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Application of RAPD in Discrimination of *Podocarpus macrophyllus* 's Sex

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Abstract: *Podocarpus macrophyllus* 's sex is hard to determine from its morphologic characteristics when they 're not in blossom. This work was a trial of application of random amplified polymorphic DNA (RAPD) approach in sex discrimination of *Podocarpus macrophyllus*. After screening over 340 kinds of 10 bp long arbitrary primers, 23 primers were found to generate reproducible polymorphic bands, but only P20 primer could generate a common 750 bp DNA fragment with DNA samples from all the female trees. Analysis of the polymorphic bands by unweighted pair group method using arithmetic average (UPGMA) was carried out and the results showed that the disparity between male and female trees was greater than that of the same sex between different variants, *Podocarpus macrophyllus* (*Thunb.*) *D. Don* and *Podocarpus macrophyllus Var. maki Endl*. In addition, the amplification results by single primer and two primers were also compared.

Keywords: *Podocarpus macrophyllus*; RAPD; sex discrimination; UPGMA
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Podocarpus macrophyllus is a dioecious plant. It is hard to discriminate the sex of *Podocarpus macrophyllus* trees when they are not in blossom. The establishment of a convenient way to discriminate the sex of *Podocarpus macrophyllus* trees will have significant benefits both for ecology and economy, and will play an instructive role in biodiversity study.

Random amplified polymorphic DNA (RAPD) was developed independently by two research groups headed by J. Williams[1] and J. Welsh[2] in 1990. RAPD analysis has several advantages over conventional genetic analysis. First, there is no need to know the nucleotide sequence of the target fragment. Second, there are enough varieties of synthetic primers for RAPD analysis. Third, RAPD analysis is much more efficient than other genetic and molecular biological approaches, such as gene cloning, Southern hybridization, etc[3]. Recently, RAPD has been widely used in genetic analysis including gene mapping, genotyping, phylogenetism analysis, etc[4-10]. Here, we report the application of RAPD approach in discriminating *Podocarpus macrophyllus* 's sex, as well as the UPGMA analysis based on RAPD results.

1　Materials and Methods

1.1　Reagents

The decamer oligonucleotides used as primers for RAPD amplification, were purchased from Zhejiang Bolian Nutrition & Project Institute (China). The primers were used as primers for the amplification of RAPD

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sequences (See Tab. 1). Taq polymerase, dNTPs were purchased from TaKaRa Biotech (Dalian, China). Other reagents used were analytical grade.

1.2 DNA samples

DNA samples were extracted from leaves of 10 *Podocarpus macrophyllus* trees planted in the main campus of Fudan University. The numbering of trees is listed in Tab. 2. DNA extraction was performed according to protocols described by ZOU Yu-Ping (1994)[11,12].

Tab. 1 Nucleotide sequences and amplification products of 23 primers of genetic polymorphism

Primer	Sequence	Polymorphic Bands' Number
A02	5′-TGCCGAGCTG-3′	9
D02	5′-GGACCCAACC-3′	9
D08	5′-GTGTGCCCCA-3′	8
E18	5′-GGACTGCAGA-3′	14
G10	5′-AGGGCCGTCT-3′	12
L05	5′-ACGCAGGCAC-3′	12
L12	5′-GGGCGGTACT-3′	15
N08	5′-ACCTCAGCTC-3′	8
N09	5′-TGCCGGCTTG-3′	13
N18	5′-GGTGAGGTCA-3′	8
P07	5′-GTCCATGCCA-3′	11
P08	5′-ACATCGCCCA-3′	10
P09	5′-GACCCTAGTC-3′	11
P15	5′-GGAAGCCAAC-3′	9
P16	5′-CCAAGCTGCC-3′	9
P20	5′-GTGGTCCGCA-3′	16
Y01	5′-GTGGCATCTC-3′	16
Y02	5′-CATCGCCGCA-3′	6
Y04	5′-GGCTGCAATG-3′	13
Y05	5′-GGCTGCGACA-3′	7
Y06	5′-AAGGCTCACC-3′	14
Y14	5′-GGTCGATCTG-3′	17
Y15	5′-GGAAGCCAAC-3′	22

1.3 PCR amplification

All PCR amplifications were performed on GeneAmp PCR System 2400 (Perkin Elemer, USA)[13]. A typical 25 μL reaction mixture contained 10 ng template DNA, 0.2 μmol/L decamer primer, 250 μmol/L each of dATP, dCTP, dGTP and dTTP, and 0.5 U Taq DNA polymerase supplemented with 1 × PCR Buffer. The concentration of magnesium chloride was controlled at 1.7 mmol/L. Taq DNA polymerase was added into the reaction mixture at 80 ℃ after 5 min preheating at 94 ℃ to ensure the reproducibility of data. Typically, DNA amplification was carried out with 45 thermal cycles of 50 s at 94 ℃, 70 s at 38 ℃ and 120 s at 72 ℃ followed by additional 10 min at 72 ℃ at the end. The PCR products were analyzed by electrophoresis on 15 g/L agarose gel and visualized under UV light after staining with ethidium bromide.

