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Linkage Relationships as a Useful Tool to State Interspecific Gene Homology: Case Study with Isozyme Loci in *Austrocedrus chilensis (Cupressaceae)*

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Summary

Linkage relationships among 12 polymorphic isozyme loci were analyzed in *Austrocedrus chilensis* (D. DON) FLORIN et BOUTELJE. Double or multiple heterozygous individuals were chosen from a general genetic inventory of 403 trees. Twenty-nine out of 66 possible pairwise combinations were found.

Between 21 to 187 macrogametophytes per tree were subjected to horizontal starch gel electrophoresis. Linkage was proved through individual and pooled data between four pairs of genes. Three of them showed a tight linkage: *Aat2~6Pgdh2* (frequency of recombination **R** = 0.122 ± 0.036), *Aat3~Sod* (**R** = 0.125 ± 0.021) and *Aat1~Pgi2* (**R** = 0.143 ± 0.030), while a moderate linkage was found with respect to the pair *Idh2~Skdh* (**R** = 0.333 ± 0.052).

The inheritance of two allozyme gene loci was additionally proved: *Aat2* and *Pgi2*, each locus with at least two alleles. Thus, two new isozyme markers are reported for this species.

Different considerations for the establishment of a correct homology of gene loci before doing interspecific comparisons are discussed. The relative migration distance of isozymes on zymograms is proven as non sufficient evidence of homology of the encoding genes between species. On the other hand, linkages are shown to be a good tool for this purpose due to the highly conservative arrangement of the genes among related species.

Key words: Gene homology, linkage, allozyme, inheritance, *Cupressaceae*, *Austrocedrus chilensis.*

Introduction

In the last two decades, the development of QTL marker methods led to the construction of genetic linkage maps for several agronomic crop plants and a few forest tree species with the aim to verify correlations between markers and QTLs of economic importance. The number of polymorphic allozyme markers is, however, usually not large enough in order to use this type of marker for the identification of QTLs.

On the other hand, it has been suggested that gene arrangements are highly conservative within a certain family or at least within a certain genus (GURIES et al., 1978; CONKLE, 1981; KING and DANCIK, 1983; STRAUSS and CONKLE, 1986; GONCHARENKO et al., 1998). Therefore, the study of linkages of allozyme markers could efficiently contribute to the understanding of the evolution and the phylogenetic relationships of different related species.

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However, before doing gene comparisons among species, it is essential to ensure the homology of the involved genes. A direct comparison of the observed zymograms considering the relative migration of the bands is not always reliable. Differences have been detected even within the same taxonomic family (CHELIAK and PITEL, 1985).

Not all families of coniferous trees have been studied using isozyme markers with the same intensity. The *Pinaceae* family appears to be the most intensively studied group. On the other hand, not many studies dealing with *Cupressaceae*'s allozymes can be found in the literature, and only three of them analyze the linkage between the previously determined isozyme loci (HARRY, 1986; PERRY and KNOWLES, 1989; XIE et al., 1991).

In 94 the first publication appeared dealing with allozymes of *Austrocedrus chilensis* (D. DON) FLORIN ET BOUTELJE (GALLO and GEBUREK, 1994), 'Ciprés de la Cordillera', an important dioecious *Cupressaceae* endemic to the Andean-Patagonian Forest (HUECK, 1978; BRION et al., 1993). Since then, two new isozyme studies have been published (FERREYRA et al., 1996; PASTORINO and GALLO, 1998), so that a total of ten allozyme gene loci has been verified for this Patagonian cypress. However none of the mentioned works analyzes synteny of those markers.

In the present work, linkages of *A. chilensis* allozyme loci are analyzed and the results discussed emphasizing that the homology of gene loci must be taken into account before comparing results with those of other conifer species.

Material and Methods

Seeds of 403 trees from 16 populations mainly from the central part of the natural distribution area of *Austrocedrus chilensis* in Argentina were the basis for this linkage analysis.

In conifers, endosperm tissue is the immediate product of meiosis, so that linkages can be revealed directly by testing the independence of single-locus segregations in macrogametophytes. Therefore, single tree offspring could be used for this linkage analysis without the need of controlled crosses.

Double (or multiple) heterozygous trees were chosen from a general genetic inventory. Up to eight trees per double-locus combination were analyzed. Between 21 to 187 macrogametophytes per tree were subjected to horizontal starch gel electrophoresis, and seven enzyme systems were revealed (*Table 1*). Except for *Pgi2* and *Aat2*, the allozyme markers contained on them were already determined in previous works (GALLO and GEBUREK, 1994; PASTORINO and GALLO, 1998).

Table 1. – Revealed enzymes in *Austrocedrus chilensis* for the linkage analysis and the utilized buffers systems.

