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Homogenous genetic structure in populations of *Taxus baccata* with varied proportions of male and female individuals

Highlights

- Polish populations of *Taxus baccata* showed a high level of genetic diversity within populations and moderate genetic differentiation between them after nSSR marker testing.
- No significant differences in the genetic variation between *T. baccata* male and female individuals were observed, and microsatellite loci neutrality was verified.
- Determining the sex ratio in *T. baccata* populations is not essential to develop a clear understanding of genetic differentiation and diversity within and between populations of this species.

Abstract

English yew (*Taxus baccata* L.) is a strictly outcrossing and dioecious species whose populations are small and isolated. It is known that sex ratios may vary in natural populations due to local environmental conditions or stochastic events. However, unbalanced sex ratios may have negative impacts on genetic diversity through enhanced genetic drift and inbreeding. The present study represents one of the first attempts to compare the genetic variation at microsatellite loci within and between populations with different gender proportions. Our results indicated that there were no significant correlations between sex ratio and the extent of genetic variation in different populations. All populations exhibited high levels of genetic diversity. Additionally, the genetic structure was characterized separately in male and female individuals. Statistical analyses of the set estimators describing the genetic structure of male and female individuals of *T. baccata* revealed no significant differences between the two groups. Molecular analysis verified that microsatellite nuclear loci neutrality developed for *T. baccata,* as there were no significant differences in the genetic variation between males and females and no evidence for any outlier loci using coalescent and hierarchical Bayesian simulations. The results demonstrate that ignoring biased sex ratios in *T. baccata* populations had no effect on the assessment of genetic differentiation and genetic diversity within and between populations of this species. These results are discussed with regards to the practical application of molecular markers in conservation programs.

1 Introduction

Dioecy is a relatively rare mating system in plants characterized by individual plants being distinctly male or female. Dioecious species comprise approximately 10% of all known species of land plants, 4–6% of which are angiosperm species (Givnish 1980; Renner and Ricklefs 1995; Soza et al. 2012; Barrett and Hough 2013). Phylogenetic studies of angiosperm dioecious plants indicate a low speciation level and higher extinction risk compared to cosexual species (Heilbuth 2000; Vamosi and Vamosi 2005). The increased extinction risk is often linked to an insufficient number of individuals of the opposite sex, particularly in small and isolated populations (Pannell and Barrett 1998). However, dioecy can be beneficial from an evolutionary point of view due to the genetic advantages associated with guaranteed outbreeding (Freeman et al. 1997).

The sex ratio parameter is frequently applied to describe the population structure of dioecious species. The theoretical sex ratio should be 1:1 (Fisher 1930) as the result of matings between individuals of the opposite sex. Among dioecious plants, there are frequent significant equality deviations in natural populations (see review Sinclair et al. 2012), which could negatively affect their genetic diversity. Environmental factors (Mercer and Eppley 2010) and stochastic events, particularly in small isolated populations (Engen et al. 2003), lead to variations in gender proportions among populations. In populations with unbalanced sex ratios, the effective population size is low, possibly leading to decreased allelic diversity owing to enhanced genetic drift (Hedrick 2000). Moreover, in such dioecious species populations, diminished reproductive efficiency due to an insufficient number of individuals of the opposite sex also affects genetic diversity (Eppley 2005; Vandepitte et al. 2009).

English yew (*Taxus baccata* L.) is a canonical example of a strictly outcrossing and dioecious species, though the opposite sexes have the same or nearly the same characteristics (Svenning and Magård 1999; Thomas and Polwart 2003; Iszkuło et al. 2009). Female *T. baccata* individuals grow slower than males, which are typically taller and have a larger trunk diameter than females (Iszkuło et al. 2009; Cedro and Iszkuło 2011). Iszkuło et al. (2009) reported that the percentage of females increases with higher precipitation, and sex ratios only reach a balance in populations located in areas with high annual rainfall. Collectively, these factors contribute to changes in sex structure (i.e., decreased number of females) observed in ageing populations (Iszkuło et al. 2009). Several studies have calculated sex ratios in natural *T. baccata* populations and found that they vary from 40% female (see review Thomas and Polwart 2003) to 71% female (Hilfiker et al. 2004a). Most studies on the genetic structure of small and fragmented *T. baccata* populations in Europe utilized different system markers and demonstrated a high level of genetic variation and significant genetic differentiation between populations, the latter resulting from genetic drift and inbreeding (Lewandowski et al. 1995; Cao et al. 2004; Hilfiker et al. 2004b; Dubreuil et al. 2010; Myking et al. 2009; Tröber and Ballian 2011; Chybicki et al. 2011; 2012; Litkowiec et al. 2013). However, those studies were conducted without consideration of the gender proportions in analysed populations.

