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Genetic variation in the endangered Florida torreya (*Torreya taxifolia* Arn.) and implications for conservation[1]

Tyler J. Dreaden,[2,3] Katherine E. Smith,[4] C. Dana Nelson,[3] Emily E. D. Coffey,[5] Lauren Eserman,[5] and Jason A. Smith[6]

[3] United States Department of Agriculture Forest Service, Southern Research Station, Forest Health Research and Education Center, Lexington, KY 40503

[4] United States Department of Agriculture Forest Service, Southern Research Station, Southern Institute of Forest Genetics, Saucier, MS 39574

[5] Conservation & Research Department, Atlanta Botanical Garden, Atlanta, GA 30309

[6] College of Natural and Health Sciences, University of Mount Union, Alliance, OH 44601

Abstract. *Torreya taxifolia* Arn. (Florida torreya) is a critically endangered (International Union for Conservation of Nature 3.1) conifer with a very limited native range in the USA, occurring in two counties in Florida and one in Georgia where it persists in ravines along the Apalachicola River. The species suffered a major decline, ~ 99% loss, beginning in the 1930s, with a total population estimated at 357,500 in the early 1900s decreasing to 1,000 individuals currently remaining in the wild. The initial decline was attributed to an unknown fungal disease with *Fusarium torreyae* (T. Aoki, J. A. Smith, L. Mount, Geiser and O'Donnell), identified in 2011 as the causal agent of a canker disease. The objectives of this work were to develop genetic markers to uniquely identify individuals, examine the structure of the *in situ* population, and use an *ex situ* germplasm resource to determine if reproduction without fertilization (apomixis) occurs. We developed a panel of microsatellite markers, sampled individuals from three natural *in situ* populations, and found population structure at all levels tested. The markers were next applied to *ex situ* mothers, potential fathers, and progeny to investigate the occurrence of apomixis. One of 29 progeny seedlings tested and its mother had identical multilocus genotypes. The probability of observing this genotype by chance, given the mother and the two possible fathers' genotypes, is approximately one in ten. Based on these data, we cannot reject the hypothesis that apomixis occurs. Our finding of population structure suggests genotypes from as wide an area as possible are required to capture the diversity of the species in conservation efforts. The multiplexed marker panel can be used to identify individual accessions and assist with managing *ex situ* collections; however, they are not informative enough to clearly differentiate closely related individuals in the *ex situ* population.

Key words: conifers, disease, forest, pathogen, reproductive strategies, SSR

Torreya taxifolia Arn. is a conifer endemic to ravines along a 35-km stretch of the Apalachicola River in Florida and Georgia, USA. Despite its very limited native range today, this medium-stature tree was once abundant. In the early 1900s, the population was estimated to be 357,500 trees > 2 cm diameter at breast height, with adult trees reaching the canopy and exhibiting abundant regeneration (Schwartz *et al.* 2000). The species suffered a catastrophic decline beginning sometime after the late 1930s that was attributed to a "fungal disease of the stem" (Godfrey and Kurz 1962), but the causative pathogen was not identified at the time. *Torreya taxifolia* was listed under the Endangered Species Act in 1984, with an estimated 99% loss of mature trees (USFWS 1984). Its listing's justification is rather unique, as habitat loss was not a factor in the decline (Wilcove *et al.* 1998, Schwartz *et al.* 2000).

The compound effect of fungal disease, caused by *Fusarium torreyae* (Aoki *et al.* 2013), and natural disasters have pushed *T. taxifolia* to the brink of extinction. The species is now limited to a population dominated by resprouts, with a 2008–10 survey finding a mean tree height of 118 cm and an average canker incidence of 93.4% (Smith *et al.* 2011). The total population was estimated at roughly 1,400 individuals in 2000 (Schwartz *et al.* 2000). Further decline of this species occurred in 2018 when Hurricane Michael, a category 5 hurricane, directly hit the heart of the species range, Torreya State Park. Surveys completed by the Atlanta Botanical Garden (ABG) documented 29% mortality directly due to

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[2] Author for correspondence: tyler.j.dreaden@usda.gov.