Enzyme	Abbreviat.	E. C. N°	Buffer System
6-phosphogluconate dehydrogenase	6-PGDH	1.1.1.44	Tris/citrate
Malate dehydrogenase	MDH	1.1.1.37	Tris/citrate
Isocitrate dehydrogenase	IDH	1.1.1.42	Tris/citrate
Shikimate dehydrogenase	SKDH	1.1.1.25	Tris/citrate
Aspartate aminotransferase	AAT (GOT)	2.6.1.1	Poulik
Superoxide dismutase	SOD	1.15.1.1	Poulik and Ashton
Phosphoglucose isomerase	PGI	5.3.1.9	Poul., Asht., T/c

Tris/citrate and Poulik buffer systems were used for the electrophoresis procedure. General recipes followed PASTORINO and GALLO (1998) with some little modifications. PVP (3%), EDTA (0.05%), sucrose (5%) and DTT (0.15%) were added to the extraction buffer. Tris/citrate electrode buffer had pH 7.4, and gels of this buffer system were prepared with 11.5% starch and 2.7% sucrose. Poulik gel buffer had pH 8.5. Both gels were prepared scarcely one hour before running. Embryos were separated from macrogametophytes by hand after imbibing the seeds during one day on moistened filter paper at room temperature. Ashton buffer system was also assayed in place of Poulik, but no good resolution was obtained in AAT.

Inheritance analysis was performed with respect to the variants of the AAT2 and PGI2 zones. Extra data were specially used for this purpose (embryos were also studied). MENDELian segregation was tested through both individual and pooled data with the log-likelihood G-test at the level of $\alpha = 0.05$ (William's correction was performed by individual data to obtain an adjusted G) (SOKAL and ROHLF, 1981). Individuals with less than 10 analyzed macrogametophytes were not considered. A homogeneity test was utilized before joining data from different trees.

Linkages were tested in individual and pooled segregation data. Firstly, the MENDELian segregation (1:1 ratio) in each locus was tested by means of the X^2 test ($\alpha = 0.05$). Then a second X^2 test ($\alpha = 0.05$) was performed in each analyzed mother tree to detect deviation from the expected two-locus segregation (1:1:1:1) of the gamete classes under the hypothesis of no-linkage. At least 20 macrogametophytes per tree were analyzed in order to assure an expected value in each class of at least five.

If the no-linkage hypothesis was rejected for at least one tree, the Bonferroni test was performed (SACHS, 1997). This test considers that if several samples of a population are tested, the probability of mistaken rejection of the hypothesis at the population level (that is to falsely reject in at least one test when all the tests are considered at the same time) is not α_i but $\Sigma\alpha_i$, where α_i is the significance level of each sample-test. If all α_i are equal, then $\Sigma\alpha_i = k\alpha_i$, where k is the number of samples tested. In this way, if the results of the tests of the samples (each tree in our study) will be extended to the population (the group of tested trees from each double-locus combination) and a certain α value is required ($\alpha = 0.05$ in our case), each sample-test must be carried out with $\alpha_i = \alpha/k$ (that is $\alpha_i = 0.05/k$). Linkage was assumed when the hypothesis was rejected through Bonferroni test in at least one tree of the group.

When linkage was proved, the two more frequent classes were considered to belong to the parental type, and the other two to the recombinant. The frequency of recombination (**R**) between loci and its Standard Error ($\mathbf{SE_R}$) were calculated after BAILEY (1961) by: $\mathbf{R = r/N}$ and $\mathbf{SE_R = [R(1\text{-}R)/N]^{1/2}}$, where **r** is the number of recombinants and **N** the total number of analyzed macrogametophytes. The genetic distances between loci (**D**) were estimated by KOSAMBI's (1944) formula in centiMorgans: $\mathbf{D = 25\ ln[(1+2R)/(1\text{-}2R)]}$.

Pooled data from all trees of each double-locus combination were utilized to calculate each **R**. Before pooling the data, the homogeneity of the distribution of data between trees had to be proved, and therefore a log-likelihood G-test (α: 0.05) (SOKAL and ROHLF, 1981) was performed. Because the arrangement of the alleles in different trees could be in different combinations (genes in some trees arranged in coupling phase and in others in repulsion), leading to the rejection of homogeneity when actually this would not mean different recombination values in different trees, data was rearranged before testing homogeneity. Parental and recombinant types were taken into account, so on the one hand the more frequent classes of each tree were summed, and on the other hand the less frequent classes. Since linkage is a phenomenon related to positions on chromosomes (chromosomal topography) and not to the genetic information itself, it was not considered which allele occupied the locus.

Results and Discussion

PGI pattern and inheritance analysis

Two single-banded zones were observed: the most anodal was monomorphic, while the other showed two variants (two or three faint bands could often be seen above or below this slower zone but were assumed as artifacts). One locus with two alleles was verified: *Pgi2-100* and *Pgi2-90* (*Table 2*). A compatible pattern was found in embryos. However, only two bands could be seen for the heterozygotes, and then equal migration velocity of the hybrid band to one of the homomeric was assumed for this dimeric enzyme under the described conditions.

