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C. Mattioni · M. Casasoli · M. Gonzalez · R. Ipinza
F. Villani

Comparison of ISSR and RAPD markers to characterize three Chilean *Nothofagus* species

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Abstract The present study is the first report of fingerprinting on three Chilean *Nothofagus* species using ISSR and RAPD markers; 61 *Nothofagus nervosa*, 32 *Nothofagus obliqua* and 32 *Nothafagus dombeyi* individual trees, sampled from collection and natural sites, were analyzed. Among 45 primers tested, the 6 ISSR and 6 RAPD primers selected for the analysis generated a total of 63 ISSR and 42 RAPD fragments. A high proportion of polymorphic bands, ranging from 97% and 80%, was found using both markers. A similar number of private and marker bands was generated by both markers in all the species examined and one discriminant ISSR fragment was obtained for *N. dombeyi*. Jaccard and Dice similarity indices were used to evaluate pairwise genetic divergence; cluster analysis of the similarity matrices was performed to estimate the intra- and inter-specific genetic diversity, and PCA analysis was employed to evaluate the resolving power of the markers to differentiate between the species. These analyses, carried out for both markers, allowed us to identify three main groups corresponding to the three *Nothofagus* species. The results of the present study can be seen as a starting point for future researches on the population and evolutionary genetics of these species.

Keywords *Nothofagus dombeyi · Nothofagus obliqua · Nothofagus nervosa · Fingerprinting*

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C. Mattioni · M. Casasoli · F. Villani (✉)
Istituto per l'Agroselvicoltura, CNR, Via G. Marconi, 2,
05010 Porano (TR), Italy
e-mail: F.Villani@ias.tr.cnr.it
Fax: +39-763-374330

M. Gonzalez
Instituto Forestal, Huérfanos 554, 3085 Santiago, Chile

R. Ipinza
Instituto de Selvicultura, Universidad Austral de Chile,
Campus Isla Teja, 567 Valdivia, Chile

Introduction

The genus *Nothofagus* (*Nothofagaceae*) is one of the most-important elements of the Southern Hemisphere flora: 40 species, evergreen and deciduous, and some natural hybrids are spread among Central and Southern Chile, Argentina, New Zealand, Australia, New Guinea and New Caledonia. In Chile nine *Nothofagus* species are present in the autochthonous forest, generally in the dominant tree layer, between latitudes 33° and 55.5 °S, from sea level up to 2,500 m above sea level (Donoso 1996). Among these, *Nothofagus nervosa*, *Nothofagus obliqua* and *Nothofagus dombeyi* are potentially very important timber producers due to their high wood quality and relative fast growth; however, indiscriminate logging in the last centuries degraded vast areas of the Chilean forest causing a serious state of deterioration of their genetic resources (Grosse 2000).

Despite its ecological and economic importance, the taxonomy and genetic structure of the genus *Nothofagus* is not entirely clarified, due also to the occurrence of natural hybridization among species.

Until now, few studies have been carried out on the phylogeny of *Nothofagus* (Hill and Jordan 1993; Martin and Dowd 1993; Manos 1997; Setoguchi et al. 1997) and on the genetic variation among populations using isozymes (Premoli 1996, 1997; Marchelli and Gallo 2000a, b) and molecular markers (Marchelli et al. 1998).

Over the last 10 years, polymerase chain reaction technology has led to the development of two simple and quick techniques called RAPD and ISSR. The former detects nucleotide sequence polymorphisms, using a single primer of arbitrary nucleotide sequence (Williams et al. 1990), and the latter permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats. (Wu et al. 1994; Zietkiewics et al. 1994).

RAPD and ISSR markers have been used both for DNA fingerprinting (Moreno et al. 1998; Blair et al. 1999; Divaret et al. 1999; Gilbert et al. 1999), for population genetic studies (Wolfe et al. 1998; Nebauer et al.

Table 1 Sampling sites and number of the *N. nervosa*, *N. obliqua*, *N. dombeyi* samples collected

Sampling sites	Locality	Region	Number of samples/species		
			N. nervosa	*N. obliqua*	*N. dombeyi*
Plantation					
1	Volpir	IX	10		
2	Neltume	X	10		
3	S. José de la Mariquina	X	9		2
Nursery					
4	Gorbea	IX	4	5	10
5	Lanchoche	IX	9		
Natural population					
6	Selva Oscura	IX	9	22	12
7	P. National Nahuelbuta	VIII	10	5	18

1999) and for phylogenetic studies (Hess et al. 2000). There are also some reports in which the capacity of RAPD and ISSR to detect polymorphism and to discriminate among taxa has been compared (Nagaoka and Ogihara 1997; Esselman et al. 1999).