Due to increased inbreeding and genetic drift, both of which have negative genetic impacts on populations, it is important to investigate and compare genetic variation within and between a large number of populations with unbalanced sex ratios. For this investigation of *T. baccata* populations, five highly polymorphic nuclear microsatellite (nSSR) markers were selected. Specifically, our aims were to (1) describe the values of parameters used to characterize genetic structure in male and female individuals, (2) determine whether microsatellite markers have a neutral character, and (3) determine the correlation between sex ratios and genetic variation in populations.

2 Material and methods

2.1 Plant material

Needles were collected from 26 natural *T. baccata* populations in Poland (Table 1). These populations covered the entire area of Poland and were part of a conservation and restoration program launched in 2006 (Fig. 1). The sex ratios (% female) in the studied populations were based on data collected by Kostrzyca Forest Gene Bank. The presence of micro- and macro-strobili and seeds or seed envelope remains were used to determine the sex of each individual. Groups of male and female individuals were defined in each population based on this information. In total, 2198 individuals, including 1040 males and 1158 females, were subjected to microsatellite analysis.

2.2 DNA extraction and microsatellite genotyping

Genomic DNA was extracted from needles using a modified CTAB protocol described by Dumolin et al. (1995). The identification of suitable markers was based on their ability to provide repeatable, high quality results, sufficient polymorphism, and unambiguous allele binding of eight nuclear microsatellite markers originally identified in *T. baccata* by Dubreuil et al. (2008). The final set of loci included five nSSRs (tax23, tax26, tax31, tax36, tax92).

PCR reactions were performed in one multiplex reaction and two single reactions. Three nSSR loci (tax26, tax36 and tax92) were simultaneously amplified in a multiplex reaction using a Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany). PCR multiplex reactions were performed in a total volume of 10 µl composed of 5 µl Qiagen Multiplex Master Mix (2X), 0.2 µl primer mix (20 µM), 1 µl Q-Solution (5X), 0.8 µl RNase-Free water, and 3 µl DNA template (approximately 10–20 ng). PCR reactions for amplifying loci tax23 and tax31 were performed in a total volume of 10 µl, containing 10–20 ng DNA template, 1X PCR, pH 8.3 (Novazym, Poland), 25 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM of each primer, 10 ng/µl BSA, and 1.25 U VivaTaq polymerase (Novazym, Poznan, Poland). PCR conditions for both the single and multiplex reactions were as follows: an initial denaturation step at 95 °C for 15 min, followed by 10 touchdown cycles at 94 °C for 30 s, 30 s at 60 °C (–1 °C/ cycle), 40 s at 72 °C, 30 cycles at 94 °C for 30 s, 50 s at 50 °C, 40 s at 72 °C, and a final extension at 72 °C for 7 min. The fluorescently labelled PCR products and a size standard (GeneScan 500 LIZ) were separated on a capillary sequencer ABI 3130 (Life Technologies, Carlsbad, CA, USA). The alleles were identified based on their size using GeneMapper software (ver. 4.0; Life Technologies).

2.3 Data analysis

Genotypic linkage disequilibrium for all microsatellite loci was verified in each population and across all populations based on a permutation test using Arlequin software (Excoffier et al. 2005). The genetic diversity within and among populations was estimated based on the following parameters: total number of alleles (A); allelic richness (AR_{25}) corrected for a minimum sample size of 25 individuals; observed heterozygosity (H_o); and unbiased expected heterozygosity (H_e), all of which were computed using FSTAT v 2.9.3 (Goudet 2001). Null alleles are known to be present in the nSSR markers used in this study (Dubreuil et al. 2008). Therefore, the frequencies of null alleles (N0) in each population were estimated based on the Individual Inbreeding Model (IIM) with a Gibbs sampler 10^5 iterations using INEST 1.0 software (Chybicki and Burczyk 2009). All parameters describing genetic variation in a population were separately estimated for male and female individuals.

Table 1. The location, code, and percentage of females in the studied populations and the number of male and female *Taxus baccata* individuals analysed.