In *Cupressus macrocarpa* HARTW. (CONKLE, 1987), *Thuja plicata* (D. DON) DONN (YEH, 1988), *Thuja orientalis* L.(XIE et al., 1991), *Cupressus sempervirens* L. (PAPAGEORGIOU et al., 1993), and *Metasequoia glyptostroboides* HU et CHENG (KUSER et al., 1997) two zones were also found of which only the slowest one revealed polymorphism. Two polymorphic zones were found by HARRY (1986) in *Calocedrus decurrens* (TORR.) FLORIN, and by MILLAR and MARSHALL (1991) in *Chamaecyparis lawsoniana* (A. MURR.) PARL. PERRY and KNOWLES (1989) also observed two zones in *Thuja occidentalis* L., but both were monomorphic.

AAT2 pattern and inheritance analysis

Three zones were already described for this enzyme system (PASTORINO and GALLO, 1998), but in the present work the analysis of further individuals revealed a variant of the middle zone, AAT2, namely a null allele. Thus, this zone which was formerly thought to be monomorphic, could be proved to be encoded by a gene locus (*Table 2*) with two alleles: *Aat2-100* and *Aat2-null*. Only two heterozygous trees of the 403 analyzed carried this extremely rare allele.

Also HARRY (1986) and XIE et al. (1991) found three variable zones for the AAT enzyme system, and in the first of these two articles a null allele was also reported for the middle locus.

Table 2. – Genetic analysis of PGI2 and AAT2 enzymes in *Austrocedrus chilensis.* Homogeneity of segregation ratios and MENDELian hypothesis proved through G-test in pooled and single tree data.

Locus	*Aat2*	*Pgi2*
Putative genotype	*100/n*	*100/90*
Nº analyzed trees	403	345
Nº analyzed populat.	16	16
Nº total heterozygot.	2	105
Nº analyzed heteroz.	2	17
Pool data segr. ratio	89:89	343:307
Gh	1.45 ns	19.1 ns
Gt	1.45 ns	21.1 ns
Individual segr. ratio	44:52	52:55
Gadj	0.66 ns	0.08 ns

ns = not significant

New interpretation of known phenotypes

MDH. In a previous paper (PASTORINO and GALLO, 1998), three loci were proven to control the inheritance of three variable zones in the MDH enzyme system. A null allele was described in the more cathodal polymorphic locus (*Mdh4*). With further analysis it was possible to see that this formerly presumed null allele actually appeared as a single band if the gel remained in the staining solution for a longer time (a couple of hours). This faint variant is slower than the common one, and it is now renamed as *Mdh4-80*.

IDH. This is a similar case as MDH. As in many other species, two loci were proven to control the mode of inheritance of IDH variants in *Austrocedrus chilensis* (PASTORINO and GALLO, 1998), each one with two alleles. At the most anodal locus one null allele was assumed. This fact makes it hard to accept the whole interpretation, because it has to be also accepted that the *null* allele would be the common one while the *100* allele is the exception (and that was scarcely one exception between 128 analyzed trees). In more recent analysis, it was possible to see in the macrogametophytes which did not carry the 100 allele a very faintly stained band faster than it. This band can be observed only in clear zymograms in any tree, but always in macrogametophytes. Thus, the formerly thought *null* allele is now assumed to be actually this faint band, which is then renamed as *100.* The former 100, which stains quite normally, is now renamed *82* according to its relative migration distance. Embryos never showed any of these variants, so *Idh1* appears to be not active in this tissue.

Linkage relationships in Austrocedrus chilensis

With 12 variable loci, 66 different pairwise combinations are possible. However, only 29 were found. This is not hard to accept when the predominant genetic profile of the species is considered: six out of the 12 loci are virtually monomorphic (*Aat2, Mdh2, Mdh3, Mdh4, Idh1* and *Idh2*).

Raw data and the results of the X^2 tests for single-locus and joint segregations can be seen in *Table 3*. Segregation distortion does not affect the linkage analysis if it occurs in only one of the two loci (BAILEY, 1961). Although the genetic control of *Pgi2* and *Sod* has been already proved, segregation distortion was registered in this two loci for one and the same tree (G4). This slight distortion could be related to the small number of macrogametophytes analyzed for it (33). Nevertheless, these data cannot be considered in the linkage analysis.

The hypothesis of non existing linkage was rejected in individual cases of eight two-loci combinations, which were then submitted to the Bonferroni test. Only four of these combinations were proven to indicate linkage: *Aat1~Pgi2* ($\alpha_i = 0.025$), *Aat3~6Pgdh2* ($\alpha_i = \alpha = 0.05$), *Aat3~Sod* ($\alpha_i = 0.017$) and *Idh2~Skdh* ($\alpha_i = \alpha = 0.05$). Frequencies of recombination, their standard errors, and KOSAMBI distances between loci are communicated in *Table 4*. Results of the homogeneity G-test are also expressed there.

