DOI: 10.1515/frp-2016-0008
Available online: www.lesne-prace-badawcze.pl

Leśne Prace Badawcze / *Forest Research Papers*
March 2016, Vol. 77 (1): 68–75

ORIGINAL RESEARCH ARTICLE

e-ISSN 2082-8926

Preliminary studies on the molecular identification of sex in Taxus baccata L.

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Abstract. The scientific objective of this research was to screen random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) primers in order to find the molecular markers enabling the distinction between male and female individuals of the European yew. This is an initial step toward understanding the mechanisms of sex determination in this species.

The study was conducted on European yew originating from two sites in Poland: the Zadni Gaj nature reserve near Cieszyn and the yew collection from the Botanical Garden of the Jagiellonian University in Krakow.

In the present study, 716 random primers (696 RAPD primers: OPA-OPAI_1-16 and 20 ISSR primers marked as UBC) were tested to identify the sex in European yew by means of a modified bulked segregant analysis (BSA) method. The work was conducted in three stages, gradually limiting the number of primers through the elimination of primers that either did not exhibit any differences between the examined groups or did not provide amplification products.

Among the tested primers, no ideal markers that would be present in all individuals of one sex but absent in the individuals of the other sex were found. However, some markers were found (A07_954, H13_729, J08_660, L12_390, U01_457, V14_527, AE03_941, AE03_1014) to occur with greater frequency in one sex. Using these, we further examined 13 band combinations (profiles) that were observed to occur only in male individuals and another 13 combinations that were observed only in female individuals, which could be used in the practical identification of sex.

This is the first report to ascertain the sex of *Taxus baccata* trees, and it may help to determine the sex at an early stage of development.

Keywords: molecular markers, RAPD, ISSR, BSA, yew.

1. Introduction

The European yew (*Taxus baccata* L.) is a shrub or small tree belonging to the gymnosperm plants, usually growing up to 20–28 m. It is a dioecious, slow-growing species, widely spread in Europe, but highly fragmented. When it grows in good light conditions, it reaches its maturity at the age of about 30–35 years; however, under heavy shadows, it does not mature until the age of 70 or even 120 years. It is a shadow-resistant species, but it can also grow in full insolation. Over recent centuries, a decrease in European yew population size has been observed across the whole of Europe, which is related amongst others to human forest management (Thomas, Polwart 2003; Hilfiker et al. 2004).

The *Taxus* genus contains both dioecious and monoecious species. Monoecy is common in *Taxus canadensis* (Allison 1991), whilst dioecy dominates in *Taxus brevifolia*; however, about 10–15% of monoecious trees are also observed in the latter (DiFazio et al. 1996; Hogg et al. 1996). *T. baccata* is a typical dioecious species in which monoecy is an extremely rare phenomenon (Iszkuło, Jasińska 2004).

In some plants, labile in sex expression (an individuals or their parts can change sex during ontogeny) could be observed, what may be a result of problems with sex control in a complex environment or it may be an adaptive ability. Sex determination in dioecious plants may be of a genetic or an environmental character. In the case of genetic determination, it may be controlled by a single locus on an autosome, many loci on autosomes or genes on heteromorphic chromosomes (Irish, Nelson 1989). The application of molecular techniques over recent decades has enabled the recognition and description of numerous floral meristem identity genes that contribute to the plant development processes (Weigel, Meyerowltzt 1994; Ainsworth 2000; Theissen 2001; Ming et al. 2011; Gschwend et al. 2012). However, it is still not fully clear what the direct effect of these genes is on sex determination in many plant species.

Received: 12.10.2015 r., reviewed: 25.11.2015 r., accepted: 22.12.2015.

