Restoration Ecology
THE JOURNAL OF THE SOCIETY FOR ECOLOGICAL RESTORATION INTERNATIONAL

RESPONSE ARTICLE

How Many Seed Transfer Zones Are Necessary for the Preservation of the Genetic Identity of *Austrocedrus chilensis* Natural Populations in Argentina?

Mario J. Pastorino[1,2,3]

Abstract

Two different studies based on isozymes that include genetic structure analysis have arrived at contrasting conclusions regarding the minimum number of seed transfer zones for Patagonian cypress (*Austrocedrus chilensis*) in Argentina that are required in order to avoid genetic contamination in restoration programs. Unfortunately, the more recent article lacks discussion on these controversial results, which is, therefore, the purpose of this article. The reliability of the markers used and the sampling performed in these studies are evaluated comparatively. The later study found higher levels of diversity and differentiation but paradoxically suggested that only two seed transfer zones would be enough to preserve the genetic identity of the natural populations of the species, whereas the earlier study concluded that at least five are necessary. Arguments are presented here for the case that definition of fewer than five genetically homogeneous groups is absolutely inappropriate and implies a probable risk of genetic contamination and maladaptation.

Key words: genetic structure, operational genetic management units, Patagonian cypress, provenance regions.

Introduction

After several years of debate in the Argentinean community, the national Native Forest Law (N° 26.331) finally came into force in 2009, establishing minimum standards for environmental protection in order to enrich, restore, preserve, manage, and make sustainable use of Argentina's native forests. This novel and promising law promotes and funds the restoration of degraded forest ecosystems, making active intervention possible. As a consequence, restoration projects are gradually being developed in the Andean forests of Patagonia, which in turn demand the definition of seed transfer zones for the main forest tree species of the region. Although there is a vast tradition of delineation of such operational genetic management units in North America and Europe, few examples exist in Latin America (Vergara 2000) and none in Argentina.

A recent genetic diversity study, based on isozymes and including structure analysis (Souto et al. 2011), has concluded that two seed transfer zones could be enough to preserve the genetic identity of *Austrocedrus chilensis* (D. Don) Pic. Ser. et Bizzarri natural populations in Argentina. However, a previous genetic diversity study also based on isozymes and also including structure analysis (Pastorino & Gallo 2009a, 2009b) concluded that at least five seed transfer zones are necessary. These controversial conclusions warrant discussion, which unfortunately was absent from the more recent article. Therefore, this discussion is the specific purpose of this article.

The Tool

Both studies are based on isozymes. However, only Pastorino and Gallo (2009a) performed genetic control analysis in order to ensure that the bands observed in the zymograms are not mere phenotypes but gene markers (Pastorino & Gallo 1998, 2001). This analysis has been recommended repeatedly in the literature (e.g. Gottlieb 1977; Crawford 1983; Bergmann & Hattemer 1998; El-Kassaby & Ritland 1998). Simple cases of monomeric enzymes encoded by a single locus (e.g. SKDH E.C.1.1.1.25 in *Austrocedrus chilensis*) can "work acceptably well" on the basis of untested assumptions regarding their inheritance mode. However, in complex cases such as MDH E.C.1.1.1.37 in *A. chilensis*, which besides being dimeric is encoded by at least three independent loci [five activity zones were reported by Pastorino & Gallo (1998)], the lack of genetic control analysis, even in haploid tissues,

[1]Unidad de Genética Ecológica y Mejoramiento Forestal, Instituto Nacional de Tecnología Agropecuaria (INTA), EEA Bariloche, CC 277, 8400 S.C. de Bariloche, Río Negro, Argentina
[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[3]Address correspondence to M. J. Pastorino, email mpastorino@bariloche.inta.gov.ar

doi: 10.1111/j.1526-100X.2012.00899.x

makes their interpretation extremely dubious. Moreover, using diploid material for electrophoreses, as Souto et al. (2011) did, doubles the complexity of zymogram interpretation. In conifers, adults can be genotyped by analyzing a set of megagametophytes, namely haploid tissue. For these complex cases in particular the use of haploid tissue is necessary. This procedure multiplies the experimental effort several times [Pastorino & Gallo (2009a) subjected at least eight megagametophytes per individual to electrophoresis], but, in these cases, is the only way to get reliable data. Furthermore, null alleles such as *Got1-n* and *Got2-n* in *A. chilensis* (Pastorino & Gallo 1998, 2001) cannot be identified at all on diploid tissue.