Comparison between results of different studies

Results of linkage analysis between isozyme loci are usually compared with those of other species, especially of the same family but not exclusively. However, not too much care is generally taken about the interspecific homology of the gene loci that are being compared. Such a carelessness can mislead to erroneous conclusions, for example about the phylogenetic relationships between the involved species.

When two or more loci are found in any enzyme system, the relative migration distance of the encoded isozymes is used to distinguish them, and they are consequently called 1, 2, 3 and so on (or A, B, C, etc.). Normally this relative position on the zymogram is considered as the evidence of homology between loci from different species. However, mutations can create alleles that invert the relative position of the corresponding isozymes on the zymogram.

This point can be illustrated with the experiment from CHELIAK and PITEL (1985) who run in the same gel five different species to compare the AAT enzyme system. The results suggest that *Aat1* and *Aat2* in *Pinus* and *Picea* are the homologues of *Aat2* and *Aat1*, respectively, in *Pseudotsuga, Larix*

Table 3. – Individual tree analysis for linkage in *Austrocedrus chilensis*.

Pair of loci	Nº gametophytes per gamete class				Segregation – X^2 test			
					Loc. A	Loc. B	Joint segregat.	
Tree	A1B1	A1B2	A2B1	A2B2	χ^2	χ^2	χ^2	P val.
Aat1-Aat2								
O22	22	17	24	19	0.20	1.22	1.41	0.702
Aat1-Aat3								
H30	6	8	9	13	1.78	1.00	2.89	0.409
Aat1-Sod								
P2	24	12	24	18	0.46	4.15*	5.08	0.166
P4	19	25	16	19	1.03	1.03	2.16	0.539
P7	11	8	14	15	2.08	0.08	2.50	0.475
P12a	11	14	14	17	0.64	0.64	1.29	0.733
O8	6	6	4	5	0.43	0.05	0.52	0.914
C20	5	10	7	3	1.00	0.04	4.28	0.233
B1	18	19	22	17	0.05	0.21	0.74	0.865
H12	18	36	19	25	1.02	5.88*	8.37*	0.039
Aat1-Pgi2								
O13	9	39	41	7	0.00	0.17	42.83*	0.000
H30	2	12	22	1	2.19	3.27	31.43*	0.000
Aat1-6Pgdh2								
O21	7	3	8	4	0.18	2.91	3.09	0.378
O22	21	19	22	20	0.05	0.20	0.24	0.970
C20	24	31	22	24	0.80	0.80	1.85	0.604
H12	8	9	4	7	1.29	0.57	2.00	0.572
Aat1-Mdh2								
P2	16	21	30	26	3.88*	0.01	4.76	0.190
P4	23	22	21	14	1.25	0.80	2.50	0.475
P9b	4	7	7	4	0.00	0.00	1.64	0.651
P12a	13	12	15	16	0.64	0.00	0.71	0.870
Aat1-Mdh4								
O13	51	46	48	42	0.26	0.65	0.91	0.822
Aat1-Idh2								
A22	36	45	38	53	0.58	3.35	4.14	0.247
Aat1-Skdh								
P12a	8	7	7	8	0.00	0.00	0.13	0.988
O13	14	15	18	15	0.26	0.06	0.58	0.901
O22	23	16	26	17	0.20	3.12	3.37	0.339
A22	13	18	16	20	0.37	1.21	1.60	0.660
Aat2-Pgi2								
G23	20	24	25	27	0.67	0.38	1.08	0.781
Aat2-6Pgdh2								
O22	8	37	35	2	0.78	0.20	47.85*	0.000
Aat2-Skdh								
O22	27	18	23	14	0.78	3.95*	4.73	0.193
Aat3-Sod								
F7	15	59	66	4	0.11	2.25	80.39*	0.000
CL3	1	12	11	0	0.17	0.00	20.33*	0.000
A13	34	7	4	34	0.11	0.11	41.35*	0.000
Aat3-Pgi2								
F7	11	11	11	8	0.22	0.22	0.66	0.883
L14	4	10	4	3	2.33	1.19	5.86	0.119
H30	10	7	13	8	0.42	1.68	2.21	0.530
Aat3-6Pgdh2								
CH12	18	26	18	14	1.89	0.21	4.00	0.261
Aat3-Skdh								
F7	13	9	6	13	0.22	0.22	3.39	0.335
G27	22	16	23	19	0.20	1.25	1.50	0.682
Sod-Pgi2								
F7	12	11	10	8	0.61	0.22	0.85	0.837
F15	21	19	32	17	0.91	3.25	6.06	0.109
G4	20	4	4	5	6.82*	6.82*		
Sod-6Pgdh2								
F15	13	28	25	25	0.89	2.47	5.84	0.120
O1	12	15	19	18	1.56	0.06	1.88	0.599
O20	9	7	4	7	0.93	0.04	1.89	0.596
C20	6	6	3	7	0.18	0.73	1.64	0.651
G4	12	10	5	3	6.53*	0.53	7.07	0.070
H12	30	21	27	45	3.59	0.66	10.17*	0.017
H15	4	5	5	8	0.73	0.73	1.64	0.651
Sod-Mdh2								
P2	42	36	40	37	0.01	0.52	0.59	0.899
P4	26	13	23	24	0.74	1.67	4.70	0.195
P12a	15	15	18	16	0.25	0.06	0.38	0.945
Sod-Idh2								
H22	13	12	12	8	0.56	0.56	1.31	0.726
Sod-Skdh								
P12a	10	5	6	9	0.00	0.13	2.27	0.519
F7	7	16	12	6	0.61	0.22	6.32	0.097
F15	14	20	16	26	0.84	3.37	4.42	0.219
G4	10	14	5	4	6.82*	0.27	7.85*	0.049
Pgi2-6Pgdh2								
F15	29	27	12	27	3.04	1.78	7.86*	0.049
G4	14	16	18	4	1.23	2.77	8.92*	0.030
Pgi2-Mdh4								
O13	26	26	31	24	0.08	0.46	1.00	0.801
Pgi2-Idh1								
CH28	11	7	16	12	2.17	1.39	3.57	0.312
Pgi2-Skdh								
F7	12	12	11	12	0.02	0.02	0.06	0.996
F15	18	30	15	17	3.20	2.45	6.90	0.075
O13	18	12	17	16	0.14	0.78	1.32	0.725
G4	14	14	10	9	1.72	0.02	1.77	0.622
6Pgdh2-Skdh								
F15	17	20	19	29	1.42	1.99	3.99	0.263
O22	25	18	24	15	0.20	3.12	3.37	0.339
G4	15	12	6	11	2.27	0.09	3.82	0.282
Mdh2-Skdh								
P9b	16	21	13	28	0.21	5.13*	6.62	0.085
P12a	7	7	8	8	0.13	0.00	0.13	0.988
P16	11	16	9	13	0.51	1.65	2.18	0.535
Mdh4-Skdh								
O13	17	22	20	17	0.05	0.05	0.95	0.814
Idh2-Skdh								
A22	20	11	16	34	4.46*	1.00	14.46*	0.002

