

# Phylogenetic Relationship Within Genus *Araucaria* (Araucariaceae) Assessed by Means of AFLP Fingerprints

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## Abstract

Highly polymorphic AFLP markers were applied to analyse the phylogenetic relationships of seven species from three sections within genus *Araucaria* (Araucariaceae) with cladistic and phenetic approaches. The objectives of the study were to compare the intrageneric relationships within *Araucaria* assessed by AFLP markers with the classification according to chloroplast DNA sequences and morphological characters. The AMOVA revealed 48% of the variation among species. The results of the principal coordinate analysis revealed three distinct groups: (1) *A. angustifolia* and *A. araucana* (= section *Araucaria*), (2) *A. bidwillii* (= section *Bunya*) and (3) *A. cunninghamii*, *A. heterophylla*, *A. rulei* and *A. scopulorum* (= section *Eutacta*). In the cladistic and phenetic analyses, phylogenetic trees were subdivided into two sister clades, one comprising the samples from section *Eutacta*, the other one was divided again into two sister clades corresponding to sections *Araucaria* and *Bunya*. These results are congruent with a previous phylogenetic study of the family Araucariaceae based on *rbcl* sequences and with the classification of genus *Araucaria* based on morphological characters. Both *rbcl* sequence data and AFLP analyses do not support section *Bunya* as one of the oldest sections within genus *Araucaria*, as suggested by the fossil record. The utility of AFLP markers for phylogenetic analyses is discussed.

**Key words:** *Araucaria*, AFLP, phylogeny, phylogenetic relationships.

## Introduction

The genus *Araucaria* de Jussieu (Family Araucariaceae, Order Coniferales) includes 19 species. Its current geographic distribution is restricted to the Southern hemisphere (GOLTE, 1993). Despite their important ecological and economical role, some species like the South American *A. angustifolia* (Bert.) O. Ktze and *A. araucana* (Mol.) K. Koch are nowadays classified as vulnerable due to intense human pressures (BEKESSY *et al.*, 2002; STEFENON and NODARI, 2003).

From an origin in the Triassic, the family Araucariaceae expanded and diversified in both hemispheres in the Jurassic and Early Cretaceous (KERSHAW and WAGSTAFF, 2001). Within genus *Araucaria*, the fossil records suggest a basal position of section *Bunya* as one of the oldest recorded sections (STOCKEY and TAYLOR, 1978).

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A phylogenetic study of the *rbcl* gene for the family Araucariaceae (SETOGUCHI *et al.*, 1998) revealed a clear structure within the genus *Araucaria*. This structure is in accordance with the taxonomic classification based on morphological characters in sections *Araucaria*, *Eutacta*, *Intermedia* and *Bunya*. However, molecular data of the *rbcl* gene did not support the early divergence of the monotypic section *Bunya* (SETOGUCHI *et al.*, 1998). These authors suggested that further molecular data should be added to enhance the statistical probability concerning the position of *A. bidwillii* Hook. (the only extant species in section *Bunya*) in the phylogenetic tree. The *rbcl* sequence is very commonly used for phylogenetic analyses. However, some studies have shown that its sequence is much conserved and sometimes not able to clarify relationships between closely related taxa (WANG *et al.*, 1999; RYDIN and WISTRÖM, 2002). According to WANG *et al.* (1999), *rbcl* tends to be conservative among some genera of the gymnosperm family Pinaceae.

AFLPs are highly polymorphic dominant markers that cover a larger proportion of the whole genome (MUELLER and WOLFENBARGER, 1999), randomly accessing both coding (rather conservative) and non-coding (not necessarily conservative) regions. Thus, they may provide many informative markers to complement the single gene *rbcl* information within genus *Araucaria*. The AFLP technique has been used to reveal evolutionary relationships at the species or genus level (KOOPTMAN *et al.*, 2001; BEARDSLEY *et al.*, 2003; BROUAT *et al.*, 2004) and is considered to be able to resolve phylogenetic relationships congruent with analyses based on morphological characters and on nuclear markers as internal transcribed spacers (ITS) or restriction fragment length polymorphisms (RFLPs) (BROUAT *et al.*, 2004). Here, we report a phylogenetic analysis of the genus *Araucaria* generated by means of AFLP markers and discuss the capacity of these markers to produce informative phylogenetic data to an 'ancient' genus of gymnosperms.