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hurricane damage (USFWS 2020). Hurricane Michael significantly altered the habitat by reducing canopy cover in ravines by 80–90% (USFWS 2020), but trees were likely killed directly from being uprooted, flooded, or covered by debris during the hurricane. Posthurricane effects are still being evaluated, as some trees were further weakened, and the combined impact of stress and *F. torreyae* might lead to additional mortality. The current posthurricane population is estimated to be no more than 1,000 individuals (USFWS 2020; E.E.D.C., personal communication).

Efforts to preserve *T. taxifolia ex situ* were initiated in the late 1980s through collaborations among botanic gardens with expertise in conifer propagation. The ABG began its conservation efforts in 1991 and now holds approximately 750 genetically distinct *T. taxifolia* accessions in their *ex situ* collections (E.E.D.C., personal communication). The major goal of a conservation collection is to preserve genetic diversity, providing a resource for reestablishment of the species following a catastrophic event (Center for Plant Conservation 2019). For similarly imperiled species, a target goal for *ex situ* conservation is 90–95% of the standing genetic diversity (Hoban 2019, Griffith *et al.* 2021). *Torreya taxifolia*, like many conifer species, has a large genome (1C = 21.60 pg; Zonneveld 2012), making some standard population genetic analysis methods such as RAD-seq or GBS cost-prohibitive. Therefore, one objective of this study was to develop informative and cost-effective genetic markers for evaluating genetic diversity. Microsatellites or simple sequence repeats (SSRs) were chosen because they are commonly used in a wide range of population genetic studies and are useful in both low- and high-throughput applications. SSR markers could be used to identify individuals in *in situ* collections and the *ex situ* population and also to study the standing genetic diversity both *in situ* and in the *ex situ* population.

A population genetics study of *T. taxifolia* using allozymes found low overall levels of genetic variation and mean heterozygosity of 0.064 (Schwartz 1993), while lacking the resolution to differentiate individuals. Given the current *T. taxifolia* population largely consists of resprouts and single-stem individuals are occasionally in proximity, some of the current resprouts may arise from the same tree. An evaluation of the *in situ* population using markers that are more informative than allozymes is needed to guide *in situ* and *ex situ* conservation strategies, that is, prioritization of conservation needs based on genetic data and confirmation that individuals are not derived from the same clonal root system. An efficient conservation strategy is of utmost importance given the limited resources available for *T. taxifolia*, ranking 884 out of 926 for endangered species expenditures (USFWS 1998), and extensive population losses following Hurricane Michael.

Torreya taxifolia is dioecious, with females producing nearly globose gray-blue fruit 2.5 to 4.1 cm long and 1.9 to 3.6 cm wide (Burns and Honkala 1990). Little is known about the dispersal of this relatively large seed except for a report of gray squirrels gathering seed as soon as the arils turn purple at the Maclay State Gardens (USFWS 1986). It is, however, unknown what role squirrels played historically or in more natural settings. A related species, *Torreya nucifera* ((L.) Siebold & Zucc.), is subdioecious, but this has never been observed in *T. taxifolia*. Viable seed from isolated *T. taxifolia* female trees has been observed in both cultivated and natural settings. This seed production, despite the lack of nearby male trees, has led researchers to question if apomixis (reproduction without fertilization) could explain such seed production. Apomictic seeds have not been reported in conifers, but apomixis features have been observed in *Picea abies* (Norway spruce) in experimental conditions, that is, "leader cells" behaving almost identically to the central cell in the archegonium (Bell 1994, Durzan *et al.* 1994). These observations led us to consider whether or not apomixis may occur in *T. taxifolia*, thus providing a means of surviving isolating conditions and the first indication of such a phenomenon occurring in a conifer.