*significant differences at the level of α = 0.05 or smaller

and *Abies*. The staining intensity of the bands as well as the linkage relationships support this idea.

When only one locus can be recognized coding for a certain enzyme, it is immediately assumed to be the homologue from other species which also reveal one locus. However, there are examples in the literature where a different number of loci are recognized for a certain enzyme in species even from the same family, and therefore, it is impossible to know which one is

Table 4. – Linkage parameters for the studied linked loci in *Austrocedrus chilensis.* Homogeneity of segregation ratios as indicated by the G-test, segregation ratios from the pool, frequency of recombination (R) and its standard error (SE_R), and KOSAMBI distance between loci (D).

Locus combination	G	Pooled segregation	R	SE_R	D [cM]
Aat1 ~ Pgi2	3.64 ns	11:51:63:8	0.143	0.030	14.69
Aat2 ~ 6-Pgdh2	--	8:37:35:2	0.122	0.036	12.44
Aat3 ~ Sod	3.88 ns	23:105:111:8	0.125	0.021	12.82
Idh2 ~ Skdh	--	20:11:16:34	0.333	0.052	40.24

ns = not significant

missing in the species with fewer loci. For example, two loci were found for SKDH in *Calocedrus decurrens* (HARRY, 1986), but only one in *Austrocedrus chilensis*. The latter does not reveal any other zone, not even a diffuse or unreliable band, just one unique band. So, which one of the two from *C. decurrens* is the homologue of *A. chilensis*? On the other hand, blurred zones might also be simply ignored and not reported when they are actually important just to establish homology with other species.

Sometimes an additional feature can be used to recognize the homology between different species. This is the case for *Aat3*, which shows a distinctive phenotype found in several conifers such as *A. chilensis*, namely a triple-banded zymogram in the haploid tissue. Considering this, MORGANTE et al. (1993) are presumably wrong when they compare the *Aat3* locus from XIE et al. (1991) to their *Aat1* (*Aat3* is undoubtedly recognized in XIE's et al. study by the triple-banded phenotype, and it is unlikely that its homologue in *Pinus leucodermis* ANT. would change its relative position from the bottom to the top of the zymogram, especially since the *Aat1* position is found rather constant in several species of the genus *Pinus*).

Comparison between A. chilensis and other conifers

Before comparing the results of the present work with those of other conifers, interspecific homology of gene loci will be, then, considered. In *Table 5* linkages proven in *Austrocedrus chilensis* are compared with results found in the literature of other species linkage analyses about the same two-locus combinations.

