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Microsatellite markers for eastern hemlock (*Tsuga canadensis*)

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Abstract

We describe polymerase chain reaction primer pairs and reaction conditions for amplification of 15 microsatellite loci from eastern hemlock (*Tsuga canadensis*). The primers were tested on 23 individuals from a natural population in southwestern North Carolina, USA. These primers yielded an average of 5.9 alleles per locus (range of 2–14), an average observed heterozygosity of 0.45 (range 0.14–0.73), and an average polymorphic information content of 0.54 (range 0.28–0.86). In addition, eight of the primer pairs were found to amplify microsatellite loci in one or more additional species of *Tsuga*.

Keywords: Canada hemlock, conservation genetics, genetic diversity, SSR markers

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Eastern hemlock [*Tsuga canadensis* (L.) Carr., also commonly known as Canada hemlock] is endemic to eastern North America. In recent years, eastern hemlock populations have been severely, negatively impacted by the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (McClure *et al*. 2003). The microsatellite markers described herein will expand the molecular resources available to characterize the genetic diversity of hemlocks for conservation and breeding efforts.

Genomic DNA from eastern hemlock tree no. 237 (sourced from Linville Falls, North Carolina, USA) was digested with *Rsa*I and *Bst*UI (New England Biolabs) and enriched for $(AC)_{12}$, $(AG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$, $(AAAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(AATC)_6$, $(ACAG)_6$, $(ACTG)_6$, $(AAAC)_6$, $(AATG)_6$, $(AGAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(AAAT)_8$, and $(ACAT)_8$ (Glenn & Schable 2005). Products were ligated into PCR 2.1-TOPO vector and transformed into Top 10 *Escherichia coli* cells (TOPO TA cloning kit, Invitrogen). We sequenced 672 clones containing inserts between 500 and 1100 base pairs using BigDye (version 3.1, Applied Biosystems) chemistry and an ABI 3730xl Genetic Analyser. Sequences were assembled and edited in Sequencher 4.2 (Gene Codes). Microsatellite repeat arrays were found in 140 contigs for which primers were designed with 5′-tails (CAG or M13R; Boutin-Ganache *et al*. 2001; Glenn & Schable 2005) using MSATCOMMANDER (Faircloth 2008) and default primer selection values. For primer synthesis and testing, we selected 100 primer pairs adding a CAG or M13R 5′-tail to one primer and a 5′-'pigtail' (GTTT) to the second primer (Brownstein *et al*. 1996).

Primers were tested using samples from 23 eastern hemlock trees collected from a natural population near the Coweeta Hydrologic Laboratory in southwestern North Carolina, USA. The species is likely to have expanded into its current range following glaciation 12 000–20 000 years ago from refugia in the southeastern USA, and allozyme studies suggest that greater diversity is found in populations of the southeastern USA (Potter *et al*. 2008). In addition, a subset of successful primer pairs were evaluated using samples of six additional *Tsuga* species: Carolina hemlock (*T. caroliniana*), Chinese hemlock (*T. chinensis*), mountain hemlock (*T. mertensiana*), northern Japanese hemlock (*T. diversifolia*), southern Japanese hemlock (*T. sieboldii*), and western hemlock (*T. heterophylla*). Template DNA was extracted from needles using the DNeasy Plant Kit (QIAGEN). Reactions contained 10 mM Tris pH 8.4, 50 mM KCl, 0.5 μM 'pigtailed' primer, 0.05 μM CAG or M13R-tailed primer, 0.45 μM dye-labelled (HEX or FAM) CAG or M13R universal primer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 U *Taq* DNA Polymerase, and 25 ng DNA in 10 μL total volume. Reactions were completed with a touchdown programme

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Table 1 Characterization of 15 primer pairs amplifying microsatellite loci from eastern hemlock (*Tsuga canadensis*) from North Carolina, USA

