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Genetic analysis of the threatened American hart's-tongue fern (*Asplenium scolopendrium* var. *americanum* [Fernald] Kartesz and Gandhi): Insights into its mating system and implications for conservation

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ABSTRACT

American hart's-tongue fern (AHTF) is one of the rarest ferns in the United States and concern over its conservation and management has highlighted the need for genetic analysis. Genetic analysis also provides insights into the species' mating system which contributes to our understanding of its rarity and persistence. We analyzed 88 individuals from 11 populations in NY and MI based on variations in 108 loci as revealed through ISSR markers using Nei's gene diversity index, percent polymorphic loci and other measures. Low genetic diversity predominates in the populations from NY, and even lower for the populations in MI. Our results also indicate that AHTF from NY and MI are genetically differentiated from each other, as well as the populations within them. There is no positive correlation between genetic and geographic distances, as well as between genetic distance and census population size. The significantly high among population genetic variation and low gene flow value are common indicators of a predominant inbreeding mating strategy within populations, limited spore dispersal, and genetic drift. Our results also indicate that each AHTF population is an important contributor to the overall genetic variation of the species and thus, represents a significant unit for conservation efforts.

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1. Introduction

The American hart's tongue fern (*Asplenium scolopendrium* var. *americanum*, AHTF) is federally-listed as Threatened under the United States Endangered Species Act of 1973 (USFWS, 1993, 2012). This evergreen, perennial fern occurs disjunctly being restricted to a few localized populations in northern Michigan and central New York, and is even more restricted and has fewer populations in subcentral Tennessee and northern Alabama (USFWS, 1993, 2012). Censuses have been done for many of the populations in the United States, particularly those in central New York where a long-term census data set (1916–2012) is available (Cinquemani et al., 1988; Brumbelow, 2014). Although many of the populations have recovered following several dramatic census fluctuations, some of the populations in each of the four states are currently in serious decline or have been extirpated (USFWS, 2012). Most of the AHTF populations in North America occur in southwestern Ontario (Canada), which

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also has reports of populations that are now locally extirpated (Austen, 2000). Nevertheless, it is listed only as a species of special concern under the federal Species at Risk Act and Ontario's Endangered Species Act of 2007 (Environment Canada, 2013). AHTF also occurs in northern Mexico but there is no information on census population size and trends (CONABIO, 2013). The closely related European hart's-tongue fern (*A. scolopendrium* var. *scolopendrium*) is widespread throughout northern Europe and the British Isles.

Concern over the conservation and management of rare species has highlighted the importance of genetic analysis. Genetic diversity is the raw material that ensures long-term survival and adaptation of a species particularly under changing environments. The genetic structure of populations is typically a reflection of many different biological processes and their interactions including mutation, genetic drift, mating system, gene flow and selection (Soltis and Soltis, 1990; Ellstrand and Elam, 1993; Schneller, 1996). Genetic diversity is one of the three forms of biodiversity recognized by the World Conservation Union as deserving of conservation. Information on genetic diversity and variation within and among populations may also provide insights on the underlying mechanisms of rarity, as well as the development of species-specific conservation and management programs. In spite of the threatened status of the AHTF, there is no published report on the genetic diversity of any of the populations in North America. Among the various molecular tools for genetic analysis, the PCR-based Inter-Simple Sequence Repeat (ISSR) markers have been widely applied in the conservation genetics of rare ferns and other rare plants (Table 1). The ISSR approach has been adapted in our study because it involves minimal protocol development, cost-effectiveness and detection of high number of genetic polymorphisms (Zietkiewicz et al., 1994; Wolfe et al., 1998). In addition, ISSR has the added advantage of rendering the data for other analysis including genotyping, parentage analysis, phylogenetics, systematics and biogeography (Gunnarsson et al., 2005; Pohjamo et al., 2008; Vicente et al., 2011).

The goal of this study is to obtain insights on the genetic diversity and structure of 11 AHTF populations from eastern United States. This information is necessary in our understanding of the predominant mating system of AHTF which will provide insights on its rarity and persistence, as well as the development of conservation and management strategies for its long-term persistence. Specifically, this study aims to: 1) determine the levels of genetic diversity within populations; 2) determine the degree of genetic differentiation among populations; and 3) correlate the levels of genetic diversity with geographic locations and census population sizes.

Table 1
Genetic diversity estimates of rare ferns and other plants that used ISSR markers.