The objectives of the presented research are: (1) a comparison of ISSR and RAPD markers for the molecular characterization of three *Nothofagus* species, *N. nervosa*, *N. obliqua* and *N. dombeyi*, collected from Chile; (2) the evaluation of the degree of polymorphism generated from each technique as a pre-requisite for their applicability to population genetics studies in *Nothofagus*.

Materials and methods

Plant material and DNA extraction

Leaves from *N. nervosa*, *N. obliqua* and *N. dombeyi* individual trees were sampled from natural sites and from collections (either nursery or plantations), where the samples were assigned to the three species after morphological characterization. A total of 61 *N. nervosa*, 32 *N. obliqua* and 32 *N. dombeyi* samples were collected (Table 1).

DNA was extracted from 100 mg of leaf tissue according to the Doyle and Doyle method (1987).

ISSR amplification

24 ISSR primers (set # 9) obtained from the University of British Columbia Biotechnology Laboratory (UBCBL) were tested on *N. nervosa*, *N. obliqua* and *N. dombeyi* samples. In order to estimate experimental reproducibility, two independent amplifications from a subset of 18 samples were carried out.

Six ISSR primers that showed a clear and reproducible band pattern were chosen for this study.

PCR amplification was performed in a 25-µl reaction volume, containing 10 mM Tris-HCl pH 8, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Perkin Elmer), 2.5% formamide, 0.75 units of *Taq* polymerase (Boehringer Mannheim, Germany), 100 µg/ml of BSA and 20 ng of template genomic DNA.

The mixture was overlaid with mineral oil and subjected to PCR on a Perkin Elmer 480 thermal cycler programmed for an initial step of 3 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 50/52 °C, 2 min at 72 °C, and a 10-min final extention step at 72 °C. PCR products were analyzed on 2% agarose gels, then stained with ethidium bromide, visualized with ultraviolet light and photographed.

RAPD amplification

Twenty one RAPD primers obtained from Operon Technologies (Alameda, Calif.) were tested and six were used. The amplifications were performed in a 25-µl reaction volume containing 10 mM Tris-HCl pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of primer, 100 µg/ml of BSA, 0.75 units of *Taq* polymerase (Boeringer Mannheim, Germany) and 30 ng of genomic DNA. Initial denaturation was for 3 min at 94 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C and a 10-min final extension step at 72 °C. PCR products were analyzed on 1.4% agarose gels and stained with ethidium bromide. As for ISSR, the experimental reproducibility of the RAPD markers was estimated by two independent amplifications on a subset of 18 samples.

Data analysis

ISSR and RAPD amplified fragments, named by the primer code and the molecular weight (bp), were scored for band presence (1) or absence (0) and two binary qualitative data matrices were constructed.

Data analyses were performed using the NTSYS-pc (Numerical Taxonomy System, Rohlf 1993) version 2.0 computer program package. Among the various similarity indices, those of Jaccard and Dice were chosen as the most appropriate ones for dominant markers, like ISSR and RAPD, since they do not attribute any genetic meaning to the coincidence of band absence. The similarities are calculated as follows:

$$\text{Dice} = 2N_{AB}/(2N_{AB} + N_A + N_B)$$

$$\text{Jaccard} = N_{AB}/(N_{AB} + N_A + N_B)$$

where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A and N_B represents fragments in sample B.

The Dice index differs from the Jaccard index for the higher weight that it gives to the coincidences of band presence with respect to the non-coincidences. That means that the two indices are almost equal for very low (< 0.1) or very high (> 0.9) similarity levels, but diverge quite a lot at intermediate values, making more significant the comparison of their analyses in that region. Similarity matrices based on these indices were calculated. Correlation between the four matrices obtained with the two indices and the two markers types was estimated by means of the Mantel matrix correspondence test using 500 random permutations (Mantel 1967). The rationale of the test is the displacement of the quantity $Z_{OBS} = \Sigma_{i<j} X_{ij} Y_{ij}$, where X_{ij} and Y_{ij} are the off-diagonal elements of the matrices X and Y to be compared, with respect to the random variate Z_{rdm} calculated after random permutations of rows or

columns in X or Y; lower is the frequency of $Z_{rdm} \geq Z_{OBS}$, higher is the correlation *r* between X and Y. The degree of fit can be interpreted as follows: $r \geq 0.9$, very good fit; $0.8 \leq r \geq 0.9$, good fit; $0.7 \leq r \geq 0.8$, poor fit; $r \leq 0.7$, very poor fit. In order to quantify the genetic divergence within and between the three species, for both markers the average intra- and inter-specific similarity values were calculated.

