tree species). Haplotypes of effective gametes were inferred by analysing side-by-side both the embryo and the endosperm of seeds collected from 20 to 27 trees per population. The allelic frequencies of effective ovules and pollen were compared in each of the three populations. The hypothesis of sexual symmetry could only be rejected in case of one locus in two out of three analysed populations. That is, most of the loci surveyed turned out to be symmetric in the three sampled populations in spite of their contrasting environmental conditions. Therefore, sexual symmetry in *A. chilensis* seems to be mainly uninfluenced by the environment. On the other hand, all loci showed Hardy-Weinberg (HW) proportions in the three populations, even those that resulted asymmetric. HW structure is usually considered as enough evidence of panmixia, what implies sexual symmetry, and consequently this result gives an example of the low reliability of indirect methods of testing genetic processes, such as the classical HW test.

Key words: reproductive system, *Austrocedrus*, isozymes, *Cupressaceae*.

Introduction

When studying the performance of seed orchards of monoecious species, it is particularly useful to reduce the definition of sexual symmetry to 'equal contribution of gametes through pollen and ovules' (CHOI *et al.*, 2004). However, under a broader sense, sexual symmetry in a population is defined as equal allelic frequencies among the reproduction effective gametes of both sexes, the term "effective" referring to those gametes that managed to merge into new zygotes forming viable seeds, also called 'successful gametes'. This balance between the genetic constitution of effective ovules and effective pollen plays an important role in the interpretation of the reproductive system of any forest tree species, which should be known on the whole if practical measures in conservation and breeding are going to be decided.

Sexual asymmetry causes an excess of heterozygotes with respect to the Hardy-Weinberg (HW) proportions (WALLACE, 1958; PURSER, 1966; ZIEHE and GREGORIUS, 1981), what, for instance, can compensate the homozygous excess that inbreed implies, thus resulting in HW structure and leaving the severity of inbreeding totally masked (ZIEHE and HATTEMER, 1985). In fact, sexual symmetry is commonly assumed for all the loci of any forest tree species, but this is an arbitrary simplification of the complexity of its reproductive system.

Three factors are involved in the determination of sexual asymmetry of a certain locus: a) a differential gamete production for each sexual type (differential fertility), b) a differential gamete survival for each sexual type (differential viability), and c) a differential gamete efficiency for each sexual type

(assorted mating system). All three factors are considered in the proportion of the alleles of a certain locus (ZIEHE and GREGORIUS, 1981). Since environmental conditions influence these factors, sexual symmetry can vary from population to population and from year to year. Thus, the sexual symmetric condition is expected to be a character of a particular locus of a species in a certain population in a given year. If symmetry or asymmetry at certain loci can be proved to be a constant feature across populations or across years instead, a strong genetic control should be assumed for this character.

Allelic frequencies of effective ovule and pollen subpopulations of the parental generation are the basis for any analysis of sexual symmetry in a population, and can easily be calculated when in its progeny the ordered genotypes of embryos are available, i.e. when the origin of each allele in embryos (originating from the male or the female gamete) is known. As early shown by MÜLLER (1976), in almost all the gymnosperms, a direct discrimination is possible through the side-by-side analysis of both embryo and endosperm (which is a haploid tissue and a maternal gamete equivalent) of each seed. This allows inferring maternal or paternal origin of each allele in the embryo, since one of the two must be equal to the haplotype of the corresponding endosperm.

The "Patagonian Cypress" ("Ciprés de la Cordillera", *Austrocedrus chilensis* (D. DON) PIC. SER. ET BIZZARRI), a dioecious (BRION *et al.*, 1993, CASTOR *et al.*, 1996) evergreen forestry species native to the Patagonian Andes, is the most important conifer (*Cupressaceae*) of the temperate forests of Argentina. Knowledge about any aspect of its reproductive system will contribute to the conservation and breeding programs of this species, the latter of which is currently being started in Argentina (INTA EEA Bariloche, Unidad de Genética Forestal).

Based on isozyme data, this study presents the results of the first analysis of sexual symmetry in *Austrocedrus chilensis* natural populations. For this species, which can be found in extremely different environmental conditions, the environment is hypothesized to be of great influence in the balance of the allelic frequencies of both gamete types in most of the loci. A direct method of testing symmetry will be additionally confronted to the results of the classical "Hardy-Weinberg (HW) test", which is supposed to be not powerful enough as to give reliable results.

