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Development of microsatellite markers for the Japanese endemic conifer Thuja standishii and transfer to other East Asian species

James Raymond Peter Worth (jrpw2326@affrc.go.jp)
Forestry and Forest Products Research Institute
K. S. Chang
Korea National Arboretum
Y.-H. Ha
Korea National Arboretum
Aili Qin
Chinese Academy of Forestry

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Abstract

Objective: Design polymorphic microsatellite loci that will be useful for studies of the genetic diversity, structure and reproduction in the Japanese endemic conifer Thuja standishii and test the transferability of these loci to the two other East Asian species, T. sutchuenensis and T. koraiensis . Results: Fifteen loci were developed which displayed 3 to 21 alleles per locus (average = 9.2) among 97 samples from three populations of T. standishii . Observed heterozygosity for all samples varied between 0.33-0.75 (average 0.54) while expected heterozygosity values were higher with an average over the 15 loci of 0.62 (0.37-0.91). Low multi-locus probability of identity values (< 0.00001) indicate that these markers will be effective for identifying individuals derived from clonal reproduction. All 15 loci amplified in 13 samples of T. sutchuenensis , the sister species of T. standishii , with 1 to 11 alleles per locus (average = 4.33) while 13 loci amplified in four samples of the more distantly related T. koraiensis with 1 to 5 alleles per locus (average = 2.15).

Introduction

Thuja is a small Cupressaceae genus consisting of five extant species with three species in East Asia and two in North America (LePage 2003). *Thuja standishii* (Gordon) Carriere (kurobe or nezuko in Japanese and Japanese arbor-vitae in English) is endemic to Japan where it has a scattered distribution from 40.67˚ N to 33.49˚ N on the islands of Honshu, Shikoku and Dogo (Shimane Prefecture). The species is most commonly a component of subalpine forests but also occurs in a variety of habitats including warm temperate forest (rarely), cool temperate forest, moorland and near the alpine zone. The species can grow as a single-stemmed tree up to 35 m tall but occurs as a multi-stemmed shrub under 1 m tall at the maximum limit of its elevational range (Worth et al. 2019). It can purportedly reach a great age with trunks up to 3.5 m in diameter (Giant Tree Database, Biodiversity Center of Japan (http://kyoju.biodic.go.jp)) and individuals over 1000 years old.

Unlike most other Cupressaceae conifers of Japan, *T. standishii* has received little research attention with basic information on its conservation (including the impact of past logging), reproductive biology, and genetic diversity, lacking. The rarity of forest dominated by *T. standishii* and its current insignificant role in forestry probably underlies this lack of research. However, the species is undoubtedly an important part of Japan's biodiversity and cultural heritage being one of the five precious timber trees (Kiso go-boku) that from the early 18th century were strictly protected from cutting in the Kiso region of central Honshu (Mertz 2011) and, in some parts of Japan, forests containing *T. standishii* are considered to be some of the most untouched forests remaining in the landscape (e.g. Wada 1994). Small population size and geographic isolation, its vulnerability to ring barking by deer and the impacts of past logging (Worth et al. 2019) has resulted in some populations being of conservation concern, especially in western Japan where the species is very rare (Worth et al. 2019). One key aspect of the species biology that is poorly understood is the role of asexual reproduction in its regeneration. However, similarly to two other Japanese Cupressaceae conifers, *Thujopsis dolobrata* and *C. pisifera* that have been proven to regenerate clonally (Hayakawa *et al.* 2004; Hasegawa *et al.* 2015), *T. standishii* also forms dense understory banks of juveniles (Worth personal observation) which may be clonally derived.

In the current age of genomics, microsatellites retain an important role in biology due to their high information content, utility in a wide range of genetic applications and cost-efficient nature (Hodel et al. 2016). This study describes the development of Expressed Sequence Tagged (EST) nuclear microsatellite markers for *T. standishii* using next generation sequencing. These markers will be useful molecular tools to inform the conservation of this species via studies of its range-wide genetic diversity, gene flow and reproductive biology. In addition, the transferability of the markers was tested in the two other East Asian species for which no microsatellite markers have yet been developed including the Chinese endemic *Thuja sutchuenensis*, the sister species of *T. standishii* (Li and Xiang 2005; Peng and Wang 2008), and *T. koraiensis*.