## Material and Methods

### *Plant material*

Plant material was collected from botanical and private gardens (see Table 1). Seven species of genus *Araucaria* (*A. angustifolia* (Bert.) O. Ktze., *A. araucana* K. Koch, *A. bidwillii* Hook., *A. cunninghamii* Aiton ex D. Don., *A. heterophylla* (Salisb.) Franco, *A. rulei* F. Muell. and *A. scopulorum* de Laub.) corresponding to three sections (*Araucaria*, *Bunya* and *Eutacta*) were investigated. *Agathis robusta* (F. Muell.) F. M. Bailey

(Araucariaceae) was used as an outgroup (see *Table 1*). Species identification of the samples was performed in the respective botanical gardens, with exception of sample 'ang5' (cultivated in a private garden in Brazil) that was identified by V. M. STEFENON. Identification of the samples was confirmed in our laboratory and doubtful samples were excluded from the analysis. Voucher specimens were deposited in the Institute of Forest Genetics and Forest Tree Breeding of the Georg-August-University Göttingen. The natural distribution of the species is shown in *Figure 1*.

#### DNA isolation and AFLP analysis

About fifty milligrams of plant material were disrupted in a 96-well block and the total DNA was extracted using the DNEasy 96 Plant Kit (Qiagen), following the instructions of the manufacturer. The AFLP reactions were performed as described by VOS *et al.* (1995), with

slight modifications as described by GAILING and VON WUEHLISCH (2004). About 150 ng of genomic DNA was incubated at room temperature for about 16 hours for the digestion with the restriction enzymes *EcoRI* and *MseI* and the ligation of the corresponding *EcoRI*- and *MseI*-adapters to the ends of the restriction fragments. A pre-selective amplification was performed with the primer pairs displaying one selective nucleotide, namely *Eco*-primer + A (E-A) and *Mse*-primer + G (M-G). The PCR protocol for the pre-selective amplification consisted of an initial step at 72 °C for 2 min followed by 20 cycles at 94 °C for 10s, at 56 °C for 30s, at 72 °C for 2 min and of a final extension step at 60 °C for 30 min. Four microliters of the diluted (1:10) pre-selective reaction were used as template for the selective amplification with the following primer combinations: E-AGA/M-GGA, E-AGA/M-GGG, E-AGC/M-GCC and E-AGC/M-GGA. The PCR protocol for the selective reaction was: a 2 min

*Table 1.* – Plant material sampled for the phylogenetic analysis and names applied.

Species (Section)	Sample name	Source
<i>A. angustifolia</i> ( <i>Araucaria</i> )		
	ang1	University of Freiburg – Germany
	ang2	University of Tübingen – Germany
	ang3	University of Gießen – Germany
	ang4	University of Oldenburg – Germany
	ang5	Cultivated in Private Garden – Lages - Brazil
<i>A. araucana</i> ( <i>Araucaria</i> )		
	ara1	University of Gießen – Germany
	ara2	University of Tübingen – Germany
	ara3	University of Oldenburg – Germany
	ara4	Free University of Berlin – Germany
	ara5	University of Göttingen – Germany
<i>A. bidwillii</i> ( <i>Bunya</i> )		
	bid1	University of Freiburg – Germany
	bid2	University of Gießen – Germany
	bid3	University of Tübingen – Germany
<i>A. heterophylla</i> ( <i>Eutacta</i> )		
	het1	University of Freiburg – Germany
	het2	University of Gießen – Germany
	het3	University of Oldenburg – Germany
<i>A. cunninghamii</i> ( <i>Eutacta</i> )		
	cun1	University of Tübingen – Germany
	cun2	University of Göttingen – Germany
<i>A. scopulorum</i> ( <i>Eutacta</i> )		
	sco	University of Tübingen – Germany
<i>A. rulei</i> ( <i>Eutacta</i> )		
	rul	University of Tübingen – Germany
<i>Agathis robusta</i> (outgroup)		
	Agathis	University of Göttingen – Germany