Materials and Methods

A genetic inventory at the embryo stage was performed in three Argentine natural populations of *Austrocedrus chilensis* arrayed in a latitudinal transect that surveys the precipitation gradient of the species' range. The environmental conditions of each one of these populations are strongly diverging. The humid extreme (1,500 mm mean annual precipitation) is represented by a population called Llao Llao (L), located 25 km westward from San Carlos de Bariloche, in North Patagonia. It

corresponds to the mixed forest type that the species usually builds with *Nothofagus dombeyi* (MIRB.) OERST. Cerro Otto population (O), which is a pure dense forest in the environs of San Carlos de Bariloche, represents the mesic site (ca. 1,000 mm of mean annual precipitation). The arid extreme (330 mm per year) is represented by an isolated population called Pilcañeu (P), which occupies an area smaller than 4 ha and is located at the top of a rocky hill surrounded by the Patagonian steppe, 50 km eastward from San Carlos de Bariloche.

Seeds were collected in the three populations described. In order to minimize the probability of sampling related individuals, approximately 30 meters were kept as the minimum distance between seed trees but in Pilcañeu population due to its reduced size.

Ordered genotyping of embryos was assayed at 10 polymorphic isozyme marker loci, whose mode of inheritance and related laboratory procedures were shown in previous studies (GALLO and GEBUREK, 1994; PASTORINO and GALLO, 1998 and 2001). The following enzyme systems were analysed: 6-phosphogluconate dehydrogenase, 6-PGDH, EC 1.1.1.14; malate dehydrogenase, MDH, EC 1.1.1.37; isocitrate dehydrogenase, IDH, EC 1.1.1.42; shikimate dehydrogenase, SKDH, EC 1.1.1.25; glutamate-oxaloacetate transaminase, GOT, EC 2.6.1.1; superoxide dismutase, SOD, EC 1.15.1.1; phosphoglucose isomerase, PGI, EC 5.3.1.9.

Six embryos from each of 27 trees from P, 20 from O and 20 from L were analyzed electrophoretically side-by-side with their corresponding megagametophytes. The sample size is 162 embryos for the first population and 120 for each of the other ones.

The allelic frequencies of both gamete subpopulations were compared in each population through a homogeneity G test per locus (SOKAL and ROHLF, 1981). A Hardy-Weinberg (HW) test was performed for each locus in the three populations at the embryo stage.

The computer program GSED (Genetic Structure from Electrophoresis Data) version 1.1d (GILLET, 1997) was utilized to calculate the allelic frequencies and to perform the homogeneity and the HW tests as well. In cases of rejection of the null hypotheses with expected frequencies of less than 5, the exact two-tailed test on binomial distribution was run using *exactst* program (GILLET, pers. com.).

Results and Discussion

Allelic frequencies of effective ovules and pollen from Pilcañeu, Cerro Otto and Liao Liao populations are presented in Table 1. Three loci have been found to be monomorphic in these three populations: *Mdh3*, *Mdh4* and *Idh2*.

Homogeneity between allelic frequencies of both gamete types could be rejected only for *Mdh2* locus in Pilcañeu population and for *Got1* locus in Liao Liao population. Consequently, sexual asymmetry was shown in no more than one locus in each population, and not in all of the analysed populations. Furthermore, the asymmetric locus was not the same in the populations in which asymmetry was found. Thus, the allelic frequencies of most of the loci surveyed show an equilibrium between both gamete types.

Since the loci that are found to be asymmetric in one population were symmetric in the others, a high influence of the environmental conditions of their sexual asymmetry should be accepted. Thus, a variation in these two loci is also expected from year to year, so that they could reach symmetry in a following mating season. This is true particularly for these two loci. However, most of the loci surveyed turned out to be symmetric in the three sampled populations in spite of their contrasting environmental conditions. Therefore, sexual symmetry in *A. chilensis* seems to be mostly uninfluenced by the environment, what refuses the original hypothesis. That is, no matter the environmental conditions, the reproductive system of *A. chilensis* by itself seems to manage to balance the allele frequencies between both gamete types as indicated by most of the loci. This is the main result of the present study.

All loci presented HW structure in the three populations, even those that turned out to be asymmetric (Table 1). Such results of HW tests are usually interpreted as enough evidence of random fusion of gametes and sexual symmetry in the populations involved.