Population	Pop. Number	Longitude/Latitude	% females [a]	Analyzed males	Analyzed females
Leszno	1	53°46′N/20°52′E	45	50	50
Choczewskie Cisy	2	54°46′N/17°45′E	47	36	46
Cisy nad Czerską Strugą	3	53°45′N/17°57′E	46	40	47
Jelenia Góra	4	53°35′N/18°06′E	50	38	36
Wierzchlas	5	53°30′N/18°06′E	33	50	50
Cisy w Czarnem	6	53°44′N/16°58′E	49	48	48
Cisy Rokickie	7	53°45′N/14°51′E	64	49	50
Nadleśnictwo Rokita	8	53°42′N/14°51′E	63	25	46
Boleszkowice	9	52°42′N/14°33′E	55	37	55
Bogdaniec	10	52°41′N/15°04′E	32	32	32
Książ	11	50°50′N/16°16′E	37	43	37
Cisowa Góra	12	50°32′N/16°42′E	53	50	50
Cisy koło Barda	13	50°32′N/16°39′E	51	50	50
Nowy Waliszów	14	50°18′N/16°45′E	51	50	50
Cisy nad Liswartą	15	50°44′N/18°46′E	54	34	55
Cisy w Hucie Starej	16	50°33′N/19°12′E	48	26	27
Jasień	17	50°59′N/19°33′E	55	41	52
Radomice	18	50°44′N/20°39′E	68	29	65
Mogilno	19	49°39′N/20°49′E	58	38	53
Wadernik	20	49°29′N/21°38′E	51	26	30
Igiełki	21	49°30′N/21°38′E	54	30	39
Kretówki	22	49°44′N/21°53′E	40	58	39
Malinówka	23	49°41′N/21°55′E	44	50	50
Pasmo Łysej Góry	24	49°33′N/21°34′E	55	42	41
Serednica	25	49°28′N/22°31′E	46	31	26
Cisy na Górze Jawor	26	49°17′N/22°17′E	55	38	34

[a] Based on data collected by Kostrzyca Forest Gene Bank
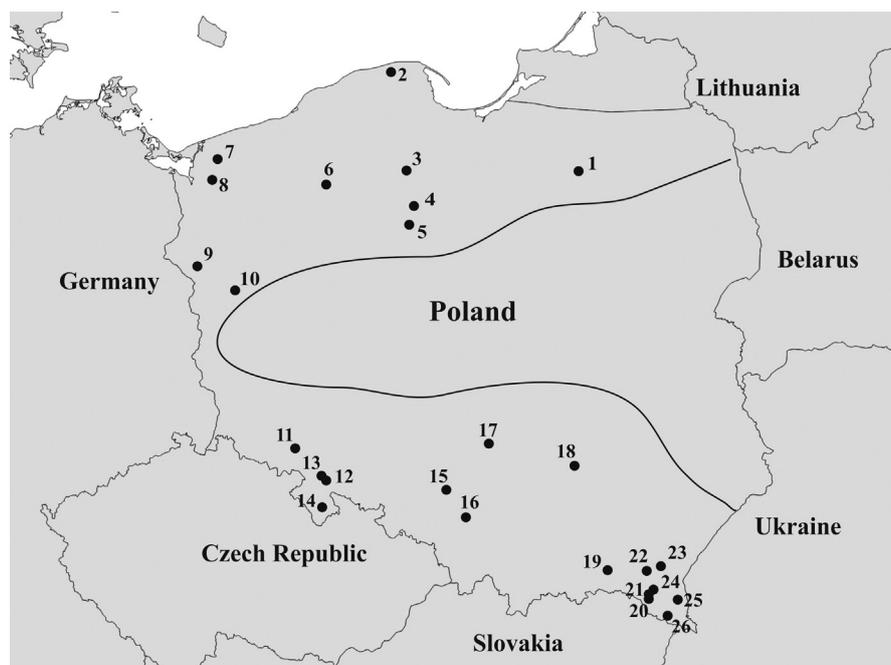
Fig. 1. Map of the geographical locations of the 26 *Taxus baccata* populations included in this study. Codes representing the named populations are listed in Table 1, and the borders of the natural *T. baccata* distribution in Poland are indicated with a black line.

The global genetic differentiation (F_{st}) among overall samples was assessed as F_{st} values (Weir and Cockerham 1984) with FSTAT v. 2.9.3 (Goudet 2001). Taking into account the presence of null alleles at all loci, FreeNA software was used to estimate F_{st} using the ENA (Excluding Null Alleles, F_{stENA}) correction method (Chapuis and Estoup 2007). The bootstrap 95% confidence intervals (CI) for global F_{st} values were calculated using 20 000 replicates over loci.