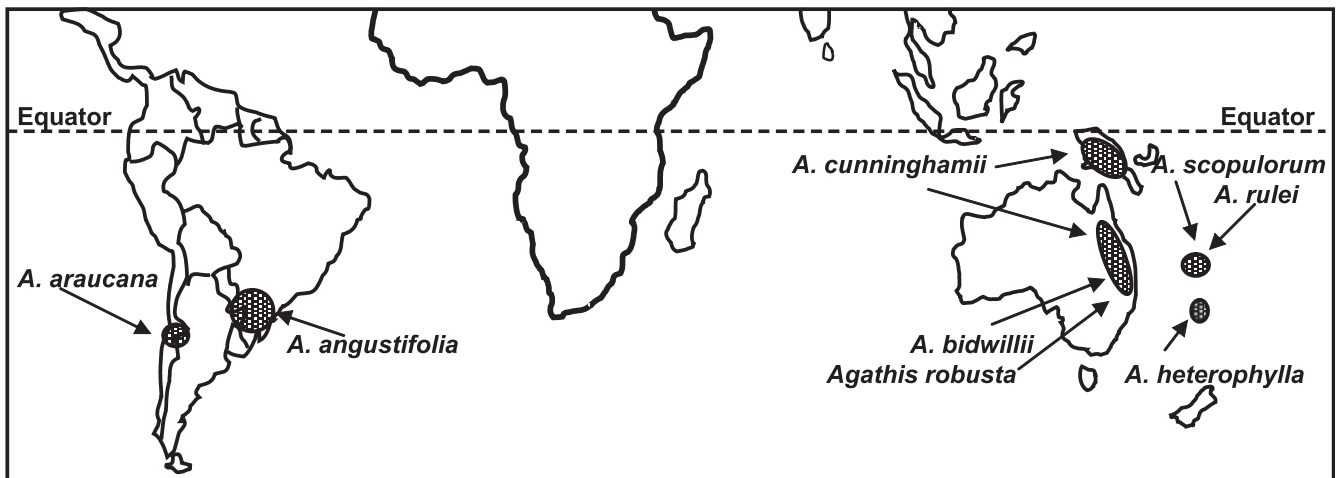


Figure 1. – Natural geographical distribution of *Araucaria* species analysed and of the outgroup species *Agathis robusta*. *A. angustifolia* (Brazil, Argentina and Paraguay), *A. araucana* (Chile and Argentina), *A. bidwillii* (Australia), *A. cunninghamii* (Australia and New Guinea), *A. heterophylla* (Norfolk Island), *A. rulei* (New Caledonia), *A. scopulorum* (New Caledonia) and *Agathis robusta* (Australia). After SETOGUCHI *et al.* (1998) and GOLTE (1993).

denaturation at 94 °C, 9 cycles at 94 °C for 10s, an annealing step at 65 °C for 30s (which was decreased by 1 °C every cycle until 56 °C was reached) and an extension step at 72 °C for 2 min. The reaction was continued with an annealing temperature of 56 °C for the last 24 cycles ending with a final extension step at 60 °C for 30 min. All PCR reactions were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research). The *EcoRI* selective primers were labelled with the fluorescent dyes NED or HEX. The fragments were separated on an ABI Genetic Analyser 3100 with the internal size standard GS 500 ROX (Applied Biosystems). The data were analysed using GeneScan 3.7<sup>®</sup> and Genotyper 3.7<sup>®</sup> software (Applied Biosystems). Bands between 50 and 350 bp (>50 rescaled peak height) were analysed. Absence (0) and presence (1) of fragments was scored and transformed into a binary matrix for data analysis. After confirming that the analysed species were monophyletic, the pre-amplified DNA of up to five samples of each species (see Table 1) was bulked and this bulked DNA served as template for a selective AFLP amplification using the same selective primer pairs and analysis parameters (see above).

#### Data analysis

Initially, genetic relatedness of species and sections were assessed for the data set of all individuals using an analysis of molecular variance (procedure AMOVA from Arlequin 2.0; SCHNEIDER *et al.*, 2000) and a principal coordinate analysis (PCO) based on Dice's coefficient of similarity (DICE, 1945) using the procedures SIMQUAL, DCENTER and EIGEN from NTSYSpc 2.0 (ROHLF, 1998). In addition, the data set of all individuals and the data set of bulked DNA were analysed with phenetic (Neighbor-Joining; NJ) and cladistic (Maximum Parsimony; MP) approaches using the software PAUP\* version 4.0b10 (SWOFFORD, 1998). The NJ analysis was performed using the genetic distance of Nei and Li (NEI and LI, 1979), which is the complement of Dice's coefficient of similarity, equalling 1 - "Dice". The parsimony heuristic tree searches were carried out under equal weight

criterion, the tree bisection-reconnection (TBR) branch swapping algorithm and the option to collapse branches at zero length. A bootstrap analysis (FELSENSTEIN, 1985) with 1000 replicates was conducted to assess the internal support for taxa in NJ and MP analyses.

The NJ and MP trees generated with the data set of all individuals were visually compared to assess the congruence between both analyses (see Figure 3). Additionally, the topology of the MP tree derived from bulked DNA samples was compared with the MP tree calculated from *rbcL* sequences after SETOGUCHI *et al.* (1998), in order to assess the congruence between AFLP and cpDNA analyses (see Figure 4).

## Results and Discussion

### Relationship among species

Following the parameters applied for markers selection, the four primer combinations generated a total of 678 polymorphic markers. From 136 to 210 markers could be analysed per primer combination (mean number = 169.5 markers).

The partitioning of the molecular variance (AMOVA) among species was calculated for the data set of all individuals and revealed that 48% of the variation reside among species ( $\Phi_{ST} = 0.48$ ;  $p < 0.001$ ).