In addition to the genetic needs, there is need for clean, pathogen-free nursery stock of *T. taxifolia* for *ex situ* conservation and to augment populations *in situ* (USFWS 2020). The inability to consistently produce pathogen-free planting stock has limited the distribution of accessions to new locations due to concern of spreading the canker pathogen that has been shown to infect other hosts in artificial inoculations (Trulock 2013). The ABG is developing an *F. torreyae* PCR assay (Dreaden *et al.* 2020) into a seedling screening method, along with fungicide and phytosanitary strategies, with the goal of producing disease-free stock for distribution. However, the ability to confirm the identities of individuals from *in situ* and *ex situ* collections is lacking and would aid ongoing and future conservation efforts.

The goals of this study were (a) to develop a short simple repeat (SSR or microsatellite) DNA marker panel for *T. taxifolia*, (b) to apply this panel to evaluate the structure of the *in situ* population, (c) to test whether apomixis occurs among seed-bearing trees in *ex situ* collections, and (d) to evaluate the markers for their ability to uniquely identify individuals *in situ* and in *ex situ* collections.

Materials and Methods. MICROSATELLITE MARKER DEVELOPMENT. *Torreya taxifolia* sequences were obtained from the 1,000 Plant Transcriptome Initiative (Carpenter *et al.* 2019). A total of 88 microsatellites were identified and primer pairs to amplify them were designed and tested following Dreaden *et al.* (2014). The primers were synthesized with the M13 sequence (CACGACGTTGTAAAAC-GAC) added to the 5′ end of the forward primer, the PIG-tail sequence (GTTTCTT) added to the 5′ end of the reverse primer. The PCR products were fluorescently labeled during PCR by including a third primer with fluorophores (VIC, FAM, NED and PET) added to its 5′ end. An M13 sequence was used for the third primer to ensure it would not hybridize to the template DNA. The primer labeling and PCR conditions followed Echt *et al.* (2011).

Torreya taxifolia leaf tissue was collected from wild individuals distributed across the study site during a 2008–10 survey (Smith *et al.* 2011). Collected leaves were stored at 4°C, in individual glass vials containing silica drying beads. Two leaves per sample were precut with scissors and placed in 2-mL Eppendorf tubes, along with lysing matrix I (MP Biomedicals, Irvine, CA) and ground using a Mini-G® tissue homogenizer (SPEX Sample Prep, Metuchen, NJ). Extraction of DNA was carried out using the Nagel-Macherey NucleoSpin® (Duren, Germany) Plant II kit and CTAB extraction buffer. Samples were eluted in 100 μL of elution buffer checked for quality using a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA) and gel electrophoresis.

The 88 designed primer pairs were prescreened using 11 individuals (nine from *in situ* populations spanning approximately 10 km and two from *ex situ* collections), to identify easily scorable polymorphic loci. Single-plex PCRs contained 2ng DNA, 1 μL 10× PCR buffer, 0.42 μL 50 mM $MgCl_2$, 0.33 μL 2mM dNTPs, 0.09 μL Taq, 2.4 μM m13 primer, 0.6 μM forward primer, 2.4 μM reverse primer, and water to 10 μL. Thermocycling conditions were 94°C 2 min, 20 cycles (94°C for 30 sec, 65°C [−0.5°C per cycle] for 30 sec, 72°C for 1 min), 25 cycles (92°C for 30 sec, 55°C for 30 sec, 72°C for 1.5 min), 72°C for 15 min, and hold at 4°C. Amplicon sizes were determined using an Applied Biosystem (Waltham, MA) 3730 DNA Analyzer with 600 LIZ™ Dye Size Standard (USDA FS SRS, Saucier, MS) and Geneious 9.1 software (Auckland, New Zealand). The 14 loci that passed the prescreening evaluation were multiplexed using Multiplex Manager (Holleley and Geerts 2009), followed by empirical testing and adjusting forward primer concentrations for more even amplification among loci. This resulted in five multiplex PCRs containing 14 loci that could be post-PCR pooled and run in a single sequencing lane. Multiplex PCR conditions were as above, except forward primer concentrations varied (as determined above), and M13 primer dye alternated between multiplexes (Table 1). Amplifications among the 14 loci in the five multiplex PCRs varied in quality and, when combined in a single sequencing lane, resulted in peaks of variable amplitudes. The uneven amplification made automated peak calling difficult, so peak calling was completed manually. Further multiplex PCR optimization is required to achieve even allele amplification allowing for high-throughput automated allele calling. The microsatellite loci were manually checked for Mendelian inheritance, confirming seedlings shared one allele with its mother and the second allele was found in one of the two potential paternal genotypes (see below) using the *ex situ* population. Loci with null alleles and ambiguous peaks were rerun in singleplex PCRs. Fragment size error rates were calculated by rerunning 10 *T. taxifolia* samples and all 14 loci in a single 96-well PCR plate, using the multiplexed panel (Table 1). Six individual sample/loci failed to amplify, with 134 matching previous measurements.