Tab. 2 Information of the plant used for RAPD analysis

Acc. no.	Species	Sex	Height/m
FD1	*Podocarpus macrophyllus* (Thunb.) D. Don	♀	2-3
FD2	*Podocarpus macrophyllus* (Thunb.) D. Don	♀	2-3
FD3	*Podocarpus macrophyllus* (Thunb.) D. Don	♂	2-3
FD4	*Podocarpus macrophyllus* (Thunb.) D. Don	♂	2
FD5	*Podocarpus macrophyllus* Var. maki Endl.	♀	5-6
FD6	*Podocarpus macrophyllus* Var. maki Endl.	♂	5-6
FD7	*Podocarpus macrophyllus* Var. maki Endl.	♂	3-4
FD8	*Podocarpus macrophyllus* (Thunb.) D. Don	♂	2-3
FD9	*Podocarpus macrophyllus* (Thunb.) D. Don	♀	4-5
FD10	*Podocarpus macrophyllus* Var. maki Endl.	♀	4

1.4 Data analysis

Polymorphic bands generated by primer E18, N18, L05, P07, P16, A02, P09, P08, P15, D02, D08, N09, N08, P20, Y01, Y02, Y04, Y05, Y06, G10, L12, N09 + A02, L05 + D02, L05 + A02, which were totally 1 405 bands, were analyzed by UPGMA. Value 1 and 0 were assigned to the presence or absence of a

DNA band at indicated position of agarose gel. Then the simple matching coefficient of similarity and genetic distance were calculated by improved formula from Nei 's(the Nei 's formula was $I = \mathrm{h}XiYi/(\mathrm{h}Xi^2\ Yi^2)^{1/2})$: $S_{XY} = 2N_{XY}/(N_X + N_Y)$, and $D_{XY} = -\ln S_{XY}$. S_{XY} represents the similarity between tree X and tree Y, N_{XY} represents the number of RAPD bands owned by tree X and tree Y commonly, N_X represents the total RAPD bands of tree X, N_Y represents the total RAPD bands of tree Y, and D_{XY} represents the genetic distance between tree X and tree Y. UPGMA cluster analysis was performed with Mega2 software based on Tab.3, and the result is shown in Fig.1.

Tab.3　Genetic distance matrix for the 10 cultivars studied

Acc. no.	FD1	FD2	FD3	FD4	FD5	FD6	FD7	FD8	FD9	FD10
FD1	0	0.633 7	0.947 9	0.995 2	0.598 4	0.962 5	0.927 3	0.941 4	0.752 9	0.527 5
FD2	0.633 7	0	0.905 5	0.902 6	0.488 1	0.901 9	0.846 0	0.827 7	0.678 1	0.589 1
FD3	0.947 9	0.905 5	0	0.758 2	0.832 2	0.689 5	0.677 6	0.652 5	0.803 2	0.764 5
FD4	0.995 2	0.902 6	0.758 2	0	0.758 8	0.754 6	0.794 4	0.704 0	0.909 0	0.728 2
FD5	0.598 4	0.488 1	0.832 2	0.758 8	0	0.751 1	0.841 1	0.729 8	0.530 6	0.504 6
FD6	0.962 5	0.901 9	0.689 5	0.754 6	0.751 1	0	0.658 5	0.620 1	0.908 6	0.680 1
FD7	0.927 3	0.846 0	0.677 6	0.794 4	0.841 1	0.658 5	0	0.561 6	0.971 3	0.814 4
FD8	0.941 4	0.827 7	0.652 5	0.704 0	0.729 8	0.620 1	0.561 6	0	0.709 1	0.784 4
FD9	0.752 9	0.678 1	0.803 2	0.909 0	0.530 6	0.908 6	0.971 3	0.709 1	0	0.679 5
FD10	0.527 5	0.589 1	0.764 5	0.728 2	0.504 6	0.680 1	0.814 4	0.784 4	0.679 5	0