FSTAT was used to compare grouped male and female samples based on various genetic variation parameters. Statistically significant differences between the overall means of the standard parameters describing genetic structure were determined using a permutation test (10 000 permutations) within FSTAT. Several parameters, including AR, A, F_{st}, mean of H_e and H_o, were compared. Due to software limitations, not all genetic diversity parameters were statistically analysed in FSTAT. Analysis of variance (ANOVA) was used to determine significant differences (p-value ≤ 0.05) between the means of male and female frequencies of null alleles.

The genetic relationship among populations was further analysed by principle coordinate analysis (PCoA) using GenAlEx v. 6 (Peakall and Smouse 2006) based on F_{stENA} values generated by FreeNA with an ENA correction.

Outlier loci, i.e., markers potentially under selection, were detected using two different approaches to minimize false positive detection. First, the Beaumont and Nichols method (1996) in the Lositan Selection Workbench (Antao et al. 2008) was used to determine whether microsatellite loci were outliers. The method uses a coalescent simulation approach based on an island model for migration to identify outlier loci displaying unusually high (i.e., putatively under directional selection) and low (i.e., potentially under stabilizing selection) values of F_{st} by comparing observed F_{st} values with values expected under neutrality. Loci with a high F_{st} value are putatively under directional selection, while loci with a low F_{st} value are considered to potentially be under stabilizing selection. The evaluation was performed using 50 000 iterations, stepwise mutation models, and all loci. The mean neutral F_{st} was used as a preliminary value. Loci lying outside a 99.5% confidence interval were considered to be potentially under selection rather than neutral. Second, the hierarchical Bayesian simulation (Beaumont and Balding 2004) in Bayescan 2.1 software (Foll and Gaggiotti 2008) was used to detect non-neutral loci. This method is based on the decomposition of locus-population F_{st} coefficients into a population-specific component (beta), shared by all loci and a locus-specific component (alpha) shared by all populations using a logistic regression model. Analyses were performed using a default set of parameters. To avoid false positives, the higher prior odds (1000) were used. The scale based on Bayes factor described by Jeffreys (1961) was used to consider a locus under selection.

Regression analyses were used to infer relationships between sex ratios and genetic diversity (A, AR_{25}, H_e, H_o). Analyses were conducted using JMP® statistical software (SAS Institute Inc., Cary, NC, USA, 1989–2007).

3   Results

All nSSR loci were polymorphic, and there was no evidence detected to indicate linkage disequilibrium. The examined populations and grouped individuals of male and female *T. baccata* samples exhibited a high level of genetic diversity, and the level was similar within populations and for each sex. The genetic diversity parameters for whole populations and the separate male and female samples are displayed in Table 2. The mean number of alleles per population (A) ranged from 2.2 in the Bogdaniec (10) to 14.6 in the Cisy koło Barda (13), with an overall average of 9.4 among all populations. The mean number of alleles ranged from 2.2 in male samples from the Bogdaniec (10) to 14.0 in male samples from the Cisowa Góra (12) and Cisy koło Barda (13), with an overall average of 9.4 among all males. A similar trend was observed in the female samples, with the lowest

Table 2. Parameters used to estimate the genetic structure of the studied *Taxus baccata* populations averaged across five nSSR loci. A – average number of alleles, AR$_{25}$ –allelic richness, H$_e$ – expected heterozygosity, H$_o$ – observed heterozygosity, N0 – null allele frequency.