IN SITU DATA SET. The genetic structure of the wild population of *T. taxifolia* was investigated by sampling three populations (north, middle, and south) spanning approximately 13.6 km (Fig. 1). Each population included all adjacent individuals in the area, 15 individuals per population, and spanned 0.35 to 1.19 km. One sample was discarded due to poor amplification, resulting in the analysis of 44 individuals in total. Poppr (Kamvar *et al.* 2014) in R version 3.6.0 (R Core Team 2022) was used for microsatellite population genetic calculations including codes for

Table 1. Microsatellite loci, primer sequences, and PCR multiplexes used to genotype *Torreya taxifolia*. Italicized loci were not used in the final analysis.

Multiplex-dye	SSR loci[1]	Repeat	Alleles	Primer sequence 5′-3′ Forward	Reverse	Forward primer μM
11-Ned	*Ttax001*	ATCT		M13-CAGATCTATAGATATGGCCC	PIG-TATAGAATCAATCTTGGCC	0.012
	Ttax016	TCC	4	M13-GGTCCTCTGTAATACTCAGC	PIG-TAATAGACACAAATGGATGG	0.03
29-Fam	Ttax003	AAAG	2	M13-TAGTTAGTCTTTGATCCTGG	PIG-TCCTACTTGTACATGTGTCC	0.012
	Ttax006	TTC	4	M13-ACCACACTTTAAAACAAGC	PIG-AGTGAGAGTAAACTTCCACC	0.012
	Ttax083	TC	2	M13-TAGGAGTCATTTTAACAAGC	PIG-TATGAAAAGCTTTTACTTGC	0.018
31-Vic	Ttax076	AC	2	M13-AAAACTTCACAGACGACG	PIG-TTTAAGACTAATTTCCATGC	0.024
	Ttax048	TC	3	M13-TCATATCTTCTGTATTTCGC	PIG-ATCATTCATGTCCTAAGAGC	0.012
	Ttax002	ATCT		M13-GTTAGATCTATCTAAGGGCC	PIG-ATATATCTTTCTAGTCGGGC	0.006
35-Pet	Ttax060	AT	4	M13-CATGAGATTGAAAGTTTAGG	PIG-AAGTAAATTAATTAGCCAGC	0.018
	Ttax026	ATT	2	M13-GTTCTGTAAATTTGTTGTCC	PIG-CAAACTACACAGACAAAACC	0.018
	Ttax013	TCC	2	M13-TATTACACCTGTCAAATTCG	PIG-TGTTAACAAAGTCCTTATGC	0.006
36-Fam	Ttax070	AT	3	M13-TCTAAAGAAGAGGAATGAGG	PIG-GATTCGTAAGTGAAAGGC	0.012
	Ttax067	AT	2	M13-CTCTGTACCATATTTTGTCG	PIG-CCTTATACAACAAAACAAGG	0.012
	Ttax005	ATT	4	M13-ACATCTCATTCTCATTCTCC	PIG-TTGTTCAATAATCTTTCCC	0.018