Cluster analysis of the similarity matrices was performed and the results summarized as UPGMA dendrograms. In order to estimate the congruence among dendrograms, cophenetic matrices for each marker and index type were computed and compared using Mantel test.

Finally a Principal Coordinate Analysis was performed in order to highlight the resolving power of the ordination.

Results

Among the total primers tested the most polymorphic pattern has been obtained using ISSR 807 $(GA)_n$, while poly$(AT)_n$ primers gave no amplification products.

The six ISSR and RAPD primers, chosen for their clear and reproducible band patterns, are reported in Table 2.

We calculated the total number of ISSR and RAPD bands, the number of polymorphic bands and the bands shared among the three taxa. We defined discriminant bands as those present only in one species with 100% percentage, private bands those present only in one species with a percentage higher than 80% but lower than 100%, and marker bands those present with a percentage higher than 60% in one species and less than 10% in at least one of the other two species.

ISSR band patterns

The six chosen primers generated a total of 63 fragments, an average of 10.5 bands per primer. The size of amplified products ranged from 178 to 1,722 bp (see Table 2).

The number of fragments shared between pairs of species were: 34 (54.0%) between *N. nervosa* and *N. obliqua*, 18 (28.6%) between *N. nervosa* and *N. dombeyi* and 18 (28.6%) between *N. obliqua* and *N. dombeyi*. A high percentage of polymorphism was detected in all the examined species: 92.0% in *N. nervosa*, 88.8% in *N. obliqua*, 97.0% in *N. dombeyi*. The ISSR primers generate one (1.6%) discriminant and six (9.5%) private fragments in *N. dombeyi*; no private or discriminant bands were obtained for *N. obliqua*, while three (4.8%) private bands were detected in *N. nervosa*. Eight marker bands (12.7%) were found in *N. obliqua*, five (7.9%) in *N. nervosa* and three (4.8%) in *N. dombeyi*. The codes and molecular weights of discriminant, private and marker bands are reported in Table 3.

RAPD band patterns

The six primers chosen generated 42 RAPD fragments, an average of seven bands per primer. The size of amplified products ranged from 190 to 1,370 bp (Table 2).

A total of 17 (40.5%) RAPD fragments were shared between *N. nervosa* and *N. obliqua*, ten (23.8%) fragments between *N. nervosa* and *N. dombeyi* and four (9.5%) between *N. obliqua* and *N. dombeyi*.

The percentage of polymorphic RAPD bands found in the three species was: 87.0% in *N. nervosa*, 80.9% in *N. obliqua* and 94.6% in *N. dombeyi*. We could not find any discriminant band using RAPD primers, but five (11.9%) and four (9.5%) private bands for *N. nervosa* and *N. obliqua* were respectively scored. Eight (19.0%) marker bands were scored in *N. obliqua*, three (7.1%) in *N. nervosa* and three (7.1%) in *N. dombeyi*. The codes and molecular weights of discriminant, private and marker bands are reported in Table 3.

Comparison of genetic relationship estimates

The Mantel test between the two Dice and between the two Jaccard similarity matrices gave $r = 0.95$ and $r = 0.97$, respectively, showing for both indices a high correlation between RAPD- and ISSR-based similarities.

Table 2 ISSR and RAPD primers used, total number of fragments scored for each primer and the size of the amplified fragments

Primer type	Sequence (5′–3′)	No. of recorded markers	Size range bp
ISSR primers			
UBC-807	AGA GAG AGA GAG AGA GT	20	298–1722
UBC-834	AGA GAG AGA GAG AGA GYT	6	178–676
UBC-836	AGA GAG AGA GAG AGA GYA	6	240–546
UBC-841	GAG AGA GAG AGA GAG AYC	6	264–780
UBC-890	VHV GTG TGT GTG GTG TG	13	272–590
UBC-891	HVH TGT GTG TGT GTG TG	12	272–600
RAPD primers			
OPD-13	GGG GTG ACG A	8	230–600
OPF-03	CCT GAT CAC C	10	190–630
OPK-04	CCG CCC AAA C	5	400–830
OPK-20	GTG TCG CGA G	9	297–959
OPM-03	GGG GGA TGA G	4	492–1107
OPM-07	CCG TGA CTC A	6	700–1370