Souto et al. (2011) revealed eight enzymes, whereas Pastorino and Gallo (2009a) revealed just seven, of which five were coincident. One of the non-coinciding enzymes in the set utilized by Souto et al. (2011) is cathodal peroxidase (Per Cat E.C.1.11.1.7). Peroxidases are known to vary occasionally in relation to the environment (Bergmann et al. 1989; Bergmann & Hattemer 1998), a feature that should be tested on the species of interest before using this kind of enzyme in population genetic studies.

Finally, Souto et al. (2011) stated erroneously that in the study of Pastorino and Gallo (2009a) only five loci were utilized, which was presented as an argument for a new study. However, both studies are based on 12 polymorphic gene loci [putative gene loci in the case of Souto et al. (2011)].

Sampling

A considerable difference in sample size appears to exist between studies, because Souto et al. (2011) collected leaves at 67 sampling points, whereas Pastorino and Gallo (2009a) only sampled 27 populations. However, those 67 sampling points can hardly be considered 67 different populations because of the small distance that separates them. From the geographical coordinates of Appendix 2 in Souto et al. (2011), it is clear that sampling was carried out in clusters. For example, sampling points 12, 18, 20, 22, and 23 are located alongside Aluminé river, with a distance of 3.300 m between the first and last one. In addition, points 11 and 19 are approximately 2.000 m distant from this group, thereby forming a cluster of seven sampling points that should be considered a single population. And this is not the only case; for example, points 26 and 27 are separated by 500 m, and the most extreme example: points 4 (39° 02′ 51.0″ S, 70° 59′ 48.7″ W) and 10 (39° 02′ 53.0″ S, 70° 59′ 48.3″ W) have almost the same geographical coordinates.

A recent study (Colabella 2011) has fitted dispersal pollen curves for *Austrocedrus chilensis* in a marginal population, estimating a mean effective pollination distance of 1.000 m. Moreover, the leptokurtic feature of the fitted curves described the occurrence of large distance pollination events of at least 10 km. Unless additional information is presented showing genetic isolation between particular forest fragments, a distance of less than 5.000 m cannot be accepted as enough evidence of isolation. Under this conservative assumption, the 67 sampling points of Souto et al. (2011) would represent only 38 populations.

Pastorino and Gallo (2009a) had already shown that for the characterization of genetic variation, more important than extensive sampling is a good sampling strategy, that is, to sample suitable populations. New populations added to a previous study (Pastorino et al. 2004) have altered neither the estimation of the amount nor the distribution of genetic variation in *A. chilensis* Argentinean populations.

Both studies distributed the sampling points across the entire Argentinean range of the species and in several cases even sampled the same populations. However, a couple of differences must be pointed out. Souto et al. (2011) did not sample the northern extreme of the Argentinean range, which has a 100 km isolation from the next forest patch (Pastorino et al. 2006), or the forest around El Bolsón city, which is one of the most conspicuous of the entire *A. chilensis* natural distribution. They also left a space of 125 km without sampling points between the northern and central populations, and in general, they sampled the dry side of the precipitation gradient more thoroughly, with some prominent gaps on the wet side of Traful, Nahuel Huapi, and Puelo basins. Pastorino and Gallo (2009a, 2009b) did not sample the riparian forest along Aluminé River, or the forests within the Futalaufquen basin, and in general the sampling points were distributed taking into account the relative relevance of each forest patch in terms of size and continuity. Thus, in the areas with small patches scattered in a steppe matrix, such as the northern region in the study of Souto et al. (2011), few points were sampled.

Results

Souto et al. (2011) found higher levels of diversity and differentiation not only at species level but also at population level. As a general result, this could be attributed to sampling differences, because Souto et al. (2011) over-represented the marginal forest patches from the steppe, which turned out to be the most diverse in both studies.