[1] SSR = simple sequence repeat.

genotype accumulation curve (genotype_curve), private alleles (private_alleles), probability of encountering a given genotype more than once by chance, (psex), nongametic phase linkage disequilibrium (ia), analysis of molecular variance (AMOVA) (poppr.amova), K-means hierarchical clustering (find.clusters), discriminant analysis of principal components (DAPC) with cross validation (xvalDapc), and minimum spanning network using Bruvo distance (bruvo.msn) (Grünwald *et al.* 2017).

Ex situ Data Set. The genetic structure of an *ex situ* population, maintained by ABG, was described by including all of the mature individuals, including seven females, two males, and six open-pollinated seedlings from each of the five female trees (Fig. 2). Data for one seedling were discarded due to poor amplification, and the two females without corresponding seedlings were excluded from further analyses, resulting in an analysis of 36 individuals in total. The probability of a seedling inheriting the same alleles as its mother, given the genotypes of the mother and two possible fathers, was calculated using parental allele frequencies and assuming unlinked loci.

Results. *In situ* Data Set. Two of the loci did not follow predicted Mendelian inheritance patterns in the *ex situ* population and were excluded, leaving 12 loci in the analyses (Table 1). The genotype accumulation curve reached a plateau at nine microsatellite loci with all 12 loci allowing for identification of all 44 individuals. The loci had two to four alleles per locus (mean 2.8) with allele evenness ranging from 0.35 to 0.92 (mean 0.73) (Table 2). The north and middle populations each contained one private and 28 total alleles, with the south having four private and 31 total alleles. The Simpson's index and Nei's gene diversity (expected heterozygosity) were 0.933, 0.929, 0.933, and 0.380, 0.360, 0.373, for the north, middle and south populations, respectively. The probability of encountering a given genotype more than once by chance was 3.800^{-8} to 1.340^{-2}, with a mean of 1.605^{-3}, or about 1 in 1,600. Nongametic phase linkage disequilibrium (LD) among loci was tested with $\bar{r}_d$ (minimum −0.11 Ttax026:Ttax048, maximum 0.21 Ttax076:Ttax006, mean 0.01) and some LD was found, $P = 0.016$.

The AMOVA found significant ($P < 0.01$) diversity at all levels tested (between populations, between samples within populations, and