Six pairs of loci can be compared to HARRY's work about *Calocedrus decurrens* (1986). It appears to be just a difference of nomenclature in two of the loci involved. Our *Aat1* seems to be the same as *Aat2* after HARRY. If his sketched zymograms are considered (those whose electrophoresis were performed with Poulik buffer system, like in the present work), it is possible to observe that similar migration distances correspond to *Aat1* and *Aat2*, namely the two zones overlap. Therefore, which one is called "1" or "2" is arbitrary. This is not the case when the zymograms resulting from the morpholine/citrate buffer system are considered (a most anodal and a most cathodal zone are undoubtedly recognized). This observation reflects that also the method should be taken into account before stating homology. The other nomenclature difference is related to the MDH enzyme system. Since we recognize five zones instead of the three of HARRY and the first one was blurred and presumably not variable, HARRY's *Mdh1* is then assumed as the homologue of our *Mdh2*.

HARRY found also no linkage between the pairs *Aat1~Aat3, Aat3~Pgi2, Aat3~Skdh1, Pgi2~Skdh1* and *Mdh2~Skdh1,* while he did find synteny in *Aat1~Pgi2* pair, just like in the present work.

RUDIN and EKBERG (1978) calculated that the minimum sample size of macrogametophytes per tree to detect linkage with a recombination frequency (R) between 0.35 and 0.40 at the level of $\alpha = 0.05$ varies between 40 and 80. For *Aat1~Pgi2* HARRY worked with not more than 20 macrogametophytes per tree, however he could prove linkage because it is very tight between these two loci (R = 0.06 for the pooled data). Here can also be mentioned *Cunninghamia lanceolata* HOOK., a member of the *Taxodiaceae* family (close related family to *Cupressaceae*) whose linkage for this combination was reported by MÜLLER-STARCK and LIU (1988) (R = 0.237). Actually they also found linkage for the combination *Aat2~Pgi*, and indeed with similar recombination rate (it is also remarkable that a unique *Pgi* locus was observed).

In *Thuja occidentalis*, PERRY and KNOWLES (1989) analyzed linkage in 23 pairs of loci, but only four of them are comparable to the present work (their *Mdh1* and *Mdh3* are assumed to be homologous to our *Mdh2* and *Mdh4*, respectively). They did not find linkage in the following pairs either: *Aat1*~Idh, *Aat1~Mdh4* and *Aat1~6Pgdh2*. Although quite weak, they reported linkage between the loci *Aat1* and *Mdh2* (R = 0.415 ± 0.033), which could not be proved in the present work.

Table 5. – Linkages proven in *Austrocedrus chilensis* and their comparison with evidence found in the literature for other species about the same two-loci combinations (R: frequency of recombination, D: KOSAMBI distance between loci).

Pair of loci	A. chilensis	Other species
Aat1-Pgi2	R = 0.143	Calocedrus decurrens: R=0.06[1]; Cunninghamia lanceolata: R=0.237[2]; Pinus taeda: R=0.024[3], D=2.5cM[4]; P. banksiana: R=0.038[5]; P. contorta: D=2.1cM[4]; P. jeffreyi: D=2.3 cM[4]; P. strobus: R=0.048[5]; P. rigida: R=0.03[6]; P. ponderosa[7]; P. attenuata: R=0.08[8]; P. sylvestris: R=0.065[9]; P. mugo: R=0.091[9]; P. nigra: R=0.00[9]; Picea mariana: R=0.043[5], R=0.250[10], R=0.14[5]; Pic. Glauca: R=0.045[11]; Pic. Abies: R=0.153[12]; Larix laricina: R=0.03[13]; Larix decidua: R=0.026[14]; Abies balsamea: R=0.03[15]; Pseudotsuga menziesii: R=0.02[16].
Aat2-6Pgdh2	R = 0.122	Pinus echinata: R=0.395[17]; P. taeda: not linked[3]; P. sylvestris: not linked[18]; P. leucodermis: not linked[19]; Picea abies: not linked[12].
Aat3-Sod	R = 0.125	Pseudotsuga menziesii: R=0.22[16]; Larix laricina: R=0.242[13].
Idh2-Skdh	R = 0.333	Pinus echinata: R=0.004[17]; Pinus leucodermis: not linked[19] (however, unlikely homology for the two species).

[1]HARRY (1986), [2]MÜLLER-STARCK and LIU (1988), [3]ADAMS and JOLY (1980), [4]CONKLE (1981), [5]cited in CHELIAK and PITEL (1985), [6]GURIES et al. (1978), [7]cited in GURIES et al. (1978), [8]STRAUSS and CONKLE (1986), [9]GONCHARENKO et al. (1998), [10]BOYLE and MORGENSTERN, [11]KING and DANCIK (1983), [12]GEBUREK and WÜHLISCH (1989), [13]CHELIAK and PITEL (1985), [14]LEWANDOWSKI (1999), [15]NEALE and ADAMS (1981), [16]EL-KASSABY et al. (1982), [17]RAJA et al. (1997); [18]SZMIDT et al. (1984); [19]MORGANTE et al. (1993)

Five double-locus combinations are comparable to the work of XIE et al. (1991) with *Thuja orientalis*. They also could not prove linkage in the pairs *Aat3~Skdh1, Aat3~6Pgdh2, 6Pgdh2~Skdh1* and *Pgi2~Skdh1*. However they reported synteny between *Aat3* and *Pgi2*, although so weak ($R = 0.46 \pm 0.02$) that it is almost meaningless (it is remarkable that in the single tree analysis only one out of 12 trees revealed linkage). It must also be stressed that no more than 41 macrogametophytes per individual could be analyzed for this pairwise combination in the present work, so according to RUDIN and EKBERG (1978) if the recombination frequency is higher than ~0.35, linkage would be revealed with a probability smaller than 95% ($\alpha = 0.05$).