Species	No. of individuals examined	No. of primers used	PPL	Nei's genetic diversity	Source
A. Rare ferns including a lycophyte and a moss					
AHTF	88	10	81	0.18	This Study
Botrychium pumicola	99	6	52	0.14[a]	Camacho and Liston 2001
Ceratopteris pteridoides	72	13	45	0.14	Dong et al., 2007
Ceratopteris thalictroides	138	12	65	0.15	Dong et al., 2008
Isoetes hypsophila	56	12	82	0.22	Chen et al., 2005
Sphagnum angermanicum	128	5	84	0.07	Gunnarsson et al., 2005
B. Rare gymnosperms and angiosperms					
Aster pyrenaeus (Asteraceae)	290	6	85	0.21	Escaravage et al., 2011
Astragalus nitidiflorus (Leguminosae)	75	8	51	0.17	Vicente et al., 2011
Changium smyrnioides (Apiaceae)	84	18	85	0.24	Qiu et al., 2004
Cycas guizhouensis (Cycadaceae)	215	11	36	0.11	Xiao et al., 2004
Dimorphandra wilsonii (Leguminosae)	21	12	40	0.12	Viana e Souza and Lovato 2010
Physaria bellii (Brassicaceae)	300	3	63	0.22	Kothera et al., 2007
Thuja sutchuenensis (Cupressaceae)	139	15	76	0.16	Liu et al., 2013

[a] Averaged from multiple populations.

2. Materials and methods

2.1. Study sites and sample collection

Eighty eight American hart's-tongue ferns (AHTF) from 11 populations in eastern United States were examined including eight populations from New York and three populations from Michigan (Table 2). The approximate geographical locations and relative geographic distances of the 11 populations are shown in Fig. 1. Since AHTF is a federally protected species, official permits were obtained from the appropriate state agencies prior to sample collections. The specific identities of the study sites cannot be revealed so as to protect the populations from unwarranted collections and so only symbols representing the populations will be used in this report. Only four (LB, LR, GC and SB) of the six populations of AHTF from Clark Reservation State Park (Onondaga County, NY) were included in this study because our preliminary genetic analysis indicated that individuals from DR and GC exhibited the same banding patterns and are very close in location to each other. We choose GC because of its larger population size. GL and most populations outside of Clark Reservation State Park were not included in this study because of their very small census population sizes. All three populations (FC, HG and CT) in Chittenango Falls State

Table 2
Populations (arranged according to levels of genetic diversity), census population sizes, and genotypes of *Asplenium scolopendrium* var. *americanum* from eastern United States.

Populations	Locations	County	State	Pop size[a]	No. of AHTF with unique genotype (n = 88[b])	No. of AHTF with shared genotype/No. of AHTF sharing the genotype	Total no. of genotype (n = 8)
SB	Clark Reservation State Park	Onondaga	NY	1191	8	0	8
LB	Clark Reservation State Park	Onondaga	NY	428	8	0	8
LR	Clark Reservation State Park	Onondaga	NY	138	5	1/3	6
GC	Clark Reservation State park	Onondaga	NY	1797	5	1/3	6
SR	Split Rock Unique Area	Onondaga	NY	152	6	1/2	7
FC	Chittenango Falls State Park	Madison	NY	107	8	0	8
HG	Chittenango Falls State Park	Madison	NY	159	6	1/2	7
CT	Chittenango Falls State Park	Madison	NY	46	6	1/2	7
TC	Hiawatha National Forest	Mackinac	MI	284	1	1/7	2
SE	Hiawatha National Forest	Mackinac	MI	283	1	1/7	2
SC	Hiawatha National Forest	Mackinac	MI	196	0	8	1

[a] Census population size from central New York and northern Michigan are based from Brumbelow (2014) and USFWS 2012, respectively.
[b] Fronds from NY and MI used in DNA isolation were collected in June 2010 and August 2013, respectively.