Table 3 ISSR and RAPD bands (discriminant, private, marker) which characterize the three *Nothofagus* species studied. Discriminant: $p = 100\%$ only in one species. Private: 80% <p> 100% only in one species. Marker: 60% <p> 100% in one species and 0<p> 10% in the others

Species	Discriminant ISSR	Private		Marker	
		ISSR	RAPD	ISSR	RAPD
N. nervosa		UBC-807-943 UBC-834-730 UBC-891-351	OPF03-492 OPF03-615 OPK04-830 OPK20-393 OPM03-790	UBC-807-560 UBC-807-1722 UBC-841-1107 UBC-891-272 UBC-891-600	OPK20-799 OPD13-369 OPF03-190
N. obliqua				UBC-807-861 UBC-807-930 UBC-834-178 UBC-836-455 UBC-890-330 UBC-890-730 UBC-891-333 UBC-891-590	OPK04-400 OPK04-615 OPK20-380 OPK20-667 OPK20-959 OPF03-451 OPM03-1107 OPM7-700
N. dombeyi	UBC-834-697	UBC-807-350 UBC-834-338 UBC-836-568 UBC-841-240 UBC-890-546 UBC-891-424	OPD13-584 OPF03-630 OPK20-492 OPM03-492	UBC-841-738 UBC-807-500 UBC-807-1353	OPK04-713 OPD13-375 OPD13-230

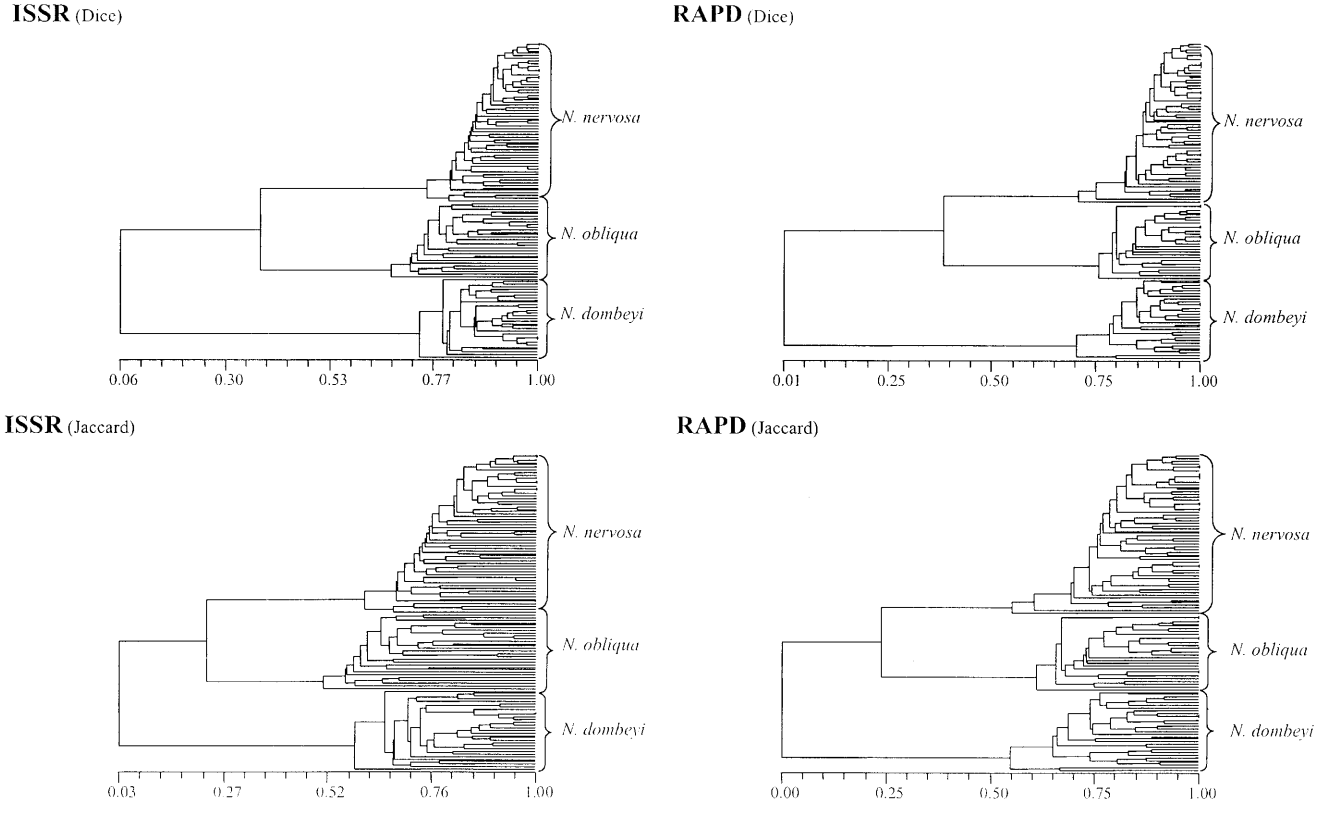

Fig. 1 UPGMA dendrograms of the three *Nothofagus* species based on Jaccard and Dice genetic indices and using ISSR and RAPD markers