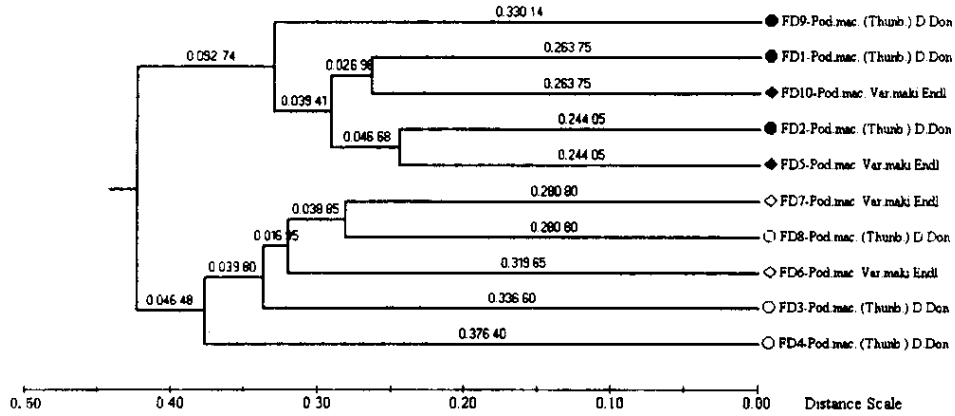

Fig.1　Dendrogram for the 10 trees of *Podocarpus macrophyllus* studied was based on cluster analysis (UPGMA) of genetic distance based the data on Tab.3

2　Results and Discussions

Totally 340 kinds of decamer nucleotides were used as primers for RAPD analysis to generate polymorphic DNA bands. As the results, 23 of them were able to amplify reproducible polymorphic DNA bands. The numbers of polymorphic DNA bands were listed in Tab.1. Only one primer, P20 (5 '-GTGGTCCGCA-3 '), was found to be able to generate a common 750 bp DNA fragment in all female *Podocarpus macrophyllus* tree 's DNA samples, as indicated by allows in Fig.2.

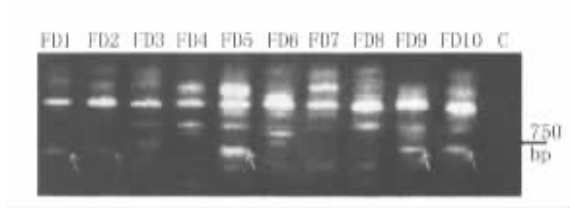

Fig.2 Agarose gel of RAPD-amplified *Podocarpus macrophyllus* DNA

DNA from each of the 10 *Podocarpus* was amplified with the primer P20 and separated on a 15 g/L agarose gel with the methods as mentioned before. Each of the samples (1, 2, 5, 9, and 10) has a fragment about 750 bp that is present in the female only. Here the number 1 to 10 represents cultivars from FD1 to FD10, C stands for negative control. The DNA molecular size markers used were 970, 750, 650, 450, 250 bp

The dendrogram obtained by unweighted pair group method using arithmetic average (UPGMA) is shown in Fig.1. To our surprise, ten cultivars were grouped into two clusters naturally by their sex. Whereas six of the ten cultivars are *Podocarpus macrophyllus* (Thunb.) D.Don (three males and three females), and four are *Podocarpus macrophyllus* Var. maki Endl (two males and two females). Our data revealed that in the case of *Podocarpus macrophyllus*, the genetic disparity between male and female trees was greater than that between two male or female trees from different variants.

Podocarpus macrophyllus Var. maki Endl is variety of *Podocarpus macrophyllus* (Thunb.) D.Don, and their morphological traits can only be separated from leaves: a leaf of *Podocarpus macrophyllus* (Thunb.) D.Don is stripped needle, mostly 7 to 12 cm long and 7-10 mm board, while that of *Podocarpus macrophyllus* Var. maki Endl is much shorter, about 2.5-7 cm in length, 3-7 mm in width, with its cusp obtuse. Other characters of the two such as bloom and fruit are the same. Though their separation in taxonomy depends solely on their morphological differences, the result of UPGMA analysis of RAPD bands demonstrates little variance of polymorphism between the species and its variety. One reasonable explanation is that the differentiation of sex happened before the appearance of variants. It is also a reasonable speculation that the expression of a certain genes or several genes may have been changed a little bit, which leads to big morphological divergence between variants, whereas the genetic basis may not have big differences. Therefore, more attention should be paid in genetic population study between closely related variants.