The PCO analysis generated three groups that were clearly differentiated and corresponded to sections *Araucaria*, *Bunya* and *Eutacta*. The first principal coordinate explained 23% and the second principal coordinate explained 13% of the total variation. The three represented sections were clearly differentiated. Samples of *A. angustifolia* and *A. araucana* (Section *Araucaria*) group together, while the monotypic section *Bunya*, represented by *A. bidwillii*, is also clearly separated in this analysis. The structure among species within section *Eutacta* was not clarified, but the four species analysed of this section form a distinct group (Figure 2).

For the data set with all individuals, ninety percent (608 out of 678) of the AFLP markers were parsimony-

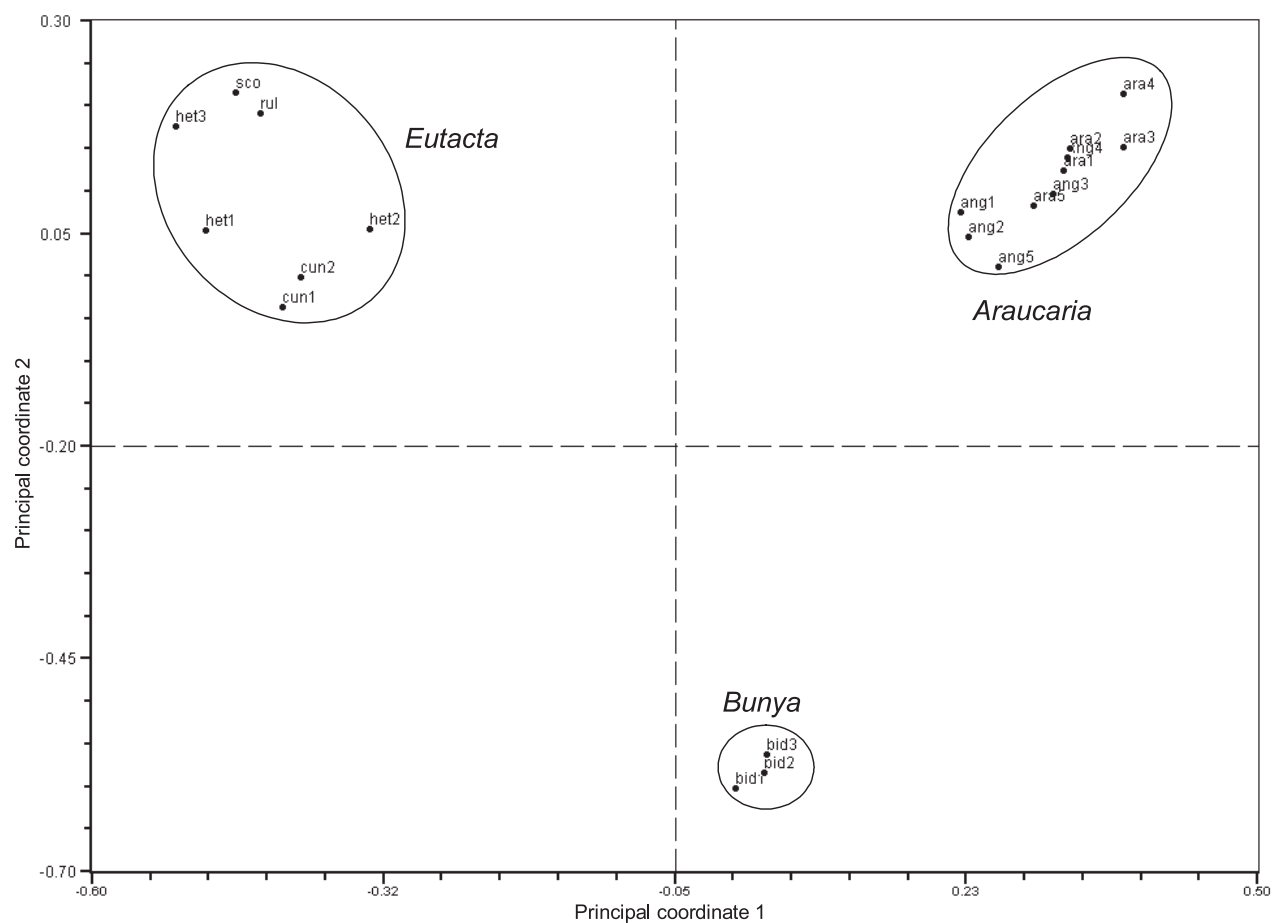


Figure 2. – Principal coordinate analyses (PCO) based on Dice's coefficient of similarity (DICE, 1945) showing differentiation among sections of genus *Araucaria*. The first coordinate describes 23% and the second coordinate 13% of the total variation. For sample codes see Table 1.