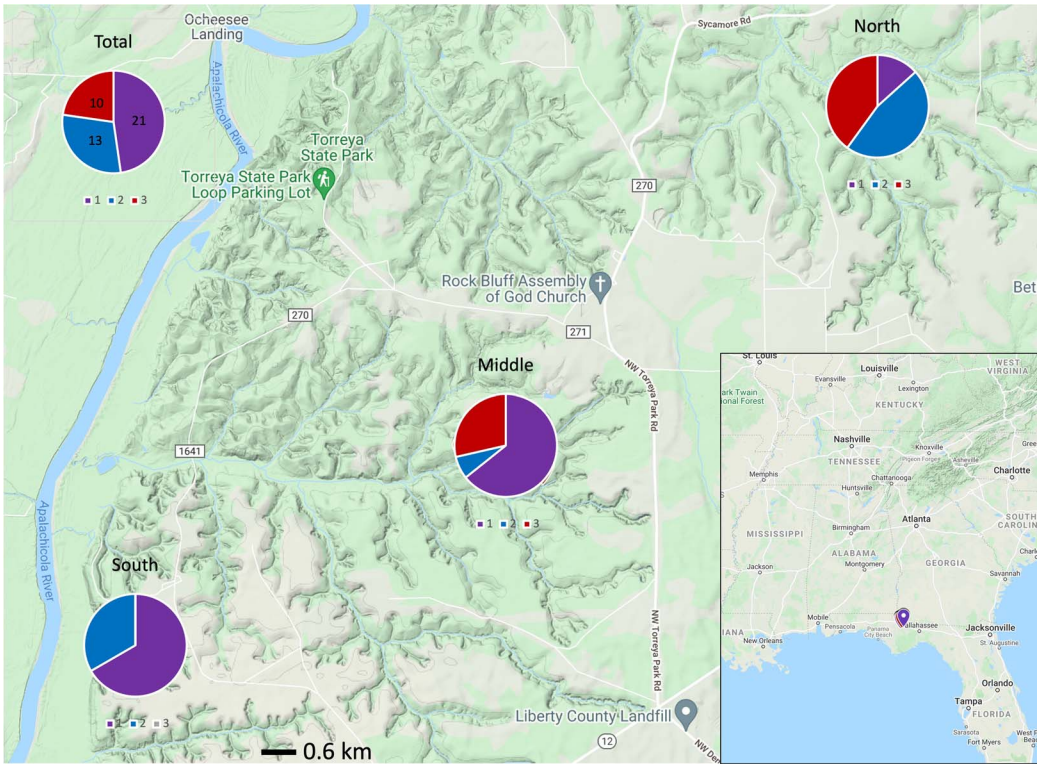

FIG. 1. Approximate location of the three *in situ Torreya taxifolia* populations sampled. Coloration of the charts represent K-means hierarchical cluster assignment (K = 3) of the 44 individuals. The lower right insert gives the location of the study site in the Florida Gulf Coast.

within samples) (Table 3). Several approaches to visualize the genetic structure were used including K-means hierarchical clustering (K = 3 in Fig. 1), minimum spanning network using Bruvo distance (Bruvo *et al.* 2004) (not shown), and DAPC (K = 2–5 in Fig. 3B). The optimal number of K-means clusters using BIC varied from two to five depending on the methods used (criterion = "diffNgroup" and "smoothNgoesup" respectively [Jombart and Ahmed 2011]) (Fig. 3A).

EX SITU DATA SET. The 12 loci had from one to three alleles per locus (mean: two), while the allele evenness ranged from 0.46 to 0.97 (mean 0.70) and eight loci were polymorphic. The genotype accumulation curve did not reach a plateau, allowing for the identification of 32 multilocus genotypes among the 36 individuals. Three pairs of siblings and one mother/seedling pair shared genotypes (Fig. 2). The probability of the seedling inheriting the same alleles as its mother, given the genotype of the mother and two possible fathers, was $P = 0.089$, or about 1 in 10.

Discussion. In the *in situ* population, the microsatellite panel enabled identification of each of the 44 individual samples with a 1 in 1,600 chance of obtaining the same genotype for a pair of trees by chance. Given the small population size, the marker panel should be adequate for future population studies and identifying individual accessions in multiple collections. We did not find evidence of current resprouts arising from the same clonal root system, as all samples were unique genotypes.

We identified structure at all levels sampled (between populations, between samples within populations, and within samples with 5, 11, and 83% of the variation explained, respectively) using the AMOVA. The south population had the largest number of private and total alleles compared to the other populations. The number of clusters found and cluster assignment (Fig. 1, 3) was not consistent between methods. However, the proportion of the clusters found in a population shifted in a general northeast to southwest direction (Fig. 1, 3), and this pattern was consistent at K = 3 to K = 5. The differentiation between populations along a