Concerning the genetic distance between male and female trees was greater than that of the same sex between different variants, we here propose an audacious supposition: in order to discriminate the gender of a posterity of a plant, we can use some species of close relative in taxonomy to make it, if its male or female parent is not at hand. But this point has not been proved.

We have also compared the results of one-primer amplification and two-primer amplification. As shown in Fig.3, in most cases the products of two-primer amplification were less than that generated by one-primer amplification. An interesting phenomenon was, the two-primer-derived bands were less polymorphic but more intense. Another feature was that there were few common bands shared by one-primer and two-primer amplifications when the same primers were used.

Our study has preliminary demonstrated that the RAPD analysis can be successfully applied to discriminate the sex of *Podocarpus macrophyllus* Trees. However, to verify our results and to confirm its potential application, examination of more *Podocarpus macrophyllus* trees should be carried out.

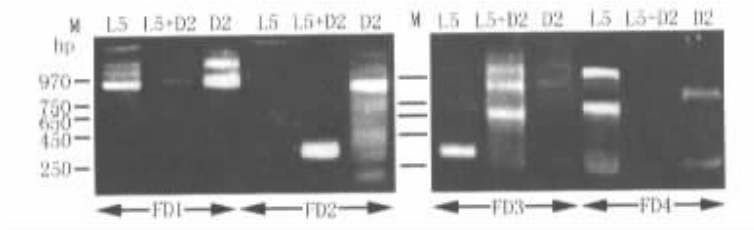

Fig.3　PCR amplifications with one primer and two primers

L5：the products were amplified with the primer L05；D2：the products were amplified with the primer D02；L5 + D2：the products were amplified with the twinned primers D02 and L05. FD1 to FD4 are the numbering of trees. The DNA molecular size markers used were 970, 750, 650, 450, 250 bp

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RAPD 技术在罗汉松性别辨别中的应用

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摘 要：部分植物(包括不少濒危物种)是雌雄异株，且在非花期仅凭形态学特征难以辨别其性别 这给品种改良、植物保护工作都带来了麻烦. 以罗汉松(*Podocarpus macrophyllus* (*Thunb.*) *D. Don*)、短叶罗汉松(*Podocarpus macrophyllus Var. maki Endl*)为具体实验材料，就 RAPD 技术在植物性别判断方面进行了尝试. 在筛选了 340 种随机引物后，发现其中 23 种引物的产物有良好的多态性 最终筛选到了可以扩增出与性别相关的特异性条带的引物 P20. 在这基础上对表现出多态性的 RAPD 产物进行了 UPGMA 聚类分析，观察到各样本在遗传多态性上，性别间的差异大于变种与原种间的差异. 同时还对单引物、双引物的 RAPD 结果进行了对比.

关键词：罗汉松；RAPD；性别判断；UPGMA

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复旦大学生物多样性科学研究所(IBSFU)

复旦大学生物多样性科学研究所是我国成立的第一个专门从事生物多样性科学研究和高级专门人才培养的机构. 生物多样性资源是人类赖以生存和发展的最重要物质基础之一，系及国家安全. 由于人口急剧膨胀，经济高速发展以及人们需求的增加，生物多样性面临巨大压力，物种灭绝速度比自然灭绝速度快 100～1 000 倍. 因此，这已引起世界各国政府的重视和公众的极大关注.

该所于 1996 年成立以后，确定以下几个研究方向：1. 生物多样性科学的理论与方法；2. 外来物种入侵生态学；3. 基因多样性与生物安全；4. 生物多样性地理信息系统及生物信息学；5. 城市生态与生态经济.

学术带头人陈家宽教授(博士生导师)、骆亦其教授(长江奖励计划讲座教授，博士生导师)、卢宝荣教授(复旦大学特聘教授，国家杰出青年基金获得者，博士生导师)、吴千红教授(博士生导师)，钟扬教授(博士生导师)、李博教授(博士生导师). 承担有国家自然科学基金“九五”重大项目《中国关键地区生物多样性保育的研究》和上海市科委《上海市及其邻近地区生物多样性维持与丧失机理的研究》等多项重要项目，与国际水稻研究所(IRRI)、WWF、加拿大女王大学、日本广岛大学、美国孟山都公司等均有广泛的合作，近五年在国内外重要刊物上发表论文近 150 篇，并与内蒙古创业集团合作组建内蒙古复旦蒙耀生物技术有限责任公司，着重开发西部极端环境下的生物多样性资源.

研究所所长：陈家宽教授 副所长：卢宝荣教授、钟扬教授、李博教授.

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