Table 2. – Pairwise genetic distances (NEI and LI, 1979) between samples of *Araucaria*. For sample codes see Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 ang1	-																				
2 ang2	0.099	-																			
3 ang3	0.085	0.073	-																		
4 ang4	0.055	0.069	0.048	-																	
5 ang5	0.080	0.035	0.070	0.058	-																
6 ara1	0.128	0.081	0.102	0.115	0.077	-															
7 ara2	0.130	0.095	0.101	0.111	0.099	0.055	-														
8 ara3	0.125	0.093	0.082	0.097	0.091	0.058	0.052	-													
9 ara4	0.123	0.123	0.086	0.089	0.123	0.097	0.073	0.080	-												
10 ara5	0.122	0.060	0.095	0.101	0.073	0.039	0.061	0.060	0.105	-											
11 het1	0.174	0.139	0.158	0.163	0.149	0.157	0.167	0.174	0.190	0.143	-										
12 het2	0.142	0.124	0.149	0.150	0.131	0.130	0.156	0.153	0.177	0.129	0.039	-									
13 het3	0.177	0.143	0.158	0.165	0.152	0.153	0.172	0.164	0.197	0.137	0.019	0.042	-								
14 cun1	0.164	0.162	0.186	0.189	0.159	0.190	0.197	0.195	0.229	0.172	0.117	0.110	0.117	-							
15 cun2	0.165	0.150	0.175	0.178	0.152	0.163	0.174	0.187	0.220	0.154	0.095	0.092	0.102	0.053	-						
16 rul	0.218	0.158	0.178	0.185	0.167	0.194	0.190	0.196	0.212	0.181	0.094	0.121	0.095	0.155	0.141	-					
17 bid1	0.142	0.119	0.129	0.146	0.112	0.149	0.145	0.144	0.172	0.122	0.145	0.142	0.158	0.150	0.152	0.195	-				
18 bid2	0.158	0.116	0.139	0.145	0.113	0.127	0.132	0.126	0.171	0.119	0.148	0.142	0.153	0.160	0.161	0.186	0.048	-			
19 bid3	0.162	0.120	0.130	0.152	0.116	0.126	0.138	0.126	0.159	0.116	0.153	0.145	0.164	0.177	0.168	0.190	0.054	0.050	-		
20 sco	0.203	0.164	0.181	0.193	0.174	0.184	0.171	0.177	0.195	0.167	0.089	0.106	0.086	0.132	0.129	0.081	0.192	0.194	0.179	-	
21 Agathis	0.228	0.180	0.219	0.223	0.169	0.191	0.210	0.224	0.326	0.168	0.216	0.195	0.218	0.204	0.197	0.261	0.192	0.194	0.200	0.234	-

informative, while the NJ tree was generated with all 678 markers. Pairwise genetic distances (NEI and LI, 1979) between samples are shown in *Table 2*. In the MP analysis heuristic search yielded two shortest trees of 2003 steps, a consistency index (CI) of 0.31 and a retention index (RI) of 0.53. The consistency index in the parsimony analysis suggests a high level of homoplasy. Nevertheless all sections and species were supported by high bootstrap values (*Figure 3*). *Araucaria rulei* and *A. scopulorum* (section *Eutacta*) that are represented only by unique samples cluster together in clade *Eutacta* and show the same position in *rbcL* and AFLP trees (*Figure 4*) suggesting that also unique samples are informative to represent the respective species.