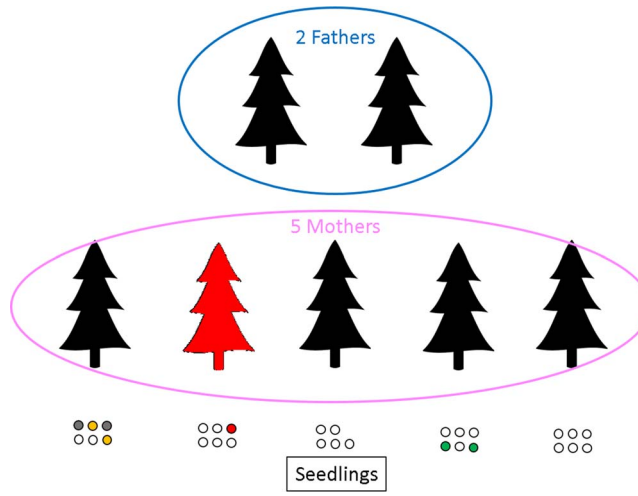

FIG. 2. Depiction of the *ex situ* population of *Torreya taxifolia* along with multilocus genotype assignment. Seedling and mother fill color represent genotypes that were recovered more than once and trees/seedlings are arranged by sample number, left to right. Note that one mother and seedling shared the same genotype (red fill), suggesting apomixis cannot be ruled out, and three other pairs of siblings had the same genotype.

northeast to southwest gradient can also be seen in the minimum spanning network (data not shown), where samples from the north population tend to be in the top portion of the network, the middle population toward the center, and the south population in the lower portion of the network. Latitude and proximity to the river might explain the gradient we recovered. Our sampled populations do not let us investigate this gradient as they covary (Fig. 1). Future work should sample additional populations and larger sample sizes within populations to examine if genetic structure is correlated with latitude or proximity to the river.

Table 2. Summary statistics for each locus.

Locus	Alleles	1-D[1]	Hexp[2]	Evenness	Hardy-Weinberg[3]
Ttax016	4	0.713	0.721	0.920	0.291
Ttax005	4	0.465	0.470	0.693	0.977
Ttax067	2	0.283	0.286	0.681	0.442
Ttax070	3	0.464	0.469	0.739	0.119
Ttax013	2	0.325	0.329	0.731	0.283
Ttax026	2	0.201	0.204	0.594	0.395
Ttax060	4	0.614	0.621	0.829	0.013
Ttax048	3	0.088	0.089	0.410	0.992
Ttax076	2	0.483	0.489	0.968	0.100
Ttax003	2	0.442	0.447	0.895	0.880
Ttax006	4	0.067	0.067	0.349	1.000
Ttax083	2	0.449	0.455	0.907	0.009

[1] Simpson index.
[2] Nei's 1978 gene diversity (expected heterozygosity).
[3] Probability of χ^2 test based on expected genotype frequences calculated from allelic frequencies.

In the *ex situ* population, we recovered 1 of 29 seedlings with the same genotype as its mother, supporting the hypothesis that *T. taxifolia* has the potential to reproduce via apomixis. The probability of this seedling obtaining the same alleles as its mother, given the genotypes of the two possible fathers, was about 1 in 10, although this calculation likely violates the assumption of no linkage among loci. Given these results, we cannot confirm or dismiss the occurrence of apomixis, although if apomixis does occur, it appears to be rare. A larger study to confirm linkage among loci, using a larger number of seedlings and more polymorphic loci, is needed to definitively determine if apomixis occurs. We found the samples T_taxifolia086 (Mother)/T_taxifolia087 (Father) and T_taxifolia069 (Mother)/T_taxifolia071(Father) are closely related, while T_taxifolia078 (Mother) and T_taxifolia094 (Mother) are less closely related. This suggests progeny from the *ex*

Table 3. Analysis of molecular variance for 44 individuals in the three *Torreya taxifolia in situ* populations.

	d.f.	Sum of squares	Mean square	*P* value
Between pop	2	25.51	12.75	0.001
Between samples within pop	41	204.79	4.99	0.009
Within samples	44	174	3.95	0.001
Total	87	404.3	4.65	