In the two loci at which sexual asymmetry was detected, a factor causing homozygous excess is obviously compensating the homozygous decrease that asymmetry causes. A previous study in Pilcañeu population indicated that random mating was not occurring (PASTORINO and GALLO, accepted). Instead, evidence of locally distributed pollen clouds was presented, what probably implies mating among related individuals and thus a certain amount of inbreeding. The same might be occur-

Table 1. – Allelic frequencies among female and male effective gametes, corresponding homogeneity G tests and zygotic Hardy-Weinberg (HW) expectations in three populations of *Austrocedrus chilensis*. Results of the tests are expressed in terms of probability.

Locus	Allele	<i>Got1</i>		<i>Got3</i>		<i>Sod</i>		<i>Pgi2</i>		<i>6Pgd2</i>		<i>Mdh2</i>		<i>Skdh</i>	
		100	97	100	50	100	115	100	90	100	78	100	72	100	94
Pilcañeu population	ovule	0.559	0.441	1.000	0.000	0.235	0.765	0.887	0.113	0.852	0.148	0.889	0.111	0.290	0.710
	pollen	0.625	0.375	0.994	0.006	0.296	0.704	0.861	0.139	0.914	0.086	0.981	0.019	0.198	0.802
	Homog. test	0.229		0.237		0.208		0.487		0.083		0.000		0.052	
	HW test	0.644		0.956		0.526		0.661		0.307		0.151		0.481	
Cerro Otto population	ovule	0.772	0.228	1.000	0.000	0.275	0.725	0.776	0.224	0.850	0.150	1.000	0.000	0.412	0.588
	pollen	0.867	0.133	1.000	0.000	0.300	0.700	0.704	0.296	0.842	0.158	1.000	0.000	0.445	0.555
	Homog. test	0.061		--		0.669		0.254		0.858		--		0.600	
	HW test	0.168		--		0.176		0.026 ¹		0.158		--		0.956	
Liao Liao population	ovule	0.981	0.019	0.974	0.026	0.242	0.758	0.725	0.275	0.866	0.134	1.000	0.000	0.374	0.626
	pollen	0.905	0.095	1.000	0.000	0.217	0.783	0.716	0.284	0.874	0.126	1.000	0.000	0.307	0.693
	Homog. test	0.013		0.040 ^{#2}		0.645		0.877		0.847		--		0.285	
	HW test	0.320		0.843		0.175		1		1		--		0.492	

[#]expected frequencies less than 5, ¹the exact test threw a probability value of 0.069, ²the exact test threw a probability value of 0.366, -- monomorphic in both subpopulations compared.

ring in Llao Llao population, but we cannot generalize such observation to all of the populations of the species.

Indirect methods, like HW test, suffer from the shortcoming of being based on too many assumptions, which can hardly be controlled in order to test them separately. Direct procedures of testing any kind of genetic processes should always be preferred. Otherwise, conclusions could be no more than speculation based on mathematical models not well adjusted to the real biological processes.

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PCR-Multiplex of Six Chloroplast Microsatellites for Population Studies and Genetic Typing in *Pinus sylvestris*

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Abstract

The major drawback of microsatellites analysis is that they are expensive to develop, labor-intensive and demand skilled personnel. However, such studies might be still simplified and accelerated by multiplexing of the markers and the use of high-throughput systems for genotyping DNA fragments. In this paper we present a single, simple and highly effective PCR-multiplex reaction composed of six chloroplast microsatellites widely used for population studies in pines but here applied to *Pinus sylvestris*. The reaction allows for rapid genotyping of large sample sizes.

Key words: chloroplast microsatellites; PCR-multiplex, *Pinus sylvestris*; polymorphism; haplotypes.

Introduction

The set of microsatellites for *Pinus* species has been developed by VENDRAMIN et al. (1996) and PROVAN et al. (1998). Since then, the chloroplast microsatellites have become an efficient and powerful marker for studying genetic diversity and differentiation among conifers (ECHT et al., 1998; GUGERLI et al., 2001; WALTER and EPPERSON, 2001). They also have proved to be useful for measuring pollen contamination in seed orchards (PLOMION et al., 2001), or for the identification of the origin of populations with uncertain history (RIBEIRO et al., 2002).

The availability of high-throughput systems for genotyping DNA fragments (automated sequencers) involving multicolor