However, higher levels of diversity were also estimated within each population. The fact that some populations are common to both studies gives us an opportunity to make a direct comparison of the intrapopulation level of variation estimated in each case. Some examples presented in Table 1 make these contrasting results evident.

Why do two different diversity studies based on the same tool produce such different results? I think there is no obvious answer to this question. I can speculate about three main reasons:

(1) The fact that Souto et al. (2011) did not perform any analysis to test the genetic control of the bands observed in their zymograms casts some doubt on the reliability of their data.
(2) Pastorino and Gallo (2009a) analyzed megagametophytes, whereas Souto et al. (2011) worked on leaf tissue. It has been shown in several plant species that isozyme profiles of a single individual may vary with organ or tissue, or be present or absent depending on the organ, tissue, or

Table 1. Intrapopulation variation parameters calculated by two different studies (Pastorino & Gallo 2009a [Pastorino] and Souto et al. 2011 [Souto]) in five populations of *Austrocedrus chilensis*.

Populations	*Reference*	n	A/L	A_e	H_e	H_o
Ao. Catan Lil	Pastorino	20	1.58	1.22	0.186	0.188
Sampling point 7	Souto	25	2.20	1.67	0.364	0.350
Confluencia	Pastorino	30	1.58	1.13	0.119	0.104
Sampling point 43	Souto	29	2.08	1.21	0.157	0.141
Ao. Chacay	Pastorino	20	1.58	1.19	0.166	0.166
Sampling point 27	Souto	28	1.90	1.48	0.226	0.143
Pilcañeu North	Pastorino	44	1.50	1.19	0.159	0.146
Sampling point 37	Souto	30	2.42	1.39	0.238	0.226
Ao. La Fragua	Pastorino	32	1.50	1.19	0.162	0.154
Sampling point 30	Souto	30	2.00	1.31	0.213	0.214

Number of sampled trees per population (n), number of alleles per locus (A/L), number of effective alleles (A_e), expected heterozygosity (H_e), and observed heterozygosity (H_o) of each population.

stage of development surveyed (Scandalios 1969; Feret & Bergmann 1976). In fact, Pastorino (2000) reported the absence of the band corresponding to the locus *Idh1* in *Austrocedrus chilensis* embryos, which is observed clearly in megagametophytes. Hence, it is possible that in spite of revealing the same enzymes, the studies have observed different zymograms.

(3) Diversity studies performed with genetic markers are based on a random sample of genes, typically including some 10 genes. This is really a sample of constrained size. Consequently, it does not seem impossible that different samples give different results. This argument is disquieting, because to my knowledge this possibility is never considered, even though it would invalidate comparisons between different studies, which are quite common in the literature. An experiment conducted with this specific purpose should be performed to test this hypothesis.

Fortunately, both studies coincided in the general pattern of genetic structure, that is, a latitudinal trend of diversity, decreasing from north to south, with the marginal populations from the steppe being the most diverse. Moreover, there is agreement on the relevance of the last glaciation in modeling the present genetic structure, and a similar agreement can also be noted regarding the role of fire if a previous article of Pastorino and Gallo (2002) is considered.

Conclusions

In spite of a similar result concerning the general pattern of genetic structure of *Austrocedrus chilensis* in Argentina, the two studies arrived at different conclusions. The analysis of genetic structure performed by Souto et al. (2011) did not combine the different factors that caused the present pattern of diversity. They lacked a method for testing different proposed population groupings, such as that used by Pastorino and Gallo (2009a), so they attempted to recognize genetically homogeneous groups of populations by an admixture analysis alone, which takes into consideration the genetic frequencies but not the geographical position of the populations and the life history implied by those locations. This procedure prevented them from recognizing that other effects are superimposed on the latitudinal pattern. Thus, they arrived at the definition of only two homogeneous groups (north and south), whereas Pastorino and Gallo (2009a, 2009b) had defined five, including: (1) northern group (populations northward to 41° 30′ S); (2) central group (populations between 41° 30′ S and 42° 30′ S); (3) southern group (populations southward to 42° 30′ S); (4) ice-border group (non-glaciated populations of the ecotone in the center of the species Argentinean range); and (5) ancient-distribution group (non-glaciated extremely isolated populations of the northern half of the Argentinean species range). The definition of fewer homogeneous groups in the study of Souto et al. (2011) is paradoxical, because they found a higher level of differentiation among populations [F_{ST} was 0.116 in Souto et al. (2011), whereas only 0.060 in Pastorino and Gallo (2009a)].