Some comparisons to the most intensively studied *Pinaceae* family are also helpful. First of all, the *Aat1~Pgi2* pair (or its equivalent in some species, namely *Aat2~Pgi2* pair) appears to be the most highly conserved gene block, since it was observed in many species and at least in three different families. CHELIAK and PITEL (1985) have already presented a list of species cited in the literature with these loci tightly linked (R from 0.020 to 0.250): *Pinus taeda* L., *Pinus banksiana* LAMB., *Pinus contorta* DOUGL., *Pinus jeffreyi* GREV. et BALF., *Pinus strobus* L., *Pinus rigida* MILL., *Picea mariana* (MILL.) B.S.P., *Picea glauca* (MOENCH) VOSS, *Larix laricina* (DU ROI) K.KOCH, *Pseudotsuga menziesii* (MIRB.) FRANCO and *Abies balsamea* (LINN.) MILL. We can add to this list *Pinus ponderosa* LAWS (cited in GURIES et al., 1978), *Pinus attenuata* LEMM. (STRAUSS and CONKLE, 1986), *Picea abies* (L.) KARST (GEBUREK and WÜHLISCH, 1989), *Pinus sylvestris* L., *Pinus mugo* TURRA, *Pinus nigra* ARN. (GONCHARENKO et al., 1998), *Larix decidua* MILL. (LEWANDOWSKI, 1999), and the three already mentioned not *Pinaceae* species, namely, *Calocedrus decurrens*, *Cunninghamia lanceolata* and *A. chilensis*.

It is interesting to note the inversion of the relative migration distance between *Aat1* and *Aat2* isozymes found in different species groups, because it can easily mislead to erroneous homology. Namely *Aat1* in the „*Pinus* group" (which include *Picea* and *Austrocedrus*) is functionally the same as *Aat2* in the „*Pseudotsuga* group" (together with *Abies* and *Larix*) and vice versa (CHELIAK and PITEL, 1985; LEWANDOWSKI, 1999). Because structural and functional divergence of successively duplicated genes is mentioned as the main evolutive cause of protein genes (FUTUYMA, 1986), these observations might have evolutive causes that still remain unknown.

MORGANTE et al. (1993) also reported these two loci, *Aat1* and *Pgi2*, to be linked in *Pinus leucodermis*, but with a really not expected value, namely $R = 0.428 \pm 0.045$. This motivated us to reanalyze their data with our method. Only one from the four presented trees shows deviation from the independent segregation hypothesis, and the analysis of the pooled data reveals no linkage at all. This result made us doubt their assumed homology, and since the relative position of *Aat1* in the zymogram was found rather constant within *Pinus* spp., suspicion falls over *Pgi2*. Additionally another unexpected result involves this locus: they reported the pair *Pgi2~6Pgdh2* to be tightly linked ($R = 0.08$), while linkage could not be proved in *Pinus taeda* (ADAMS and JOLY, 1980), *P. sylvestris* (SZMIDT et al., 1984), *P. rigida* (O'MALLEY et al., 1986), *Pinus thunbergii* PARL. (SHIRAISHI, 1988), *Picea abies* (GEBUREK and WÜHLISCH, 1989), and *A. chilensis* in our paper.

RAJA et al. (1997) could not prove linkage for the pair *Aat1~Pgi* in *Pinus echinata* MILL. Nevertheless, one must notice that they observed only one *Pgi* locus in their gels, and therefore, it is not possible to be sure of its homology to *Pgi2* locus in the other species. On the other hand, they analyzed for this pair only 58 macrogametophytes from a unique tree, and then, a sampling shortcoming is also thinkable.

The double-locus combination *Aat3~Sod* has been also previously reported to be linked as in the present work in *Pseudotsuga menziesii* (EL-KASSABY et al., 1982) and *Larix laricina* (CHELIAK and PITEL, 1985).

As opposed to the present work, the pair *Aat2~6Pgdh2* was reported not to be linked in *Pinus taeda* (ADAMS and JOLY, 1980), *P. sylvestris* (SZMIDT et al., 1984), *P. leucodermis* (MORGANTE et al., 1993) and *Picea abies* (GEBUREK and WÜHLISCH, 1989). However, in the first of these studies, although linkage could not be proved, these two loci were found to belong to the same linkage group (both loci are slightly linked to a third one, namely *Ap2*). Moreover, RAJA et al. (1997) found a result for this pair consistent with our in *A. chilensis*. They reported linkage for these two loci in *Pinus echinata*, although with a recombination frequency rather contrasting to our species ($R = 0.395 \pm 0.029$).