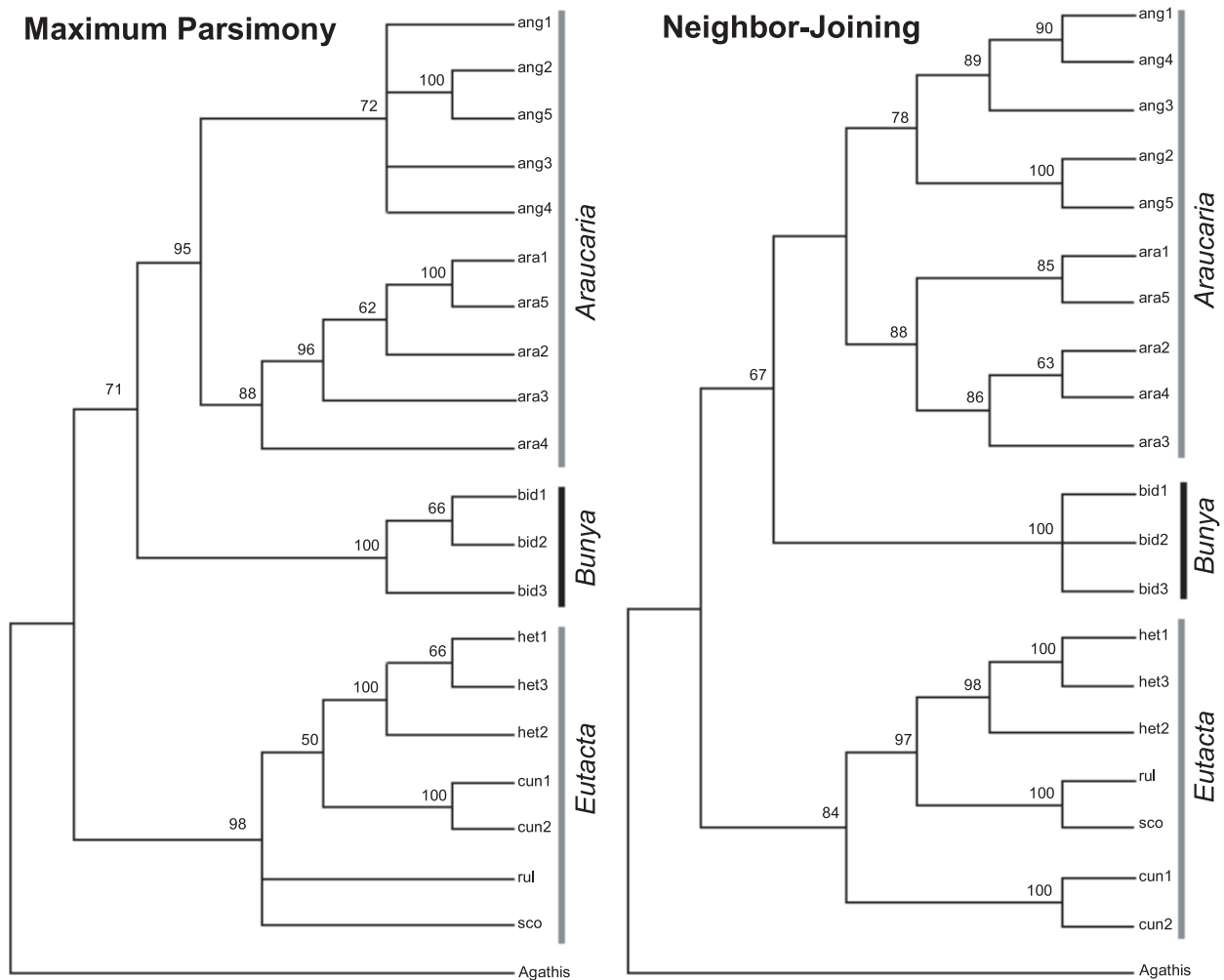
Comparing cladistic and phenetic analyses no difference was observed in the general topology of the generated trees (see *Figure 3*). The monotypic section *Bunya* revealed to be sister group to section *Araucaria* and the *Eutacta* clade is sister to the *Araucaria/Bunya* clade. Thus, the basal position of *Bunya* as one of the oldest recorded sections, as indicated by the fossil record (STOCKEY and TAYLOR, 1978), is not supported by our analysis. Congruence with *rbcL* data (SETOGUCHI *et al.*, 1998) and more recent paleobotanical evidence

(STOCKEY, 1994) suggest that many fossils formerly named as *Bunya* should be re-evaluated.

According to the NJ tree *A. cunninghamii* is sister to the other species of section *Eutacta*. *A. heterophylla* forms a well supported sister clade to *A. rulei* and *A. scopulorum*. In the MP tree these species form a polytomy. The clade comprising *A. heterophylla* and *A. cunninghamii* as sister species has only 50% bootstrap support.

Phylogenetic trees calculated for individual samples (*Figure 3*) and from bulked DNA (*Figure 4*) show the same topology and are congruent with the *rbcL* Maximum Parsimony analysis from SETOGUCHI *et al.* (1998). *Figure 4* shows a comparison between the AFLP phylogram generated from bulked DNA (678 AFLP markers, 430 parsimony-informative markers, tree length = 1152 steps, CI = 0.48, RI = 0.36) and the *rbcL* phylogeny.

Section *Araucaria* (*A. angustifolia* and *A. araucana*) and the monotypic section *Bunya* (*A. bidwillii*) that group together in the AFLP tree also share important taxonomic characters. Both sections are characterized by large and flat leaves, hypogeal germination, fleshy seedlings and two cotyledons that are long-stalked dur-



*Figure 3.* – Parsimony and Neighbor-Joining (Nei and Li's genetic distance; NEI and LI, 1979) phylogenetic trees generated with 678 AFLP markers for individuals of seven species of *Araucaria* from South America (*A. angustifolia* and *A. araucana*) and Australasia (*A. cunninghamii*, *A. heterophylla*, *A. rulei*, *A. scopulorum* and *A. bidwillii*). Bootstrap values for 1000 replicates are shown for each node. For sample codes see *Table 1*.