The definition of genetically homogeneous groups is relevant for several purposes and has direct consequences on the selection of seed sources in restoration programs. I find it extremely inappropriate to consider fewer than five genetically homogeneous groups. The marginal populations from the steppe cannot be merged with the continuous wetter forest of the west, not only because of their possible adaptation to the quite different current environmental conditions but also because of substantial differences in their life histories. This distinction is relevant because it is easier to collect seeds in the former because of their short, ramose and productive trees, which would probably be the preferred source. Lack of such a distinction can lead to "genetic contamination" and "maladaptation processes" (McKay et al. 2005) when restoring the continuous wetter forest. Similarly, Pastorino and Gallo (2009a, 2009b) were able to distinguish two groups (central and south) in the area where Souto et al. (2011) defined only one. This particular result may be due to the inadequate sampling of Souto et al. (2011) of the extensive, continuous forest around El Bolsón city. The diversity level observed there leads to the discrimination of those populations from populations of the southern extreme of the species range, which are clearly less diverse.

Finally, as suggested by Souto et al. (2011), knowledge of genetic variation in adaptive traits will be the key to the delineation of definitive operational genetic management units for the Patagonian cypress. These steps are now in progress. Several contributions have already been made (Aparicio et al. 2010, 2012; Pastorino et al. 2010), and others have studies underway.

Implications for Practice

- The definition of only two seed transfer zones for Patagonian cypress in its Argentinean range is not enough to preserve the genetic identity of its natural populations.

- At least five seed transfer zones must be delineated in order to avoid the risks of genetic contamination and maladaptation in active restoration programs.
- Isozymes are a useful tool for the characterization of the pattern of genetic diversity of Patagonian cypress, which can contribute to the delineation of operational genetic management units such as seed transfer zones, but an analysis of genetic control of the zymograms is necessary for reliable data.

Acknowledgments

I am grateful to P. Marchelli and L. A. Gallo for their critical reading of the manuscript and their helpful comments.

LITERATURE CITED

Aparicio, G. A., M. J. Pastorino, and L. A. Gallo. 2010. Genetic variation of early height growth traits at the xeric limits of *Austrocedrus chilensis* (Cupressaceae). Austral Ecology **35:**825–836.

Aparicio, A. G., S. M. Zuki, M. J. Pastorino, A. G. Martinez-Meier, and L. Gallo 2012. Heritable variation in the survival of seedlings from Patagonian cypress marginal xeric populations coping with drought and extreme cold. Tree Genetics & Genomes, DOI: 10.1007/s11295-011-0465-4.

Bergmann, F., and H. H. Hattemer 1998. Isozymes in forest genetics research. Pages 227–238 in A. K. Mandal and G. L. Gibson, editors. Forest genetics and tree breeding. CBS Publishers & Distributors, New Delhi, India.

Bergmann, F., H.-R. Gregorius, and F. Scholz 1989. Isoenzymes, indicators of environmental impacts on plants or environmentally stable gene markers?. Pages 19–27 in F. Scholz, H.-R. Gregorius and D. Rudin, editors. Genetic effects of air pollutants in forest tree populations. Springer, Berlin, Heidelberg, New York, London, Paris, Tokyo, Hong Kong.

Colabella, F. 2011. Análisis de flujo polínico en una población fragmentada de Ciprés de la Cordillera (*Austrocedrus chilensis*). Page 69 in Tesis de Licenciatura en Biotecnología, Universidad Nacional de San Martín, Escuela de Ciencia y Tecnología. San Carlos de Bariloche, Argentina.