Materials And Methods

Total RNA was extracted from an individual of *T. standishii* collected from the Forestry and Forest Products Research Institute Arboretum using a plant RNA isolation mini kit (Agilent Technologies, USA). An RNA-seq data set was constructed by the Beijing Genomics Institute on an Illumina HiSeq 4000 platform. The *T. standishii* RNA-seq data consisted of 38,076,160 paired-end reads of 100 bp length. *De novo* assembly was undertaken in CLC Genomics Workbench 8.5.1 and the 53,614 resultant contigs (*N*50 = 1,503 bp) were mined for microsatellite regions. Primers were developed bordering these regions with default settings using

PrimerPro (http://webdocs.cs.ualberta.ca/~yifeng/primerpro/). Microsatellites were selected if the number of tandem repeat units was greater than eight and if the microsatellite was located less than 25 bp from the beginning or end of the contig. These criteria resulted in 64 microsatellite primer pairs which were trialled for amplification in four samples. A total of 36 primer pairs successfully amplified and were subsequently tested for size heterogeneity in eight samples representative of the species range. For all loci, the forward primer was synthesized with one of three different M13 sequences (5' GCCTCCCTCGCGCCA 3', 5' GCCTTGCCAGCCCGC 3', and 5' CAGGACCAGGCTACCGTG 3'), and the reverse was tagged with a pig-tail (5' GTTTCTT 3'; (Brownstein *et al*., 1996)). The PCR reactions were performed following the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany), and consisted of a 10 uL reaction volume, containing approximately 5 ng of DNA, 5 uL of 2x Multiplex PCR Master Mix, and 0.06 uM of forward primer, 0.1 uM of reverse primer, and 0.08 uM of fluorescently labelled M13 primer. The PCR thermocycle consisted of an initial denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 30 sec, 60°C for 3 min, 68°C for 1 min; and a 20 min extension at 68°C. The PCR products were separated by capillary electrophoresis on an ABI3130 Genetic Analyzer (Life Technologies, Waltham, MA, USA) with the GeneScan 600 LIZ Size Standard (Life Technologies) and genotyping was done in GeneMarker (SoftGenetics, LLC, PA, USA). Overall, 15 loci were found to amplify reliably, display polymorphism and were readily scorable. The genetic variability of these 15 markers were tested in three populations from Atebi Daira Small Bird Forest, Mt Chausu Nature Park, in Nagano Prefecture (35.2286° N, 137.6673° E), Mt Torigata in Kouchi Prefecture (33.4936° N, 133.0638° E) and Mt Yamizo in Fukushima Prefecture (36.9343° N, 140.2679° E). The 15 primer pairs were also tested in 13 samples of *T. sutchuenensis* and four of *T. koraeinsis* (Table S1). Genetic analyses were undertaken in GenAlEx 6.5 (Peakall and Smouse, 2006) and Genepop 4.2 (Raymond and Rousset 1995). In addition, a similarity search of the contigs containing the 15 loci was conducted by the BLASTX algorithm (Altschul et al. 1990) against the National Center for Biotechnology Information (NCBI) non-redundant protein sequences (nr) database.

The multi locus probability of identity (PID) for the 15 markers, that is, the probability that two individuals drawn at random from a population will have the same genotype (Waits, et al. 2001), was calculated in Gimlet version 1.3.3 (Valiere 2002) using all 97 samples from the three population of *T. standishii*. Three PID estimates outlined by Waits et al. (2001) were estimated: biased PID which assumes individuals mate randomly; unbiased PID which corrects for sampling a small number of individuals and, sibs PID which assumes the population is composed of siblings

Results

In *T. standishii*, the 15 loci (Table 1) displayed 3 to 21 alleles over the three populations with an average of 9.2 alleles per locus (Table 2). Overall observed heterozygosity varied between 0.33-0.75 (average = 0.54) while expected heterozygosity values were generally higher (0.37-0.91 with an average of 0.62). At the population level, the number of alleles observed per locus varied from 2-15 (average = 5.43) with six loci showing more than four alleles in each of the three populations. No significant deviations from Hardy–Weinberg equilibrium expectations were detected for any loci except for Kurobe_4219 in the Atebi Daira and Mt Yamizo populations (P = < 0.0004). Additionally, allele frequencies appeared independent among loci with no significant linkage disequilibrium detected after Bonferroni correction.