Pop. number	A			H$_e$			H$_o$			AR$_{25}$			N0		
	Male	Female	Pop	Male	Female	Pop	Male	Female	Pop	Male	Female	Pop	Male	Female	Pop
1	8.2	9.8	9.0	0.733	0.746	0.740	0.505	0.579	0.542	7.5	6.6	7.0	14.7	11.6	13.5
2	7.0	7.4	7.2	0.761	0.755	0.758	0.514	0.453	0.484	6.3	6.4	6.4	15.0	17.3	16.1
3	11.0	12.8	11.9	0.799	0.834	0.816	0.436	0.519	0.477	10.1	9.3	9.7	8.3	14.4	11.3
4	10.4	6.4	8.4	0.757	0.646	0.702	0.516	0.487	0.501	5.8	8.5	7.1	14.0	12.1	13.0
5	13.4	13.0	13.2	0.808	0.828	0.818	0.466	0.516	0.491	9.8	10.1	10.0	8.3	12.3	10.3
6	12.8	13.2	13.0	0.804	0.820	0.812	0.591	0.590	0.591	10.4	9.8	10.1	13.2	16.1	14.6
7	9.4	8.2	8.8	0.799	0.787	0.793	0.451	0.460	0.456	6.9	7.6	7.2	12.8	15.6	14.2
8	9.0	10.6	9.8	0.795	0.781	0.788	0.594	0.528	0.561	8.9	8.5	8.7	13.5	13.5	13.5
9	4.8	4.8	4.8	0.636	0.630	0.633	0.558	0.486	0.522	4.2	4.2	4.2	10.2	11.4	10.8
10	2.2	2.2	2.2	0.441	0.441	0.441	0.263	0.263	0.263	2.2	2.2	2.2	16.6	16.4	16.5
11	13.6	11.8	12.7	0.844	0.824	0.834	0.614	0.503	0.558	10.4	10.9	10.6	13.4	18.0	15.7
12	14.0	15.0	14.5	0.827	0.839	0.833	0.501	0.513	0.507	11.1	10.8	10.9	17.2	15.4	16.3
13	14.0	15.2	14.6	0.833	0.845	0.839	0.506	0.577	0.541	11.4	10.6	11.0	16.2	14.8	15.5
14	8.2	8.6	8.4	0.640	0.683	0.661	0.606	0.516	0.561	6.9	6.4	6.6	5.5	12.1	8.8
15	9.4	8.8	9.1	0.802	0.734	0.768	0.533	0.501	0.517	7.5	8.6	8.0	14.9	13.8	14.3
16	7.6	7.0	7.3	0.785	0.773	0.779	0.423	0.449	0.436	6.7	7.3	7.0	19.3	18.3	18.8
17	11.8	12.2	12.0	0.765	0.758	0.761	0.445	0.423	0.434	9.3	9.3	9.3	21.2	16.6	18.9
18	7.0	6.4	6.7	0.746	0.717	0.732	0.514	0.479	0.496	5.2	6.4	5.8	14.3	13.2	13.7
19	10.0	12.4	11.2	0.786	0.815	0.800	0.479	0.555	0.517	9.6	8.4	9.0	10.9	13.5	12.2
20	4.4	5.2	4.8	0.543	0.619	0.581	0.412	0.430	0.421	4.9	4.1	4.5	12.9	13.5	13.2
21	7.2	9.0	8.1	0.666	0.679	0.672	0.448	0.423	0.435	7.7	6.8	7.2	14.5	14.6	14.5
22	13.0	12.6	12.8	0.706	0.706	0.706	0.425	0.419	0.422	10.1	9.5	9.8	16.0	14.8	15.4
23	11.6	10.4	11.0	0.670	0.694	0.682	0.429	0.462	0.446	8.4	8.9	8.6	14.2	12.0	13.1
24	10.8	9.0	9.9	0.807	0.777	0.792	0.475	0.478	0.477	7.5	9.0	8.3	15.2	12.4	13.8
25	6.0	5.4	5.7	0.607	0.566	0.587	0.431	0.355	0.393	5.2	5.5	5.4	13.2	15.4	14.3
26	7.6	8.6	8.1	0.658	0.693	0.676	0.512	0.492	0.502	7.3	6.5	6.9	12.4	21.8	17.1
Overall mean	9.4	9.5	9.4	0.731	0.730	0.731	0.487	0.479	0.483	7.7	7.8	7.8	13.8	14.7	14.2

number of alleles in females from the Bogdaniec (10; A = 2.2) and the highest in females from Cisy koło Barda (13; A = 15.2), with an overall average of 9.5 in all females. Allelic richness (AR) was estimated for the smallest number of individuals (25) with an assigned sex, and the overall value was 7.8; the highest value was in Cisowa Góra (12; AR = 10.9) and Cisy koło Barda (13; AR = 11.0), while the lowest value was in the Bogdaniec (10; AR = 2.2). The average AR for male samples was 7.7. The lowest AR was observed in Bogdaniec (10; AR = 2.2), while the highest values, 11.1 and 11.4, were in the Cisowa Góra (12) and Cisy koło Barda (13) populations, respectively. The values of these parameters in female groups ranged from 2.2 in the Bogdaniec (10) to 10.9 in the Książ (11).

The level of overall observed heterozygosity (H_o) and per population (mean 0.483, range: 0.263–0.591) was considerably lower than the level of expected heterozygosity (H_e) (mean 0.731, range: 0.441- 0.839). The average H_e value in the male and female grouped samples was similar at 0.731 and 0.730, respectively. H_e in males ranged from 0.441 in the Bogdaniec (10) to 0.844 in the Książ (11) and from 0.441 in the Bogdaniec (10) to 0.845 in the Cisy koło Barda (13) in the female groups. The average H_o value in male and female grouped samples was much lower than the H_e averages (H_o: 0.487 vs. H_e: 0.479). The lowest H_o level for both male and female samples ($H_o = 0.263$) was in the Bogdaniec (10) population, while the highest H_o values for males was in the Książ (11) ($H_o = 0.614$) and in the Cisy w Czarnem (6) ($H_o = 0.590$) for females.