The UPGMA dendrograms, obtained from the cluster analysis of each of the four index × marker similarity matrices gave similar results, with the identification of three main clusters corresponding to the three species (Fig. 1).

In all dendrograms the species *N. nervosa* and *N. obliqua* were closer than *N. dombeyi*. In order to estimate the correlation level between dendrograms, a new set of cophenetic matrices were calculated and compared using the Mantel test. The correlation between Jaccard- and Dice-based cophenetic matrices was $r = 0.98$ for RAPD and $r = 0.99$ for ISSR, indicating a very good agreement between dendrograms. The three-dimensional ordination confirms the cluster analysis results, showing that *N. nervosa*, *N. obliqua* and *N. dombeyi* are sharply separated (Fig. 2).

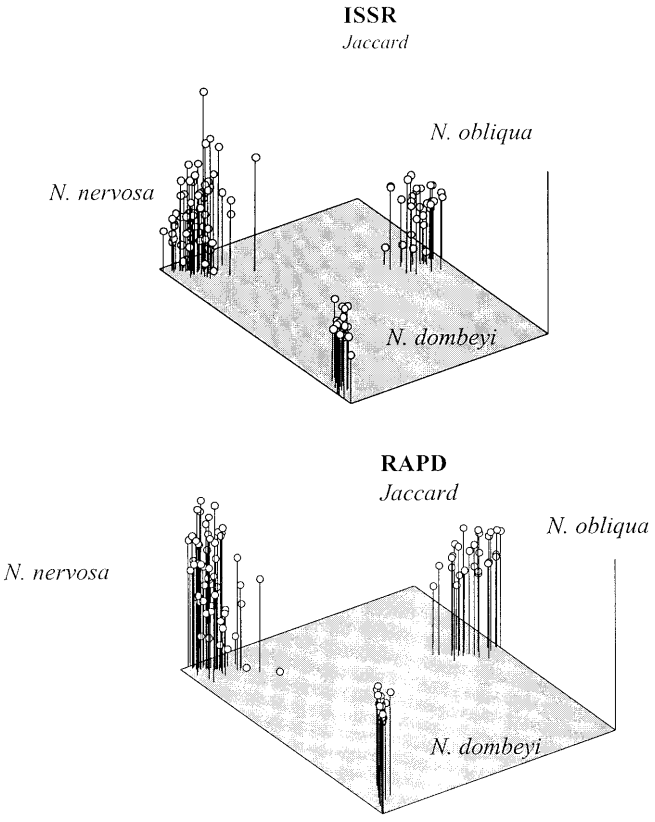

Fig. 2 Principal Coordinate Analysis using ISSR and RAPD markers and the Jaccard similarity index

Table 4 Means of similarity within and among species calculated from Jaccard matrices. Standard deviations in brackets

Species	*N. nervosa*	*N. obliqua*	*N. dombeyi*
ISSR			
N. nervosa	0.723 (± 0.097)		
N. obliqua	0.232 (± 0.061)	0.606 (± 0.092)	
N. dombeyi	0.022 (± 0.019)	0.053 (± 0.031)	0.712 (± 0.099)
RAPD			
N. nervosa	0.751 (± 0.095)		
N. obliqua	0.241 (± 0.049)	0.712 (± 0.104)	
N. dombeyi	0.003 (± 0.013)	0.002 (± 0.009)	0.685 (± 0.119)

The average within-species genetic similarity based on the Jaccard index was similar for all species and for both markers (values ranged between 0.606 and 0.751). These values decreased considerably when an inter-specific comparison was made. Particularly, the similarity of *N. nervosa* vs *N. obliqua* was about 1/3 of the intra-specific ones, and *N. dombeyi* vs the other two species fell to very low values. Moreover, in the latter case the ISSR similarities were about one-order of magnitude higher than the RAPD ones (Table 4).