Multi-locus probability of identity values across all 15 loci were below the threshold value of 0.01 considered by Waits et al. (2001) to be required to reliably distinguish between individual genotypes, even under the sibs PID (Table S2). This indicates that our markers will be effective for both identifying individuals derived from clonal reproduction and sexually derived individuals in populations even where inbreeding is prevalent.

All 15 loci amplified in *T. sutchuenensis* (two loci being monomorphic) with 1 to 11 alleles per locus (average = 4.33) while observed and expected heterozygosity were 0.43 and 0.48, respectively (Table 3). On the other hand, only 13 loci amplified in *T. koraiensis* with three loci being monomorphic. In this species, 1 to 5 alleles per locus (average = 2.15) were observed with observed and expected heterozygosity of 0.44 and 0.31, respectively (Table 3).

Discussion

The development of EST microsatellites for *Thuja standishii* will enable new genetic research into this important Japanese endemic conifer including studies of range-wide level genetic diversity and gene flow and also stand-level processes including inbreeding and clonality. The development of molecular markers may help to foster research into this species, which because of its wide

ecological range, from warm temperate forests to near the alpine zone, is an ideal species to investigate ecological and genetic processes under strongly contrasting climates.

The transferability of the 15 loci was consistent with the phylogenetic relationships of the East Asian *Thuja* (Li and Xiang 2005; Peng and Wang 2008). Thus, all 15 loci successfully amplified in the sister species of *T. standishii*, *T. sutchuenensis* and displayed considerable allelic diversity with up to 11 alleles per locus. These loci, therefore, may be particularly applicable for use in genetic studies of this geographically restricted endangered species (Yang et al. 2013). In contrast, two of the fifteen loci did not amplify in the more distantly related *T. koraiensis* and the number of alleles per locus (ranging from 1 to 5 alleles) was low although this low allelic diversity is likely to be due to the low number of samples tested.

Limitations

- The number of published microsatellite markers may be too low for optimal performance of some genetic analyses.
- These microsatellite loci have not been tested in the two North American species, *plicata* and *T. occidentalis*.
- We did not afford much time optimizing loci, therefore some polymorphic loci that may have worked with further effort may have been excluded.

Abbreviations

bp: base pair; EST: expressed sequence tag; RNA: ribonucleic acid.

Declarations

Authors' contributions

JRPW, KC, YH and AQ devised the study and did the sample collection. JRPW designed the primers, undertook fragment analysis and genotyping. JRPW did the data analysis and wrote the manuscript. All authors participated in the draft and read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The 38,076,160 paired-end reads are deposited in the NCBI BioProject Database, Accession number: PRJNA554973. The genotype data for every individual are available on demand to the corresponding author.

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable

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References

Altschul S F, Gish W, Miller W, Myers EW and Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215: 403-410.

Brownstein MJ, Carpten JD and Smith JR. Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. Biotechniques, 1996; 20: 1004–1006.

Hasegawa Y, Takata K, Tsutomu Y, Gaku H and Saitoh T. Clone identification among saplings in a natural forest of *Thujopsis dolabrata* var. *hondae* using multiplex EST-SSR analysis (in Japanese). J Japanese For Soc. 2015; 97: 261–265 ()

Hayakawa T, Tomaru N and Yamamoto S. Stem distribution and clonal structure of *Chamaecyparis pisifera* growing in an old-growth beech-conifer forest. Ecol Res. 2004; 19:411-420.

LePage BA. A new species of *Thuja* (Cupressaceae) from the Late Cretaceous of Alaska: implications of being evergreen in a polar environment. Am J Bot. 2003; 90:167-174.

Li JH and Xiang QP. Phylogeny and biogeography of *Thuja* L.(Cupressaceae), an eastern Asian and North American disjunct genus. J Integr Plant Bio. 2005; 47: 651-659.

Mertz M. (2011) Wood and traditional woodworking in Japan (2nd edition). Kaiseisha Press, Otsu City, Shiga Prefecture.

Peakall ROD and Smouse PE. Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 2006; 6: 288-295.

Peng D and Wang XQ. Reticulate evolution in *Thuja* inferred from multiple gene sequences: implications for the study of biogeographical disjunction between eastern Asia and North America. Mol Phylogenet Evol. 2008; 47:1190-1202.