Null alleles (N0) were detected at moderate frequencies per population, ranging from 8.8% in the Nowy Waliszów (14) to 18.9% in the Jasień (17), with an overall mean of 14.2%. Similar N0 frequencies were obtained in most of the male and female samples (average values: 13.8% vs. 14.7%). In some cases, however, the null allele frequencies for male and female individuals within a single population significantly varied. For example, the N0 frequencies were 5.5% and 12.1% for males and females, respectively, in the Nowy Waliszów (14) population (Table 2).

Given the presence of N0, the global genetic differentiation (F_{st}) level was estimated separately in males and females with and without ENA correction. These two approaches for estimating the F_{st} level gave similar results. F_{st} values not using ENA vs. F_{st} using ENA for males were 0.152 [confidence interval, CI = 0.134–0.164] and 0.134 [CI = 0.123–0.146], respectively, and were 0.149 [CI = 0.134–0.170] and 0.135 [CI = 0.121–0.151], respectively, for females. These results indicate that the classical measures of population differentiation are slightly biased by the presence of null alleles.

No significant difference (p-values > 0.1) were observed in the averages of the genetic variation estimators between male and female individuals (Table 3). The difference in the F_{st} in male and female samples (0.152 and 0.149, respectively) was not statistically significant (p-value = 0.901) (Table 3). These relationships are well illustrated in the PCoA plot where male and female individuals share a common subgroup for each population. The first and second principal coordinates accounted for 26.51% and 19.51% of the total variation, respectively (Fig. 2).

Table 3. Parameters used to estimate the level of genetic diversity and differentiation in male and female individuals A – average number of alleles, AR_{25} – allelic richness, H_e – expected heterozygosity, H_o – observed heterozygosity, N0 – null allele frequency, F_{st} –genetic differentiation, p–values of the statistical significance of differences between the two sample groups.

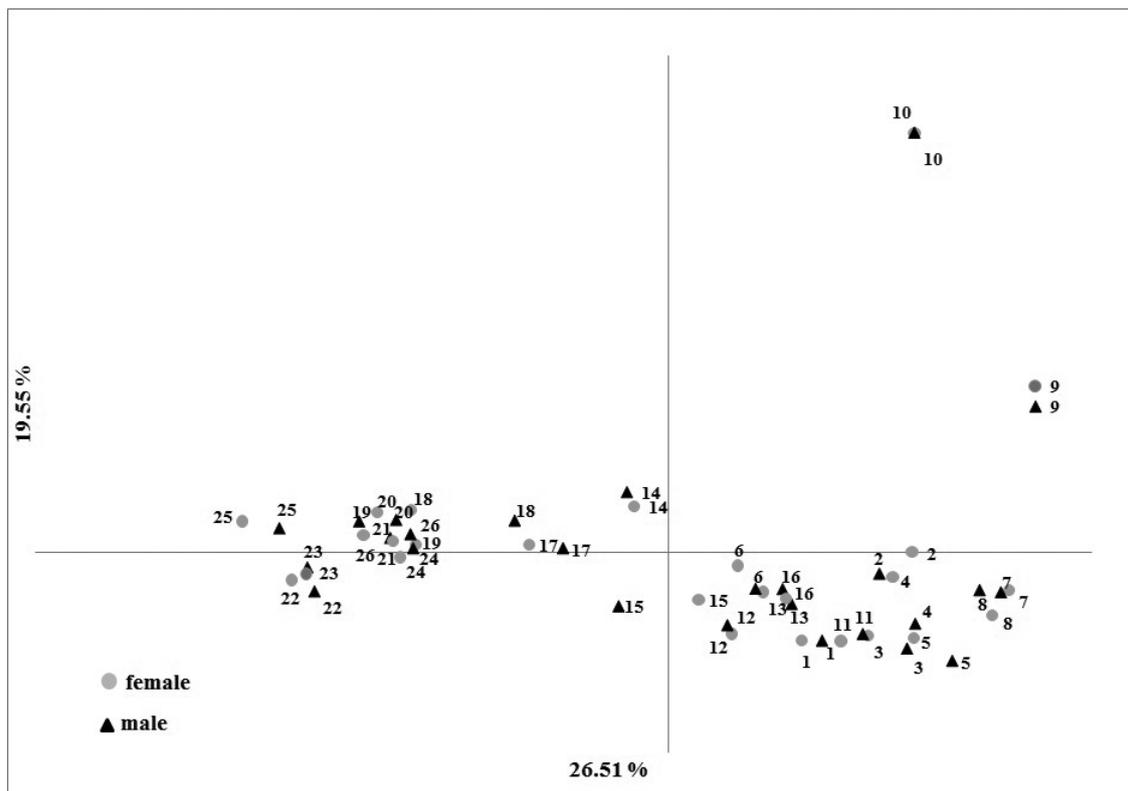| Parameter	Female samples	Male samples	p-value
A	9.512	9.469	0.945
AR25	7.769	7.744	0.965
He	0.730	0.731	0.911
Ho	0.479	0.487	0.876
N0	0.146	0.137	0.275
Fst	0.149	0.152	0.901