Locus	Primer sequence (5′–3′)	GenBank Accession no.	Repeats in cloned allele	*N*	No. of alleles: size range (bp)	H_O	H_E	P_{HW}	PIC
Tcn10A05	m: CAATATGAAATGTGTAAAATCTA p: GGAGAAACACCCAAAATAATAG	EF660516	$(AC)_{26}$	22	11: 194–238	0.727	0.702	0.734	0.663
Tcn10A12	p: AGTCATGGGGCCTCTTTGC m: CTCAGACCAGCACTCCAG	EF660517	$(AG)_{19}$	23	14: 144–184	0.696	0.888	0.006	0.858
Tcn10E06	p: GTGGGTAGCATGGACACC m: CTGAAACATGATCCGGGC	EF660518	$(ATGT)_4$	22	2: 205–209	0.273	0.406	0.270	0.318
Tcn10B01	p: CACCTCGATCATACATCGGTC c: GTCAGTCTTGCTTTCGTTTGG	EF660519	$(GT)_{26}$	23	9: 190–208	0.652	0.755	0.102	0.702
Tcn12C01	c: GAACAACAGAAGGACCCATC p: AGCCCACCGTCTCTCTAAG	EF660520	$(AAG)_7$ ... $(AAG)_{12}$	23	4: 357–368	0.609	0.658	0.724	0.572
Tcn2C05	c: CTCACTGTATTGTTCACTCAAG p: CACGCATTCACTGCTAGTTTG	EF660521	$(AC_{)15}$	23	6: 207–227	0.478	0.692	0.016	0.620
Tcn2C08	p: ACTCCACCCCTTTTAGCCC m: GGTGGGTGGTTTCTTGAAGTC	EF660522	$(AC)_9$ ... $(AC)_7$	23	5: 242–250	0.304	0.609	0.002*	0.513
Tcn2G11	p: GGTTTAAGAGCTGCGACCC c: TTTGGATCTTTGGCTATTTAAG	EF676027	$(TG)_{14}$	23	5: 270–288	0.435	0.589	0.204	0.489
Tcn3E02	p: GTGCAAGGTTAAGGCCACG m: GCCACCATAGAGCTGAGG	EF660524	$(AG)_{16}$	21	5: 365–377	0.333	0.583	0.009	0.514
Tcn3H04	c: GGAACCAACTTCGTGCGAG p: GTGGTTGGTCTCTTTCACTGG	EF660525	$(GT)_{16}$ ... $(AG)_{11}$	23	5: 301–313	0.478	0.712	0.016	0.659
Tcn8B06	c: TGGGGATCTAGGGGAAAGC p: GGGCATACAGGCATTAGGG	EF660526	$(GT)_{21}$	23	4: 300–306	0.304	0.409	0.071	0.375
Tcn2B04	m: CATGTACCGGTCCTCCTG p: AGAGGCCCTTCTTGAACCC	EU125392	$(TG)_{12}$	22	2: 168–170	0.136	0.384	0.006	0.305
Tcn7H12	p: GTTCACCTCTCATGAACAATGC m: GAGGCATAGGGGAATGTG	EU125393	$(CA)_7$ ... $(CATA)_7$	21	2: 205–216	0.333	0.345	1.000	0.280
Tcn10A07	p: GGTGAAGAAACCGGGGAATG c: TGGGGAGTTGATCACTGGG	EU125394	$(TACA)_7$	22	3: 410–426	0.364	0.427	0.572	0.360
Tcn10D07	p: GAGGGGGTAGAGAAAGAAC m: GATTGAGCCCTAGAAGTG	EU125395	$(GA)_{25}$	22	11: 176–193	0.636	0.852	0.014	0.812

Sequences used to introduce sites for the dye-labelled universal primer are given at the 5′ end as 'm' for M13R [GGAAACAGCTATGACCAT] or 'c' for CAG [CAGTCGGGCGTCATCA]. Pigtailed (GTTT) primers are indicated with a 5′-end p:. *N*, number of individuals genotyped at each locus; number of alleles, allele size range (base pairs, bp); H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HW}, probability that genotype proportions conform to Hardy–Weinberg equilibrium; and PIC, polymorphic information content. Astersik (*) indicates Hardy–Weinberg disequilibrium following sequential Bonferonni correction.

(Don *et al*. 1991): 21 cycles of 95 °C for 20 s; 65 °C for 20 s minus 0.5 °C per annealing cycle; and 72 °C for 30 s followed by 24 cycles of 95 °C for 20 s; 49.5 °C or 54.5 °C, respectively, for 30 s; 72 °C for 30 s; and a final extension period of 10 min at 72 °C.