Discussion

The RAPD technique has been widely used both for studies on wild plants (Yeh et al. 1995; Khasa and Dancik 1996; Owuor 1997; Fornari et al. 1999; Nebauer et al. 1999) and for studies on cultivated plants (Sharma et al. 1995; Divaret et al. 1999; Moeller et al. 1999). By contrast researches employing the ISSR technique have mainly focused on cultivated species (Moreno et al. 1998; Wang et al. 1998; Blair et al. 1999). However few recent papers have demonstrated the high potential of these markers for population and species-level studies (Esselman et al. 1999; Ge and Sun 1999; Clausing et al. 2000; Joshi et al. 2000).

In this work we compared the applicability of ISSRs and RAPDs as genetic markers to characterize the three Chilean *Nothofagus* species. Our results indicate these markers having a similar potential to discriminate between the three species. In all the UPGMA dendrograms, we obtained the same sample distributions: a sharp definition of the three species, with *N. nervosa* and *N. obliqua* closer than *N. dombeyi.*

The minor genetic divergence between *N. nervosa* and *N. obliqua* is demonstrated by the higher number of ISSR and RAPD bands shared between the two species, as compared to those shared with *N. dombeyi*.

These results are coherent with some morphological and physiological characters; for instance, *N. dombeyi* is an evergreen, while the other two species are deciduous. Some hybrids have been identified between *N. nervosa* and *N. obliqua* (Donoso 1996; Gallo et al. 1997; Gallo et al. 2000a, b, Marchelli and Gallo 2000c) and no hybrids are known between *N. dombeyi* and the other two species. Moreover a closer phylogenetic relationship between *N. nervosa* and *N. obliqua* was also demonstrated by studies based on rDNA spacer sequences (Manos 1997), in which these two species were grouped in the same subgenus, *Lophozonia*, while *N. dombeyi* was assigned to the subgenus *Nothofagus*.

Despite the great and similar discriminating power of both markers, some differences between the two could be detected: (1) genetic similarity values lower for RAPDs than for ISSRs, when highly divergent species were compared (*N. dombeyi* vs *N. nervosa* and *N. obliqua*); (2) a number of total, polymorphic and discriminant fragments higher for ISSRs than RAPDs. Although further investigations should be made to verify such a preliminary observation, one possible explanation could be found in the different background on which such differentiation estimates were based. In fact the inter-simple sequence repeats are regions lying within the microsatellite repeats, have a high capacity to reveal polymorphism and offer great potential to determine intragenomic and intergenomic diversity as compared to other arbitrary primers, like RAPDs (Zietkicwicz et al. 1994).

In wheat it was demonstrated that ISSR primers produce several-times more information than RAPD markers (Nagaoka and Ogihara 1997); ISSR markers detected more diversity than RAPD markers in *Calamagrostis*

porterii populations (Esselman et al. 1999). Such characteristics, which were confirmed in the present study, makes the ISSR markers a better potential tool to carry out future population genetics studies and to detect possible natural hybrids among the three *Nothofagus* species.

Problems of the reliability and repeatability of RAPD markers are well known (Ellsworth et al. 1993). Nagaoka and Oigihara (1997) in their studies found that ISSR primers, compared with RAPD primers, produce more reliable and reproducible bands. However, in our experiments, once the PCR conditions were well set up, we obtained a high reproducibility for both RAPD and ISSR markers; only very faint fragments were not reproducible and such fragments were discarded. As previously pointed out, during the ISSR screening we obtained good amplification products from primers based on $(GA)_n$ and $(GT)_n$ repeats, while $(AT)_n$ primers gave no amplification products, despite the fact that poly (AT) dinucleotide repeats are thought to be the most abundant motifs in plant species (Morgante and Olivieri 1993; Depeiges et al. 1995). Similar results were obtained in rice (Blair et al. 1999), in chestnut (Casasoli et al. 2001), in grapevine (Moreno et al. 1998) and in wheat (Nagaoka and Ogihara 1997). A possible explanation of these results is that ISSR primers based on AT motifs are self-annealing, due to sequence complementarity, and would form dimers during PCR amplification (Blair et al. 1999). The results of the present study can be seen as a starting point for future researches aimed at defining the level of intra- and inter-specific genetic diversity and to detect hybrids among these species. For this purpose, a larger number of natural populations of the three species collected from the whole distribution area should be analysed and additional primers tested.

Furthermore, in order to design new diagnostic primers more effective in genetic discrimination among species, discriminant/private bands could be cloned and sequenced, following the approach used in *Quercus* (Bodénès et al. 1997).

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