Fig. 2. Results of Principal Component Analysis based on five nuclear SSR markers in male and female *Taxus baccata* individuals.

The outlier tests performed using both analytical approaches (Lositan, Bayescan) showed no evidence for any outlier loci among the five microsatellite loci studied. This analysis confirmed that the loci have a presumed neutral character (Fig. S1, Table S1, available as a supplementary file at http://dx.doi.org/10.14214/sf.1236).

Regression analyses between sex ratio and the amount of genetic diversity (A, AR_{25}, H_e, H_o) in the *T. baccata* populations were estimated, and there were no significant correlations between sex ratio and mean number of alleles per population ($r^2 = 0.024$, $p = 0.445$). Furthermore, no significant correlations were found between sex ratio and allelic richness ($r^2 = 0.010$, $p = 0.611$), sex ratio and expected heterozygosity ($r^2 = 0.034$, $p = 0.364$), and sex ratio and observed heterozygosity ($r^2 = 0.071$, $p = 0.188$).

4 Discussion

This study represents one of the first attempts to compare the level of genetic variation and genetic differentiation in *T. baccata* populations with unbalanced sex ratios using nuclear microsatellite analysis. Moreover, the values for a set of parameters characterizing the genetic structure were estimated for individuals of each sex. This type of comparison is valuable because there are known biased sex ratios in *T. baccata,* which may have a negative genetic impact on the small and fragmented populations.

Several earlier studies have reported a high level of genetic variation and a moderate level of genetic differentiation in natural *T. baccata* populations (Lewandowski et al. 1995; Hilfiker et al. 2004b; Myking et al. 2009; Zarek 2009; Dubreuil et al. 2010; González-Martínez et al. 2010;

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Tröber and Ballian 2011; Chybicki et al. 2011, 2012). In the present study, a corresponding high level of genetic diversity was observed in all of the examined populations when considering varied gender proportions. The present results are in agreement with previous *T. baccata* genetic studies employing a variety of genetic marker systems, including nuclear microsatellites, RAPDs (Random Amplified Polymorphic DNA), AFLPs (Amplified Fragment Length Polymorphism), and isozymes (Lewandowski et al. 1995; Hilfiker et al. 2004b; Myking et al. 2009; Zarek 2009; Dubreuil et al. 2010; González-Martínez et al. 2010; Klumpp and Dhar 2011; Tröber and Ballian 2011; Chybicki et al. 2011; 2012). The results are also in accordance with those reported for other outcrossing, wind-pollinated, conifer forest tree species characterized by a high level of genetic diversity and a low to moderate level of genetic differentiation between populations (Hamrick et al. 1992; Petit et al. 2005). Similar trends to those of *T. baccata* at the level of genetic variation within and among populations have been described for the long-lived, dioecious common juniper (*Juniperus communis* L.). Utilizing three polymorphic microsatellite nuclear loci, genetic analysis of nineteen *J. communis* populations originating from twelve regions of Ireland indicated a high level of heterozygosity, ranging from 0.406 to 0.765 (Provan et al. 2008).