Genotyping was performed using an ABI 3730xl Genetic Analyser with GeneScan 500 ROX size standard and GeneMapper software (Applied Biosystems). We calculated observed and expected heterozygosities and polymorphic information content for each locus using Cervus 2.0 (Marshall *et al*. 1998). GenePop 3.4 (Raymond & Rousset 1995) was used to test for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD). We conducted a posteriori sequential Bonferroni correction (Rice 1989).

Table 1 summarizes characteristics of 15 successful primer pairs designed from *T. canadensis*. Only locus Tcn2C08 deviated from HWE following sequential correction. No significant LD was detected after sequential Bonferroni correction. Consistent heterozygote deficiency across most loci suggests that the cause may be population-specific rather than primer-related. This observation is consistent with an overall deficiency of heterozygotes reported in a study using 19 allozyme markers (Potter *et al*. 2008). Cross-species amplification results are shown in Table 2. Eight of the primer pairs amplified loci in at least one of the six tested species. *T. chinensis* was the most readily amplified species (six out of eight), while *T. heterophylla* was the least amplified (two out of eight).

These markers appear to be quite informative both within the source species, *T. canadensis*, as well as across *Tsuga*, but especially for *T. chinensis* and *T. caroliniana*. Ongoing efforts to characterize the genetic variation in the

Table 2 Marker cross-utilization results for eight *Tsuga canadensis*-derived markers tested with six *Tsuga* species of various sample sizes (n): observed number of alleles and allele size range in base pairs; dashes (—) indicate no amplification or difficult-to-interpret amplification products

Locus	*T. caroliniana* $n = 5$	*T. chinensis* $n = 5$	*T. mertensiana* $n = 3$	*T. diversifolia* $n = 3$	*T. sieboldii* $n = 2$	*T. heterophylla* $n = 4$
Tcn10A07	1: 419	1: 448	1: 431	1: 441	—	2: 432–435
Tcn10A12	—	—	2: 160–162		—	—
Tcn10D07	—	—	—	1: 176	—	—
Tcn12C01	1: 309	1: 305	—	—	2: 303–313	3: 297–303
Tcn2B04	—	2: 166–168	—	—	2: 166–170	—
Tcn2C05	1: 193	4: 166–196	1: 174	1: 194	—	—
Tcn3E02	1: 367	3: 360–392	—	2: 364–368	2: 358–364	—
Tcn8B06	1: 263	1: 297	—	1: 276	1: 297	—

eastern North American hemlocks and to develop new HWA-tolerant varieties through interspecies backcross breeding will likely benefit from these new marker resources (Pooler *et al*. 2002; Potter *et al*. 2008).

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References

Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *BioTechniques*, **31**, 24–28.

Brownstein MJ, Carpenter JD, Smith JR (1996) Modulation of non-templated nucleotide addition by *Taq* polymerase: primer modifications that facilitate genotyping. *BioTechniques*, **20**, 1004–1010.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research*, **19**, 4008.

Faircloth BC (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources*, **8**, 92–94.

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. In: *Methods in Enzymology 395, Molecular Evolution: Producing the Biochemical Data, Part B* (eds Zimmer EA, Roalson EH). Academic Press, San Diego, California.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.

McClure MS, Salom SM, Shields KS (2003) Hemlock woolly adelgid. *FHTET-2001–03. Forest Health Technology Enterprise Team*, US Department of Agriculture Forest Service, Morgantown, West Virginia.

Pooler MR, Riedel LGH, Bentz SE, Townsend AM (2002) Molecular markers used to verify interspecific hybridization between hemlock (*Tsuga*) species. *Journal of the American Society of Horticultural Science*, **1227**, 623–627.

Potter KM, Dvorak WS, Crane BS *et al*. (2008) Allozyme variation and recent evolutionary history of eastern hemlock (*Tsuga canadensis*) in the southeastern United States. *New Forests*, **35**, 131–145.

Raymond M, Rousset F (1995) GenePop (Version 1.2) population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.