Raymond M and Rousset F. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered. 1995; 86:248-249

Valiere N. GIMLET: a computer program for analysing genetic individual identification data. Mol Ecol Notes. 2002; 2:377-379.

Wada K. The vegetation of Mt Nagisodake at the south-western part of Nagano Prefecture. Bulletin of the Institute of Nature Education in Shiga Heights, Shinshu University. 1994; 31: 21-26.

Waits LP, Luikart G and Taberlet P. Estimating the probability of identity among genotypes in natural populations cautions and guidelines. Mol Ecol. 2001; 10:249-256.

Worth JRP. Current distribution and climatic range of the Japanese endemic conifer *Thuja standishii* (Cupressaceae). Bulletin of FFPRI. 2019; 18:275-288.

Yang Y, Li N, Christian T and Luscombe D. *Thuja sutchuenensis*. The IUCN Red List of Threatened Species. 2013; 3.T32378A2816862. Downloaded on 22 July 2019.

Tables

Table 1 Characteristics of the 15 microsatellite markers developed for *Thuja standishii*

Locus		Primer (5'-3')	Repeat motif	Allele size range (bp)	BlastX Top Hit Description	E-value	Genbank Accession
Kurobe_18480	F:	TCAGGACCCCAACAAATAAA	(AT)9	135-189	unknown [*Picea sitchensis*] ABR17033.1	3E-95	MN170833
	R:	GATGCTGGAGATGGTATTTCG					
Kurobe_2969	F:	ACACAATCTTGCCCAGAAGC	(TA)8	269-291	WRI2 [*Larix gmelinii* var. *olgensis* x *L. kaempferi*] AJP75043.1	0	MN170828
	R:	ACACAATCTTGCCCAGAAGC					
Kurobe_23700	F:	CCGACGCCTCTACATTCATT	(TCT)10	250-268	hypothetical protein MARPO_0043s0052 [*Marchantia polymorpha*] PTQ39804.1	2E-39	MN170835
	R:	ATGGAAAAGAGGCAGAGCAA					
Kurobe_44557	F:	GATCCCTTTTGATGGGTTCA	(TC)11	313-349	-	-	MN170841
	R:	TTATCCCCAACTTCAGCGAG					
Kurobe_15129	F:	TTAACATCCACCAATGGCAA	(TC)12	205-233	unknown [*Picea sitchensis*] ABK22564.1	9E-66	MN170831
	R:	CAAATGCATGGAATGTGGTC					
Kurobe_16758	F:	TGCAACGTCATAACCACGAT	(CT)8	272-283	CYP866B7 [*Taxus chinensis*] ATG30002.1	0	MN170832
	R:	CACCCTTCTAGAGCTGGTCG					
Kurobe_6943	F:	CTACGTCTTAGCCTCCACGC	(CT)11	159-169	early nodulin-like protein 2 [*Amborella trichopoda*] XP_011628802.2	4E-37	MN170830
	R:	ATGCCATACTGGGGTCTGAG					
Kurobe_23263	F:	CCAGGCCCTAATTTCCACTC	(AG)10	290-314	-	-	MN170834
	R:	CCATGGCACATGGATACAGA					
Kurobe_51603	F:	CAAAGCAACAGAGCCAACAA	(TG)13	162-201	-	-	MN170842
	R:	AAATAGGGTGCTGCAGGTTG					
Kurobe_31302	F:	TCCCAAGTCTGGGGCAGT	(GT)8	264-274	-	-	MN170836
	R:	GTCACAGTCGGGAGCAATTT					
Kurobe_38308	F:	TTTGCTGAAACCCAGGAATC	(TG)11	272-297	-	-	MN170837
	R:	CCCCATATGGAAGAAGATCC					
Kurobe_41636	F:	TTGAACTTTGCAGTGGGAAA	(CA)8	157-165	-	-	MN170839
	R:	GAACCAGACAGGAATCGGAA					
Kurobe_42400	F:	TTCACACAAGGGCCTACTCC	(TC)9	254-272	-	-	MN170840
	R:	GCCGACAGGTCTCTTAGCAC					
Kurobe_40825	F:	TTATCCCATGAGCGTGCTTT	(CT)8	232-276	-	-	MN170838
	R:	TAGGTGCGGTGACTATGCAG					
Kurobe_4219	F:	ACGCATCGGACAGTCGTATT	(TC)8	276-306	MACPF domain-containing protein At4g24290 [*Morus*	0	MN170829

		notabilis] XP_010087924.1
	R: CCGTGCAGAACCAAAGAAAT	

– denotes that no BlastX hits were found

Table 2 Genetic diversity of the 15 polymorphic nuclear microsatellites assessed across the three populations of *Thuja standishii*.