Statistical analyses of the set of estimators describing the genetic structure of male and female *T. baccata* individuals revealed that there were no statistically significant differences between the two groups. Cao et al. (2004) previously studied genetic variation in *T. baccata* populations in Germany using isoenzyme markers and compared the genetic structure in male and female individuals. Their analysis revealed minimal significant differences in the values of the tested parameters (H_e, H_o, A/L), which they suggested to be due to the different number of male and female individuals in the populations and the influence of genetic drift on allele frequencies in the populations. Additionally, Nosrati et al. (2012) measured levels of genetic diversity for male and female individuals of the dioecious atlantic pistachio (*Pistacia atlantica* Defs.) using RAPD markers. Their study showed that males exhibited a greater level of genetic variation than females. In contrast, Ukwubile et al. (2014) recently determined the genetic characterization of several willow (*Salix* spp.) genotypes using both RAPDs and nSSRs. They suggested that a set of parameters describing genetic variation revealed no differences between the individuals of *Salix* spp. grouped according to sex.

A growing number of studies have relied on the identification of outlier loci with distinctly different F_{st} from those with neutral expectations (Beaumont and Nichols 1996; Vitalis et al. 2001; Beaumont and Balding 2004) as evidence for the presence of non-neutral microsatellite loci in plants (Acheré et al. 2005; Lazrek et al. 2009; Shi et al. 2011). Some nSSRs may be linked to adaptively crucial genes (e.g., sex-linked genes in dioecious species), and their neutral character should be confirmed for the investigation of natural genetic processes leading to genetic depletion (Beaumont and Nicholas 1996; Ohsawa and Ide 2008). Indeed, Cherif et al. (2013) distinguished three potentially sex-linked nSSR loci located in noncoding regions, which are reliable markers of date palm sex. They showed significantly higher genetic differentiation between sexes than other nSSR loci used in their study. These sex-linked loci are heterozygous only in date palm males, and they significantly differed from Hardy-Weinberg (H-W) equilibrium. In contrast, females showed no significant deviation from the H-W expectation. Male-specific alleles allowed the authors to identify the gender in 100% of date palm individuals. Nevertheless, the analysis in the present study confirmed the neutrality of microsatellite nuclear loci originally identified in *T. baccata* using two different methods: a calescent-based simulation approach performed in Lositian and a hierarchical Bayesian simulation implemented in Bayescan. Because no differences were observed in the level of genetic diversity and genetic differentiation between male and female individuals, the absence of a significant difference indirectly suggests that the tested loci are not sex-linked genetic markers.

There are frequent significant deviations from the equality of sex ratios among natural dioecious plant populations (see review Sinclair et al. 2012). Evidently, unbalanced sex proportions

in natural populations have been presumed to affect the pattern of genetic variation in future generations due to genetic drift and increased inbreeding because of non-random mating (see review Sinclair et al. 2012), especially in a case of outcrossing, long-lived dioecious species with repeated episodes of reproduction, such as *T. baccata* (Iszkuło 2009; Chybicki et al. 2011). Furthermore, the genetic mechanism of sex determination can be labile systems. Environmental factors that determine a fixed sex expression switch on specific genes (Korpelainen 1998). Several authors have reported that an individual *T. baccata* tree can change its sex (Hartzell 1991), and cosexual yew trees carrying both female and male reproductive structures exist (1%) in the Caucasus Mountains (Pridnya 1984). Furthermore, a low level of genetic variation has been reported for small-female-biased *T. baccata* populations in Switzerland using RAPD markers (Hilfiker et al. 2004a). These findings indicated that such populations have undergone a higher degree of genetic drift than large populations. Another investigation reported that genotypic diversity of dioecious clonal forest herb dog mercury (*Mercurialis perennis*) decreased with male-biased sex ratios (Vandepitte et al. 2010). Therefore, the sampling design of collected material should be taken into consideration, as the proportion of male and female individuals in a given population may change due to many environmental factors and stochastic events related to small and isolated populations. The present study was also performed using *T. baccata* individuals derived from populations where sex ratios deviate from the 1:1 equilibrium expectation, containing between 32–68% females in the population. The number of individuals of opposite sex subjected to analyses also varied in the different populations. However, it was demonstrated that ignoring sex ratios in *T. baccata* populations had no impact on the assessment of their genetic structure because no correlation was observed between sex ratios and genetic variation parameters or any statistically significant differences in the level of genetic diversity between male and female individuals.

In spite of the fact that the differences at the level of genetic variation between male and female yews are unexpected, this hypothesis was confirmed using microsatellites. The results of the present study are relevant to the practical application of molecular markers in the conservation management of *T. baccata* genetic resources. The results also demonstrate that the consideration of the *T. baccata* population sex ratio is not essential to develop a clear understanding of the level of genetic differentiation and diversity within and between populations. It would be very helpful to identify sex-specific for *T. baccata* markers because there is currently no way to identify the sex of each shrub before reproductive age, and the sex-determining mechanism is still unclear.

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