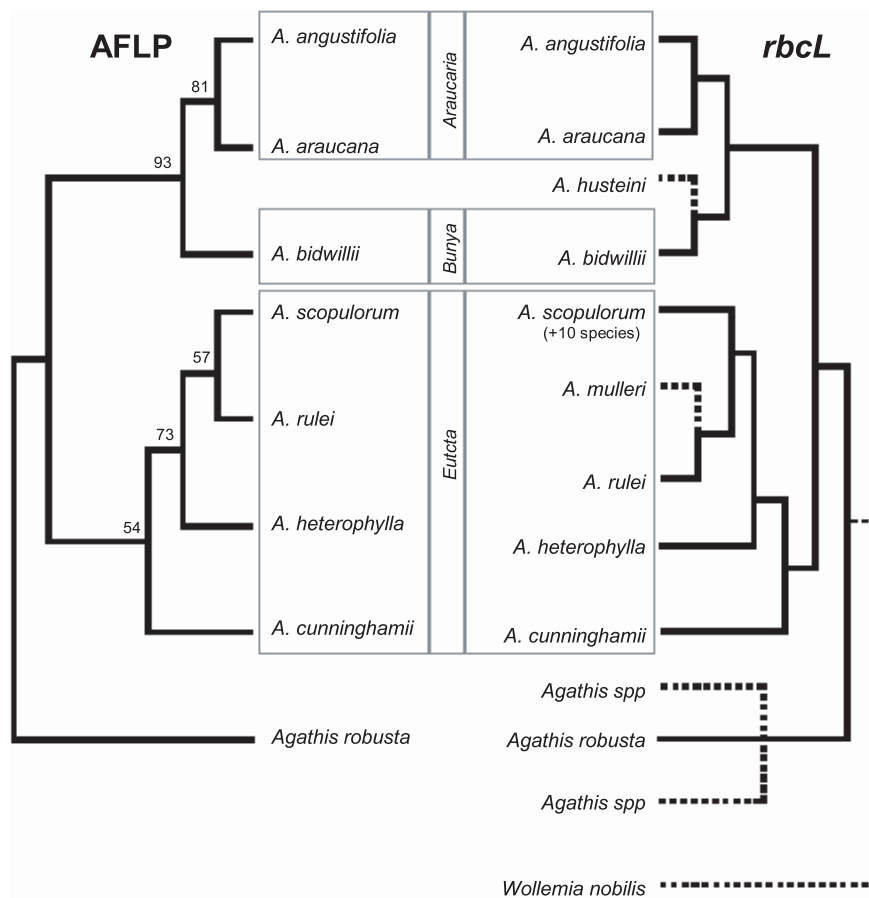


Figure 4. – Comparison between AFLP (bulked DNA) and *rbcL* gene sequences Maximum-Parsimony phylogenies of *Araucaria*. Numbers at each node in the AFLP tree represent bootstrap values (1000 replicates). Dotted branches in the *rbcL* tree show species not included in the present AFLP study. The *rbcL* phylogenetic tree is adapted from SETOGUCHI *et al.* (1998).

ing germination and retained in seed coats (STOCKEY, 1982; GOLTE, 1993). Species from section *Eutacta* display smaller leaves, epigeal germination, four sub-sessile cotyledons that are freed from seed walls at germination and no fleshy seedlings (STOCKEY, 1982; GOLTE, 1993).

Within section *Araucaria* a considerable differentiation supported by high bootstrap values was observed among individuals within species *A. angustifolia* and *A. araucana* suggesting that the individuals sampled in Botanical Gardens originated from different geographic locations.

In conclusion, the relationship among species of *Araucaria* revealed by AFLPs are in accordance with prior classifications based on molecular (*rbcL* sequences; SETOGUCHI *et al.*, 1998) and morphological (STOCKEY, 1982) studies.

#### Usefulness of AFLP technique

Despite the wide use of AFLP markers for genetic studies, there are doubts of using this technique to determine phylogenetic relationships. In order to establish phylogenetic relationships among taxa, the character analysed must show homologous similarities (modification by descent). One of the weaknesses of AFLP markers to assess phylogenetic relationships is the fact

that fragments of related taxa may have the same length, but a different sequence and are therefore not orthologous. With increasing genetic differentiation among taxa fragments of the same size are more likely to be not orthologous (MECHANDA *et al.*, 2004).

Furthermore, it is known that there are many duplication events during species evolution resulting in paralogs that constitute a general problem in deducing phylogenies. This problem is even more acute, if single genes from a multigene family (and not single copy genes) are analysed.