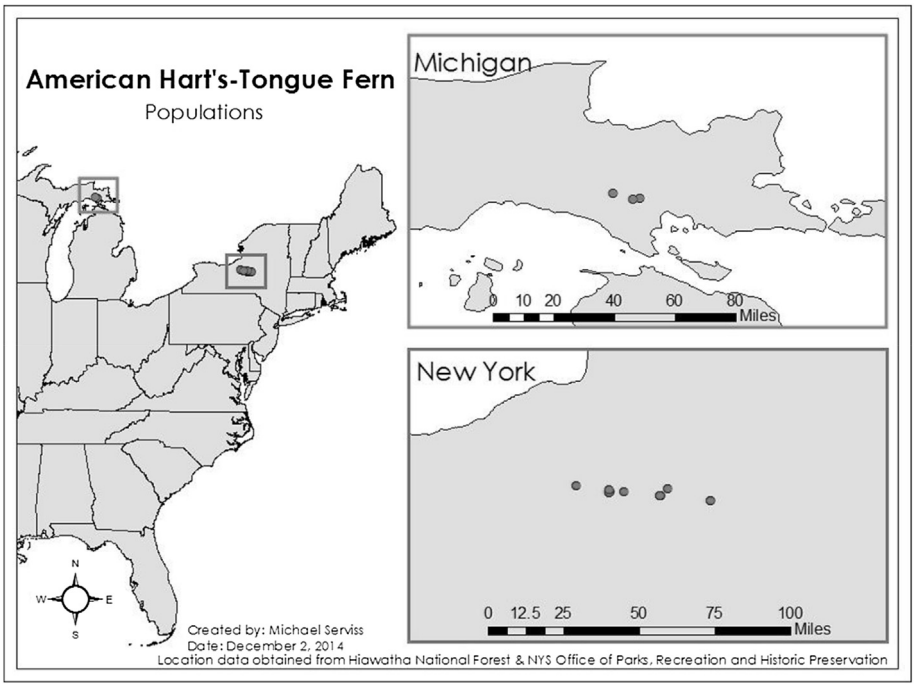

Fig. 1. Map showing the approximate location of the populations of *Asplenium scolopendrium* var. *americanum* in eastern United States.

Park (Madison County, NY) and the sole population in Split Rock Unique Area (Onondaga County, NY) were included in this study. Only three populations (TC, SE and SC) from the Hiawatha National Forest (Mackinac County, MI) were sampled also because of their relatively larger census population sizes.

Eight immature fronds were collected per population (Table 2 and Fig. 1). One frond per individual was collected at an approximately 1 m distance to the next individual along a line transect. Fronds were collected only from individuals that were relatively vigorous (i.e., those that have formed at least five new fronds at time of collection) so as not to cause drastic damage

to the plants that were less vigorous (i.e., had fewer than five new fronds). The sampling system used in this study allowed a general representation of the entire population and avoided collection of closely spaced individuals that might be genetically similar. This system of sampling also allowed correlation of the spatial distribution of the individuals and their respective genotypes to obtain an insight on the extent of genetically similar individuals in a population.

2.2. Inter-Simple Sequence Repeat (ISSR)

The ISSR primers used in this study were based on those designed from the University of British Columbia, Vancouver, Canada (UBC Primer Set 9, 2006). Out of the 36 ISSR primers that were initially screened, 10 produced clear and consistent banding patterns which were then used in this study (Table 3). Genomic DNA was extracted from fresh immature AHTF fronds using DNeasy Plant Mini kit (Qiagen Inc., Carlsbad, CA). PCR was carried out using a MasterCycler Gradient thermocycler (Eppendorf Inc., Hauppauge, NY). ISSR amplifications were conducted in a volume of 10 μL containing 5 μL HotStarTaq MasterMix (Qiagen Inc., Carlsbad, CA), 1 ng template DNA, 0.1 μM primer and PCR grade water, following the PCR conditions and agarose gel electrophoresis optimized by Discenza (2011; Table 3). To assess the reproducibility of the banding patterns for each primer, PCR reactions were repeated at least twice for all 88 samples and only bands that did not vary between dilutions were considered in the analysis. Negative controls (no template DNA) were included in all PCR runs to test for possible DNA contamination. DNA fragment sizes were determined using LabWorks software version 4.0 (UVP Inc., Upland, CA) based on a 1 Kb Plus DNA Ladder (Life Technologies, Grand Island, NY). The 10 primers produced multiband patterns in each of the samples, no band variation was detected in the duplicate runs, and no band was detected in any of the negative controls. Loci were named according to their fragment sizes (molecular weight).

The banding patterns of all 88 individuals against all 10 primers were manually examined and compared. A data matrix was constructed with the information on the presence (1) or absence (0) of bands. Although ISSR bands of the same size may represent two different loci, this interpretation also implies the presence of more polymorphisms and thus higher estimate of genetic diversity. It is also possible that co-migrating bands may represent the same locus and this interpretation results in a conservative estimate of genetic diversity, which is the approached used in this study. The number of individuals and primers used in our study represent close to average of what has been used in previous studies that used ISSR markers (Table 1).

2.3. Genetic diversity

The binary matrix was analyzed under Hardy–Weinberg Equilibrium using POPGENE software version 1.32 (Yeh et al., 1997) to determine several measures of genetic diversity including percentage of polymorphic loci (PPL), Nei's (1973) gene diversity (h) using corrected allele frequency (Lynch and Milligan, 1994), and Shannon information index (I) (Lewontin, 1972). These genetic diversity measures were analyzed at various hierarchical levels including population, groups, region and species (Table 4A–E). Population level analysis generated genetic diversity measures for each of the 11 populations (Table 4A). Two types of group level analyses were done: 1) The four-group level analysis was composed of Clark Reservation (CR) (including the four populations under study in Clark Reservation State Park), Chittenango Falls (CF) (including all three populations from Chittenango Falls State Park), Split Rock (SR) (the sole population at Split Rock Unique Area) and Hiawatha (HW) (including the three populations from Hiawatha National Forest) (Table 4B); 2) The five-group level analysis (Table 4C) was done since the SB population showed a very different level of genetic diversity as compared to any of the populations in Clark Reservation State Park based on our preliminary results and the present study. Therefore, SB was designated as a distinct group to determine how it separately affected the level of genetic diversity and distribution of genetic variation of AHTFs at Clark Reservation State Park. CR was used as one of the group designations to represent the other three (LB, LR and GC) populations from Clark Reservation State Park. The other three groups were designated as in the four-group level analysis (i.e., CF, SR and HW). The region level analysis generated two sets of genetic diversity measures, one for NY and another for MI (Table 4D). The species level analysis included all individuals from all populations resulting in a single value for each of the genetic diversity measures (Table 4E).