One of the dioecious species for which the mechanism of sex determination is still unknown is the European yew. There is also no method enabling male and female individuals to be distinguished at an early stage of development. The possibility of distinguishing individuals appears only at the moment of sexual maturity, when they start to create generative reproduction organs, a process that occurs quite late in the case of yew. For several reasons, the possibility of yew sex recognition at a juvenile age is a highly significant issue. Yew is used as a medicinal plant of high significance in cancer treatment, and some studies (Iszkuło et al. 2013) have indicated a higher concentration of active substances in female individuals. Also, in the case of decorative greenery, female individuals are more appreciated because of their decorative arils. In natural populations, the rate of mature male to female individuals is about 1:1; however, these results relate to mature tree stands, because currently studies may only be conducted after the appearance of generative organs on the trees. Owing to the impossibility of distinguishing male individuals from female ones in younger age classes, no studies have been conducted so far into the ratio of male to female individuals in populations before they reach maturity. Knowledge of the sex of young individuals is also significant for species protection. The yew restitution programme assumes the reintroduction of the species into the forests, but it is important to introduce a suitable number of male and female individuals so that the populations have the chance to function properly. Sex proportion imbalance decreases the effective population size and, in a long time scale, increases the number of homozygous individuals and all negative effects of inbreeding. In some extreme cases, it may even lead to the extinction of the whole introduced population.

Bulked segregant analysis (BSA) is a rapid procedure for identifying markers in specific regions of the genome and is first described by Michelmore et al. (1991). The method involves comparing two pooled DNA samples of individuals from a segregating population that originates from a single cross. Within each pool, or bulk, the individuals are identical for the trait or gene of interest but are arbitrary for all other genes. Two pools with a different trait of interest are analysed to identify markers that distinguish them. Markers that are polymorphic between the pools are genetically linked to the locus determining the trait used to construct the pools (Peters et al. 2003). This technique has been successfully used to identify random amplified polymorphic DNA (RAPD) markers linked to disease-resistance genes and sex-linked genes in several plants such as *Pistacia vera* L. (Hormaza et al. 1994), *Asparagus officinalis* L.) (Jiang, Sink 1997), *Salix viminalis* L. (Alstrom-Rapaport et al. 1998; Gunter 2003), *Actinidia chinensis* Planch. (Harvey et al. 1997; Gill et al. 1998), *Silene latifolia* Poir. (Zhang et al. 1998), *Cannabis sativa* L. (Mandolino et al. 1999), *Piper longum* L. (Banerjee et al. 1999), *Myristica fragrans* Houtt. (Shibu et al. 2000), *Eucommia ulmoides* Oliv. (Xu et al. 2004), *Encephalartos natalensis* R.A.Dyer & I.Verd. (Prakash, Staden 2006), *Carica papaya* L. (Urasaki et al. 2002; Chaves-Bedoya, Nuñez 2006) and *Simmondsia chinensis* (Link) Schneid. (Agrawal et al. 2007). Despite some disadvantages, RAPD markers are still very often used in such kinds of research because of the relatively low cost, the possibility of testing a high number of primers and searching both DNA coding and non-coding regions. Other effective type of molecular markers is intersimple sequence repeat (ISSR) that has been successfully used, for example, in *Humulus lupulus* L. (Danilova, Karlov 2006), *Humulus japonicus* Siebold & Zucc. (Aleksandrov et al. 2011), *Simmondsia chinensis* (Link) Schneider (Sharma et al. 2008), *Phoenix dactylifera* L. (Younis et al. 2008), *Carica papaya* L. (Gangopadhyay et al. 2007) and *Calamus tenuis* Roxb. (Sarmah, Sarma 2011) for the identification of sex polymorphism.

The aim of this study was to find molecular marker that could distinguish male and female in early stage of development and test whether RAPD or ISSR markers provide information about sex of an individual. To date, there is no information about sex determination mechanisms in *T. baccata*. This is the first step in the understanding of this process.

2. Material and methods

2.2. Plant material

Plant material was collected for the study within two sites of European yew: the Zadni Gaj reserve near Ustroń and the collection of the UJ Botanical Garden in Krakow. In Zadni Gaj, needles were collected from 12 male and 12 female fully developed, mature trees after sexual phenotypic identification, whilst in the Botanical Garden material was collected and determined from 8 male and 8 female individuals. The individual samples were stored at −80°C before use.