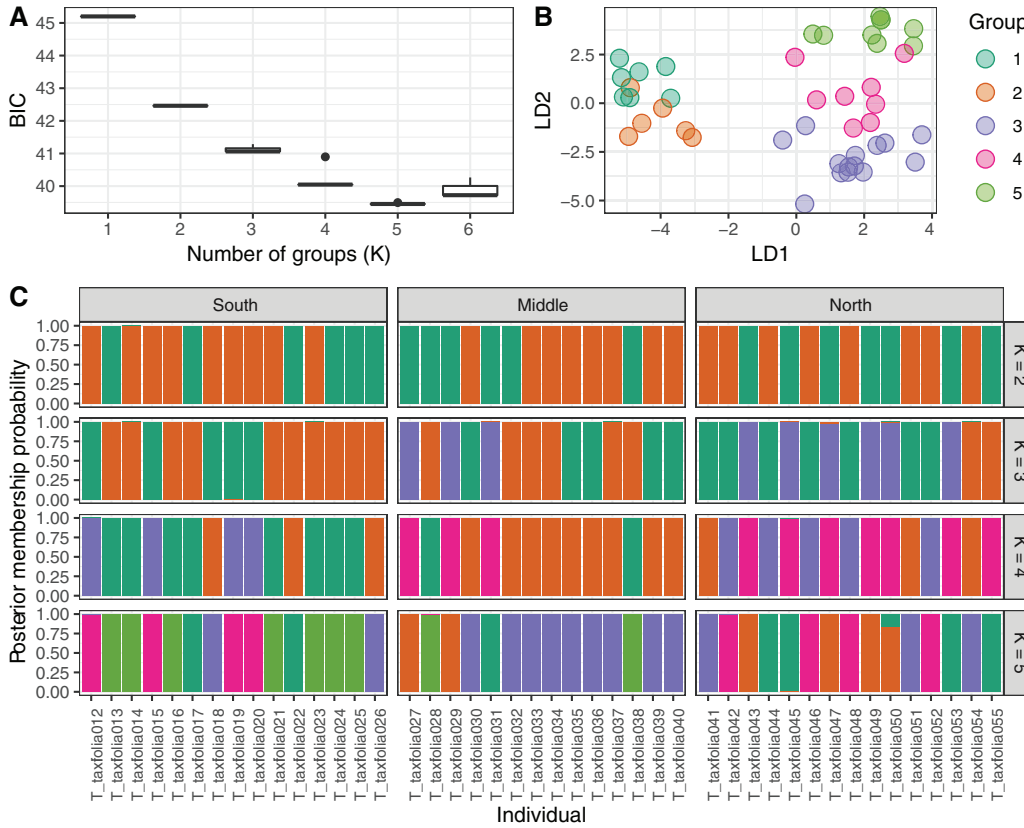

FIG. 3. Plots showing the number of clusters found in the *in situ* population of *Torreya taxifolia* and the assignment of each sampled tree. (A) Bayesian Information Criterion (BIC) values for K-means clustering with two to five optimal groups, depending on selection criteria; (B) Discriminant Analysis of Principal Components (DAPC) plot showing the relationship between groups; (C) bar plots giving the probability of each sample's assignment to each group at K = 2–5.

situ collection used in this study might be of unequal conservation value and thus best used in different settings as they vary in relatedness, and genetic paternal testing should be used to maximize conservation efforts.

Conclusions. The microsatellite panel developed here allows for the amplification of 12 loci in five multiplex PCRs and subsequently analyzed in a single sequencing lane. This strategy reduces costs for genotyping individuals in *in situ* and *ex situ* collections and confirming their identities within and between collections. Structure in the *in situ* population was found at all levels sampled and tested, implying that the best way to maintain the species' genetic diversity is to preserve the individuals from as many populations as possible rather than focusing on specific locations or populations. The question of whether apomixis occurs is inconclusive, as we could not exclude it using the data (as one seedling was the same genotype as its mother tree). Progeny from this *ex situ* collection might be of unequal conservation value because of relatedness of the parents, and genetic testing is recommended before they are used in conservation efforts.

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