However, the strongest advantage of AFLP markers to infer phylogenetic relationships is that they sample from many regions of the genome, generating a large number of markers (MUELLER and WOLFENBARGER, 1999; WEISING *et al.*, 2005). These genome-wide data sets may provide high power in testing specific phylogenetic relationships (ROKAS *et al.*, 2003).

If a large number of AFLP markers are investigated, many of them are likely to be orthologous. Indeed, ROUPPE VAN DER VOORT *et al.* (1997) found 19 identical sequences out of 20 putatively homologous AFLP markers sequenced in potato. PARSONS and SHAW (2001) sequenced ten AFLP fragments co-migrating in cricket species (genus *Laupala*) and found a degree of sequence

similarity of the same-sized bands between 97 and 100%. They suggested that same-sized AFLP fragments can be confidently considered as homologous. Thus due to the large number of markers analysed any bias in phylogenetic inference are likely to be small and the results will accurately reflect the genetic relationships among taxa (PARSONS and SHAW, 2001).

Since the relative amount of homoplastic AFLP fragments and their effect on reconstructing phylogenetic relationships is difficult to assess, the application of either phenetic or cladistic approaches when using AFLP markers has been discussed (KOOPMAN *et al.*, 2001; LARA-CABRERA and SPOONER, 2004).

KOOPMAN *et al.* (2001) suggested that, if topologies of the phenogram and the cladogram generated by AFLP fingerprints are identical, homoplasies do not influence the cladistic analysis and will not affect conclusions of species relationships. Besides, in bootstrap or jackknife branch support analyses, the presence of internal conflict caused by homoplasies will lead to an exclusion of these branches as uninformative and they will not affect the conclusions on species relationships (KOOPMAN *et al.*, 2001).

Despite the potential limitations for the use of the AFLP technique in phylogenetic analyses, in particular false fragment homology, congruence has been reported between AFLP and single gene sequence phylogenetic analyses (present study; SPOONER *et al.*, 2005), between AFLP and ITS/ETS phylogenetic analyses (KOOPMAN *et al.*, 2001; BEARDSLEY *et al.*, 2003; SPOONER *et al.*, 2005) and between AFLP and morphological characters analyses (present study; SPOONER *et al.*, 2005). Furthermore, analyses of just one or few sequences, as well as analyses of a large number of "biased" genes are likely to produce incorrect phylogenetic trees with even high bootstrap support (ROKAS *et al.*, 2003). Thus, AFLP fingerprints can be a useful technique to complement the information about phylogenetic relationships among related taxa.

Since the present AFLP phylogenetic analysis of genus *Araucaria* showed high congruence with morphological and cpDNA sequence classifications, AFLP markers can be used to confirm or complement the information about phylogenetic relationships among ancient taxa, especially if DNA sequence variation is limited or sequence information of only few loci is available.

## Conclusions

In the present study, species within genus *Araucaria* proved to be well separated from each other with strongly supported monophyletic sections. The relationship between the South American species *A. angustifolia* and *A. araucana* (section *Araucaria*) is also clearly resolved. Within section *Eutacta* from Australasia the species relationships are only resolved in the NJ tree. In addition, our data and previous reports (STEFENON *et al.*, 2003; STEFENON and NODARI, 2003) suggest that AFLPs provide suitable molecular markers to study the relationships among species within genus *Araucaria* and also within *Araucaria* species. In an ongoing project the

usefulness of AFLP markers to distinguish *A. angustifolia* populations from different geographic origins in Brazil will be tested.

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## Short Note: Crossability Between *Pinus uliginosa* and its Putative Parental Species *Pinus sylvestris* and *Pinus mugo*

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### Abstract

Results of artificial fertilization of *P. uliginosa* with *P. sylvestris* and *P. mugo* are presented and discussed. *P. sylvestris* and *P. mugo* are thought to be the parental species of *P. uliginosa*. Two grafts of one *P. uliginosa* clone from Arboretum of the Institute of Dendrology in Kórnik, Poland were used as mother individuals. One individual of *P. sylvestris* and one individual of *P. mugo* were the pollen donors. Three mature cones were obtained as the result of artificial pollination of *P. uliginosa* with *P. mugo* pollen. Out of 107 seeds, 68 were filled what gives 63%. *P. uliginosa* conelets pollinated

with *P. sylvestris* pollen were all aborted. Based on obtained data, close phylogenetic relationship between *P. uliginosa* and *P. mugo* complex is suggested.

*Key words:* *P. uliginosa*, *P. sylvestris*, *P. mugo*, hybridization, pollination, crossability.

### Introduction

Dwarf mountain pine complex (*Pinus mugo* Turra complex) is well known for its taxonomically perplexing pattern of phylogenetic relations and contradictory views on taxonomic rank of taxons belonging to this