2.2. DNA isolation

Total genomic DNA was isolated using the modified cetyltrimethylammonium bromide (CTAB) method from about 200 mg of frozen needle tissue from male and female individuals separately (Khanuja et al. 1999). DNA concentrations were detected with a Synergy™ 2 Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) and diluted in double-distilled sterile water to a final concentration of 80 ng/µl. For further analysis, DNA samples were stored at a temperature of −20°C.

2.3. Bulked segregant analysis (BSA)

The BSA was designed in two variants: a main (Figure 1) and an alternative pathway (Figure 2). Variant choice depended on on-going results obtained from particular stages. Analysis was conducted in three stages. Stage I (common for both variants) involved preparation of three mixed samples from DNA of 12 male individuals and three mixed samples from DNA of 12 female individuals (four different individuals per sample), all derived from the Zadni Gaj reserve. Each individual was represented by the same amount of DNA. At this stage, 716 of the 10-nucleotide primers selected from the Operon set (http://www.operon.com/products/downloads/OperonsRAPD10merSequences.xls) (Operon Technologies, Alameda, CA, USA) (set OP: A-Z, AA- AI 1-16) were tested on six mixed DNA samples (three male pools and three female

pools), as were 20 ISSR primers marked as UBC (University of British Columbia kit) of the following sequences (Table 1).

Primers that generated bands occurring in all mixed samples of one sex and were absent in opposite sex samples (main pathway) or demonstrated differences in the frequency of occurrence between the sexes (alternative pathway) were transferred to stage II.

At stage II, the selected primers were verified not in the mixed samples but individually on 12 male and 12 female individuals sourced from the Zadni Gaj reserve. Primers with bands characteristic entirely for male or female groups (main pathway) or differing in terms of the frequency of occurrence between the examined groups (alternative pathway) were transferred to stage III.

Stage III involved verification of selected primers on 15 male and 15 female individuals of *T. baccata* belonging to two populations (Zadni Gaj and UJ Botanical Garden). The expected final result of the main pathway was obtaining an 'ideal' marker or markers enabling the classification of a given individual to a specified sex in a quick and unequivocal manner, irrespective of its origin. In the case of the alternative pathway, the expected final effect was obtaining profiles of markers differing in terms of the frequency of occurrence in male and female individuals, unequivocally identifying examined individuals within a given sex, using the lowest number of primers possible.

2.4. DNA amplification

RAPD and ISSR analysis were used to screen a total of 716 primers of arbitrary sequences obtained from Sigma-Aldrich (Sigma-Aldrich Co. LLC., St. Louis, MO, USA). The amplification was conducted in sterile 0.2-ml Eppendorf test tubes in 10 µl of a reaction mixture containing PCR 10X DreamTaq Green Buffer, 2 mM $MgCl_2$, 200 µM each of deoxynucleotides (dNTPs), 0.5 µM of primer, 0.25 U of DreamTaq™ DNA polymerase (Fermentas, Burlington, Canada), 80 ng of genomic DNA and two tracking dyes.

Amplification was conducted in a Biometra T3 thermocycler (Göttingen, Germany) following the thermal profile: one cycle consisting of 6 min at 95°C, 45 s at 35°C and 2.5 min at 72°C followed by 36 cycles of 1 min at 95°C, 45 s at 35°C and 2.5 min at 72°C. The final elongation was conducted for 8 min at 72°C.

The amplification products were separated electrophoretically in a 1.5% agarose gel (Prona, Madrid, Spain) with a 100-bp internal size standard (GeneRuler DNA Ladder Plus, Fermentas) and stained with Midori Green DNA Stain (Nippon Genetics Europe GmbH, Duren, Germany). The results were analysed using Bio-1D++ computer software (Vilber Lourmat, Torcy, France).