	Atebi Daira (32)			Mt Torigata (31)			Mt Yamizo (34)			All (97)		
Locus	*Na*	*Ho*	*He*	*Na*	*Ho*	*He*	*Na*	*Ho*	*He*	*Na*	*Ho*	*He*
Kurobe_18480	13	0.75	0.77	11	0.81	0.82	8	0.38	0.43	21	0.64	0.77
Kurobe_2969	6	0.53	0.45	5	0.52	0.60	7	0.85	0.77	9	0.64	0.75
Kurobe_23700	5	0.78	0.77	5	0.42	0.38	6	0.59	0.57	7	0.60	0.66
Kurobe_44557	14	0.68	0.89	9	0.68	0.73	15	0.88	0.88	21	0.75	0.91
Kurobe_15129	7	0.66	0.71	2	0.03	0.03	5	0.68	0.69	8	0.46	0.63
Kurobe_16758	4	0.50	0.51	3	0.29	0.26	4	0.85	0.73	7	0.56	0.60
Kurobe_6943	3	0.28	0.30	3	0.65	0.51	4	0.35	0.33	7	0.42	0.50
Kurobe_23263	2	0.41	0.46	3	0.48	0.44	3	0.38	0.51	4	0.42	0.52
Kurobe_51603	7	0.75	0.75	3	0.55	0.54	8	0.82	0.70	9	0.71	0.72
Kurobe_31302	3	0.63	0.55	3	0.52	0.51	2	0.12	0.11	3	0.41	0.42
Kurobe_38308	6	0.59	0.66	3	0.55	0.45	5	0.50	0.58	9	0.55	0.61
Kurobe_41636	2	0.41	0.36	2	0.39	0.46	3	0.21	0.21	4	0.33	0.37
Kurobe_42400	7	0.50	0.48	5	0.58	0.53	6	0.38	0.36	9	0.48	0.46
Kurobe_40825	7	0.50	0.63	6	0.71	0.71	5	0.68	0.58	11	0.63	0.73
Kurobe_4219	4	0.28	0.51	6	0.61	0.57	6	0.62	0.70	9	0.51	0.61
Average	6.00	0.55	0.59	4.60	0.52	0.50	5.80	0.55	0.54	9.20	0.54	0.62

Note: *Na* = the number of alleles, H_o = observed heterozygosity and H_e = expected heterozygosity.

Table 3. Genetic diversity of the 15 microsatellite loci in *T. sutchuenensis* and *T. koraiensis*.

	T. sutchuenensis (13)			*T. koraiensis* (4)		
Locus	*Na*	*Ho*	*He*	*Na*	*Ho*	*He*
Kurobe_18480	7	0.46	0.80	1	0.00	0.00
Kurobe_2969	7	0.61	0.79	2	1.00	0.50
Kurobe_23700	3	0.15	0.14	2	0.25	0.22
Kurobe_44557	11	0.61	0.89	3	0.50	0.41
Kurobe_15129	6	0.69	0.76	2	0.25	0.22
Kurobe_16758	4	0.69	0.56	-	-	-
Kurobe_6943	1	0.00	0.00	-	-	-
Kurobe_23263	3	0.38	0.33	2	0.50	0.50
Kurobe_51603	4	0.38	0.38	5	1.00	0.75
Kurobe_31302	1	0.00	0.00	2	0.75	0.47
Kurobe_38308	6	0.61	0.69	2	0.25	0.22
Kurobe_41636	2	0.46	0.43	2	0.25	0.22
Kurobe_42400	2	0.31	0.26	1	0.00	0.00
Kurobe_40825	6	0.69	0.80	1	0.00	0.00
Kurobe_4219	2	0.38	0.39	3	1.00	0.59
Average	4.33	0.43	0.48	2.15	0.44	0.31

- Denotes that the loci did not amplify

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