Crawford, D. J. 1983. Phylogenetic and systematic inferences from electrophoretic studies. Pages 257–289 in S. D. Tanksley and T. J. Orton, editors. Isozymes in plant breeding and genetics. Elsevier, Amsterdam, Oxford, New York.

El-Kassaby, Y. A., and K. Ritland 1998. Isozymes: techniques, interpretation and application to forest genetics. Pages 211–226 in A. K. Mandal and G. L. Gibson, editors. Forest genetics and tree breeding. CBS Publishers & Distributors, New Delhi, India.

Feret, P. P., and F. Bergmann 1976. Gel electrophoresis of proteins and enzymes. Pages 49–77 in J. P. Miksche, editor. Modern methods in forest genetics. Springer, Berlin, Heidelberg, New York.

Gottlieb, L. D. 1977. Electrophoretic evidence and plant systematic. Annals of the Missouri Botanical Garden **64:**161–180.

McKay, J. K., C. E. Christian, S. Harrison, and K. J. Rice 2005. "How local is local?" A review of practical and conceptual issues in the genetics of restoration. Restoration Ecology **13:**432–440.

Pastorino, M.J. 2000. Genetic variation and reproduction system of *Austrocedrus chilensis* (D.Don) Florin et Boutelje, a cypress endemic to the Andean-Patagonian Forest. Page 166 in Doctoral dissertation, Georg-August University of Göttingen, Göttingen.

Pastorino, M. J., and L. A. Gallo 1998. Inheritance of isozyme variants in *Austrocedrus chilensis* (D.Don) Florin et Boutelje. Silvae Genetica **47:**15–20.

Pastorino, M. J., and L. A. Gallo 2001. Linkage relationships as a useful tool to state interspecific gene homology: case study with allozyme loci in *Austrocedrus chilensis* (Cupressaceae). Silvae Genetica **50**:233–239.

Pastorino, M. J., and L. A. Gallo 2002. Quaternary evolutionary history of *Austrocedrus chilensis*, a cypress native to the Andean-Patagonian Forest. Journal of Biogeography **29**:1167–1178.

Pastorino, M. J., and L. A. Gallo 2009a. Preliminary operational genetic management units of a highly fragmented forest tree species of southern South America. Forest Ecology and Management **257**:2350–2358.

Pastorino, M.J. and L.A. Gallo. 2009b. Definición de regiones de procedencia para las especies nativas de mayor potencial de domesticación: resultados preliminares en Ciprés de la Cordillera. Congreso Forestal Mundial. Buenos Aires, 18–23 Octubre 2009.

Pastorino, M. J., L. A. Gallo, and H. H. Hattemer 2004. Genetic variation in natural populations of *Austrocedrus chilensis*, a cypress of the Andean-Patagonian Forest. Biochemical Systematics and Ecology **32**:993–1008.

Pastorino, M. J., M. M. Fariña, D. Bran, and L. A. Gallo 2006. Extremos geográficos de la distribución natural del "Ciprés de la Cordillera." Boletín de la Sociedad Argentina de Botánica **41**:307–311.

Pastorino, M. J., S. Ghirardi, J. Grosfeld, L. A. Gallo, and J. G. Puntieri 2010. Genetic variation in architectural seedling traits of Patagonian Cypress natural populations from the extremes of a precipitation range. Annals of Forest Science **67**:508–518.

Scandalios, J. G. 1969. Genetic control of multiple molecular forms of enzymes in plants: a review. Biochemical Genetics **3**:37–79.

Souto, C. P., K. Heinemann, T. Kitzberger, A. C. Newton, and A. C. Premoli 2011. Genetic diversity and structure in *Austrocedrus chilensis* populations: implications for dryland forest restoration. Restoration Ecology, DOI: 10.1111/j.1526-100X.2011.00829.x.

Vergara, R. 2000. Regiones de procedencia de *Nothofagus alpina* y *Nothofagus obliqua*. Pages 121–132 in C. R. Ipinza, C. B. Gutierrez and S. V. Emhart, editors. Domesticación y Mejora Genética de Raulí y Roble. Instituto Forestal, Universidad Austral de Chile, Chile.