3. Results

Seven hundred and sixteen primers were tested as a part of the study. Each of them allowed 0–15 bands of lengths ranging from 200 to 3000 bp to be obtained. It was observed just at stage I that none of the examined primers gave bands that would be both present in all three examined bulk mixtures from a given group and completely absent in the bulks from the opposite sex (Figure 1). Owing to the results obtained at this stage, the decision was taken to continue the study using variant II, that is, alternative pathway. The results obtained at stage I enabled the division of the primers into four groups: primers providing some differences between male and female groups (polymorphic), primers providing no differences between the examined groups (monomorphic), primers providing no amplification products (empty), and those providing a smeared image, preventing the distinction of particular bands and a readout of their length (Figure 2). The most abundant group (74.44%) was represented by monomorphic primers, 8.38% of the primers gave no amplification products and 1.26% provided a smeared image. The above primers were rejected. Hundred and fourteen primers (15.92%) that appeared to be polymorphic between the examined groups were qualified for stage II. The number of individuals from a given group in which a previously observed marker occurred was verified in stage II on one population example (Zadni Gaj). At that stage, two markers seemed to be the most promising: primer

Table 1. Sequences of the ISSR primers from UBC group used in the research

Primer	Sequence	Primer	**Sequence**
UBC881	GGGTGGGGTGGGGTG	UBC891	HVHTGTGTGTGTGTG
UBC882	VBVATATATATATATAT	UBC892	TAGATCTGATATCTGAATTCCC
UBC883	BVBTATATATATATATA	UBC895	AGAGTTGGTAGCTCTTGATC
UBC884	HBHAGAGAGAGAGAGAG	UBC899	CATGGTGTTGGTCATTGTTCCA
UBC885	BHBGAGAGAGAGAGAGA	UBC900	ACTTCCCCACAGGTTAACACA
UBC886	VDVCTCTCTCTCTCTCT	UBC880	GGAGAGGAGAGGAGA
UBC887	DVDTCTCTCTCTCTCTC	UBC879	CTTCACTTCACTTCA
UBC888	BDBCACACACACACACA	UBC878	GGATGGATGGATGGAT
UBC889	DBDACACACACACACAC	UBC877	TGCATGCATGCATGCA
UBC890	VHVGTGTGTGTGTGTGT	UBC876	GATAGATAGACAGACA

Table 2. The sequences of markers obtained in stage III of experiment and their frequency of occurrence in male and female individuals of *Taxus baccata*

Marker	Primer sequence	Frequency of occurrence in male [%]	Frequency of occurrence in female [%]
A07_954*	GAAACGGGTG	46.6	80
A07_1411	GAAACGGGTG	26.6	46.6
B01_730	GTTTCGCTCC	46.6	40
B03_590	CATCCCCCTG	26.6	6.6
C07_540	GTCCCGACGA	66.6	66.6
H13_729*	GACGCCACAC	26.6	40
I07_642	CAGCGACAAG	13.3	26.6
J08_660*	CATACCGTGG	13.3	53.3
K14_780	CCCGCTACAC	53.3	53.3
L12_390*	GGGCGGTACT	73.3	33.3
L12_790	GGGCGGTACT	66.6	53.3
U01_457*	ACGGACGTCA	53.3	13.3
V14_527*	AGATCCCGCC	66.6	93.3
AC02_560	GTCGTCGTCT	46.6	46.6
AE03_941*	CATAGAGCGG	33.3	66.6
AE03_1014*	CATAGAGCGG	20	46.6
AE04_493	CCAGCACTTC	33.3	26.6
AE15_467	TGCCTGGACC	13.3	13.3
AF08_877	CTCTGCCTGA	20	20

* Markers selected for construction of sex-specific band profiles

Table 3. Combinations of 8 markers (profiles of bands) observed entirely in male or female individuals

Marker	male													female										
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
A07_954		*						*	*		*		*		*	*	*	*	*	*		*	*		*	*
H13_729	*									*	*	*			*			*			*	*			*	*
J08_660								*		*						*	*					*	*	*	*	
L12_390			*		*	*	*	*	*	*	*	*	*						*		*			*	*	*
U01_457					*	*		*	*	*		*	*	*										*		
V14_527			*	*		*	*		*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*
AE03_941		*			*		*				*						*	*	*	*	*	*	*			*
AE03_1014				*								*	*							*			*	*	*	*

The probability of finding sex-linked markers in dioecious plants depends on the genome size. The larger the genome is, the more random primers should be screened to find a sex-specific marker (Jiang et al. 2003). The haploid genome size of *Carica papaya* is 0.74×10^9 bp (Bennett, Smith 1991) and only 40 primers were used to obtain two male-specific markers (Banerjee et al. 1999). In *Actinidia chinensis,* the genome size is 4.36×10^9 bp (Bennett et al. 2000) and 500 RAPD primes were needed to isolate two sex-specified markers (Gill et al. 1998). Finally, for *Ginkgo biloba,* which has a large genome of about 9.9×10^9 bp (Murray 1998), 1200 primers were used to find one sex-linked male marker (Jiang et al. 2003).