On the other hand, RAJA et al. (1997) make presumably a mistake when comparing their findings with those from NEALE and ADAMS (1981) on *Abies balsamea*, because, as said, *Aat2* from *Abies* is not homologous to *Aat2* from *Pinus* but to *Aat1*. Consequently, except for the current results on *A. chilensis*, this pair was shown to be linked in only one species.

Thus, *A. chilensis*' results serve to shed light on the apparent controversy arising from *P. echinata* results. The failure on proving linkage in the four former species does not necessary mean that the loci belong to different chromosomes but that they are at least far enough as to segregate independently. Therefore, it seems to be enough evidence as to sustain that these two isozyme genes are located in the same chromosome in conifers, although separated by a large distance in the *Pinaceae* family and probably tightly linked in the *Cupressaceae* family.

At a first sight they also seem to be opposite to our *A. chilensis* some observations in other species related to SKDH enzyme system. For the pair *Idh2~Skdh1* MORGANTE et al. (1993) did not find linkage in *Pinus leucodermis*. SHIRAISHI (1988) proved linkage for the combination *Aat2~Skdh2* ($R = 0.40 \pm 0.05$) in *P. thunbergii*, and GEBUREK and WÜHLISCH (1989) proved the pair *Aat2~Skdh1* to be linked in *Picea abies* ($R = 0.343 \pm 0.065$). On the other hand, the linkage showed by RAJA et al. (1997) for the pair *Idh~Skdh2* in *P. echinata* seems to agree with our findings. Because only one SKDH enzyme appears in *A. chilensis* gels, it is not possible to assign an acceptably sure homology between their *Skdh1* or *Skdh2* and our *Skdh* (this concept is valid for all the comparisons cited above about the SKDH enzyme system, and also applies to *Idh* and *Pgi* in the study of RAJA et al. (1997)). On the contrary, considering the conservative character of linkage pairs between close related species and assuming therefore the homology according to linkage relationships, it can be inferred that *Skdh1* from *P. leucodermis* and from *Picea abies*, and *Skdh2* from *P. thunbergii* would not be homologous to *A. chilensis*' *Skdh*. Instead the respective alternative SKDH isozymes could be the corresponding homologues. To really prove this inference, it is necessary data from the respective alternative SKDH isozymes, but unfortunately they are not available in the mentioned studies (either no double heterozygote was found for the desired locus combination or the locus has just no variation). A further deduction can be drawn from those findings under the same considerations. Keeping in mind that *Aat2* from *Pinus* and *Picea* are homologous (both belong to that "*Pinus* group" respect to AAT enzyme system), it is logic to deduce that *Skdh2* from *P. thunbergii* is the homologue of *Skdh1* from *Pic. abies*.

This example shows how linkages can be utilized to postulate homology of gene loci between species.

Our data appears to be also controversial to SZMIDT et al. (1984) with respect to the pair *Aat2~Pgi2*, where they found in *Pinus sylvestris* a slight linkage (R = 0.438). Also linkage was shown for the pair *Aat2~Pgi* in *Cunninghamia lanceolata* (R = 0.263) (MÜLLER-STARCK and LIU, 1988) and *P. echinata* (R = 0.435 ± 0.024) (RAJA et al., 1997), although, as already mentioned, in those two cases one must note that only one *Pgi* locus was revealed. But there are other works which consistently support our results about this pair (*P. taeda*, ADAMS and JOLY, 1980; *P. attenuata*, STRAUSS and CONKLE, 1986; *Picea abies*, GEBUREK and WÜHLISCH, 1989).

Conclusions

If interspecific gene comparisons are going to be made with any purpose, homology between the analyzed genes must be ensured. Otherwise, comparisons are meaningless. A proper method to establish a correct homology should be performed. False homology can lead to mistakes when comparing genes between different species. The relative migration distance of isozymes on the electrophoretic zymograms was shown not to represent enough evidence of homology of the encoding genes, not even when the compared species belong to the same family. On the other hand, linkages were shown to be a very good tool to state homology of gene loci because gene arrangements have been found to be highly conservative between related species. Linkages act as a stable reference system, and they are consequently proposed to be used when stating interspecific homology. The homology between *Aat1* and *Aat2* loci of the „*Pinus*" and „*Pseudotsuga*" groups respectively, both linked with *Pgi2* locus, represents an emblematic case in this sense.

Austrocedrus chilensis shows linkage relationships between allozyme loci consistent with other species of the *Cupressaceae* and also of the *Pinaceae* families. The results on *A. chilensis* add firm evidence to solve the apparent controversy in the *Pinaceae* family about the *Aat2~6Pgdh2* pair, confirming the conservative character of gene arrangements between related species. Differences firmly stable between groups of species (rather than species alone) can be, therefore, evidence of phylogenetic divergence.

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