T. baccata is a coniferous tree and has a relatively large genome size as compared to most other plant species, including many angiosperm tree species. The size of *Taxus* genome is a little larger than that of *Ginkgo* and has 10.9×10^9 bp (Ahuja, Neale 2005). The size of the yew genome seems to be another factor impeding the search for a sex marker/markers. Thus, further testing of subsequent series of primers seems to be necessary.

Another problem in the studies on *T. baccata* is that some individuals had both male and female 'flowers'. Co-sexual individuals could be found with many male structures and with only single female ones or sometimes with equal numbers of male and female structures dispersed in the crown of the whole tree (Iszkuło, Jasińska 2004). This may be caused both by some instability of the sex determination process and also by individuals of various sexes fused in one trunk with an age as a result of growing close together. Individuals with only one branch of a different sex have been observed in the nature, and also individuals on which one annual increment on the same branch demonstrated the features of a different sex (Zarek, unpublished). Therefore, it is extremely important in such kinds of analysis to qualify the examined individual unequivocally to a given sex, which may multiple verifications during field observations. Sex instability observed in *Taxus* could be an argument for environmental basis for sex determination in this species. Some of dioecious plants have exclusively genotypic sex determination (GSD); but in other cases, environmental sex determination (ESD) is also observed. In ESD, sex development is controlled by environmental factors such as temperature, pH and hormones. Often a mixture of GSD and ESD leads to a mismatch between genotypic and phenotypic sex. According to Stehlik et al (2008), one of the most direct ways for changing sex ratios in progeny is sex determination induced by environment. Plant species that are generally very plastic in gender expression show environmentally influenced sex-allocation plasticity or sex inconstancy during flowering. But it is still unclear how environmental influences interact with genetic sex determination mechanisms.

According to Gschwend et al. (2012), species with autosomal determination mechanisms may in some circumstances create the mutations causing a return to the androgynous stadium, whilst species that form chromosomes of both sexes stay dioecious as long as these chromosomes exist. In turn, according to Ming et al. (2011), all dioecious species may potentially create sex chromosomes and also transform again from dioecy to various forms of monoecy (such as monoecy, gynodioecy or androdioecy), especially at early stages of the evolution of sex chromosomes.

It may be supposed, based on our study and literature data, that sex determination in the European yew is of a multi-gene character and may be related to the occurrence of homomorphic sex chromosomes. Finding a practical marker identifying the sex, especially before the period of individual maturity, is for now still an unresolved issue. The marker profiles proposed by us may be useful at this stage of research; however, they require verification of their frequency of occurrence in a larger number of populations.

To the best of our knowledge, this was the first attempt to find sex-specific markers for the identification of sex in *Taxus*. Further studies should be continued to increase the number of RAPD and ISSR primers screened in an effort to find a marker flanking the putative sex-determination locus as well as try to use more sophisticated methods such as next-generation sequencing (NGS) that provide very high genome coverage. The next step should be to generate an SCAR marker (sequence characterised amplified regions) because polymorphic RAPD and ISSR markers transformed to SCAR markers can be more advantageous in commercial breeding programmes if a quick assay can be developed to detect the presence or absence of the product. In addition, sequence-specific markers could potentially be used to screen more *T. baccata* populations and other *Taxus* species.

Conflict of interest

None declared.

Acknowledgements and financial support

This Research was financed by the Ministry of Science and Higher Education of the Republic of Poland. The author gratefully wish to thank the Director of the UJ Botanical Garden in Krakow for providing yew collection.

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