Table 3
Features of the ISSR primers used in the analysis of genetic diversity of *Asplenium scolopendrium* var. *americanum* from eastern United States.

Primer	Sequence (5′–3′)	Annealing Temp (°C)	No. of bands	Size range of bands (bp)
ISSR-807	$(AG)_8TG$	56.0	9	500–1450
ISSR-815	$(CA)_6GC$	57.5	10	300–1300
ISSR-816	$(CA)_6TC$	57.5	14	175–1400
ISSR-818	$(CA_)_6GG$	57.5	14	250–1500
ISSR-835	$(AG)_8CC$	56.0	8	275–1000
ISSR-844	$(CT)_6GC$	56.0	8	375–1300
ISSR-845	$(CT)_8GG$	58.2	12	350–1100
ISSR-847	$(CA)_6CC$	59.0	12	325–1400
ISSR-850	$(GT)_8CC$	56.0	12	190–1250
ISSR-851	$(GT)_8CG$	59.0	9	480–1550

Table 4
Genetic diversity statistics generated using POPGENE 1.32 for *Asplenium scolopendrium* var. *americanum* in eastern United States under various hierarchical levels (A – Eleven Populations, B – Four Groups, C – Five Groups, D – Two Regions, and E – Species) (PPL = percent polymorphic loci; *h* = Nei's gene diversity; *I* = Shannon's information index; *Gst* = Genetic differentiation, *Nm* = Gene flow).

	Number of polymorphic loci (n = 108)	PPL	*h* (mean)	*h* (SD)	*I* (mean)	*I* (SD)	*Gst*	*Nm*
A. Population								
SB	65	60	0.251	0.214	0.364	0.306		
LB	13	12	0.051	0.145	0.073	0.204		
LR	10	9	0.039	0.128	0.056	0.181		
GC	6	6	0.023	0.101	0.033	0.142		
SR	6	6	0.024	0.103	0.034	0.145		
FC	14	13	0.058	0.152	0.082	0.216		
HG	10	9	0.042	0.133	0.059	0.188		
CT	10	9	0.032	0.109	0.048	0.159		
TC	2	2	0.005	0.045	0.008	0.066		
SE	1	1	0.001	0.012	0.002	0.023		
SC	0	0	0	0	0	0		
B. Group (4)								
CR	79	73	0.219	0.152	0.346	0.227		
SR	6	6	0.024	0.103	0.034	0.145		
CF	19	18	0.060	0.138	0.091	0.205		
HW	14	13	0.002	0.018	0.005	0.043		
C. Group (5)								
SB	65	60	0.251	0.214	0.364	0.306		
CRX	26	24	0.071	0.136	0.112	0.208		
SR	6	6	0.024	0.103	0.034	0.145		
CF	19	18	0.060	0.138	0.091	0.205		
HW	14	13	0.002	0.019	0.005	0.043		
D. Region								
NY	80	74	0.153	0.129	0.261	0.193	0.577	0.366
MI	14	13	0.002	0.018	0.005	0.043	0.958	0.022
E. Species								
AHTF	87	81	0.180	0.154	0.295	0.216	0.735	0.181

Pairwise comparison of Nei's (1978) genetic distance and similarity for each of the 11 populations were also generated using POPGENE 1.32. To visualize the differences among the populations, consensus dendrograms using Unweighted Pair Group Method with an Arithmetic Average (UPGMA) were generated using POPGENE 1.32 based on genetic distance (Fig. 2). A dendrogram showing all of the 88 AHTF individuals was also generated but not shown because its large size made the labels for individuals difficult to read. The dendrograms were viewed using TreeView X program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (Page, 1996). Figures on the nodes of the dendrograms represent percentage bootstrap support values based on 1000 replicates. Branch lengths in the dendrogram correspond to the scaled genetic distances.

Mantel test (Mantel, 1967) was used to determine the correlation between genetic and geographic distances among populations. Values for Mantel test were generated using ARLEQUIN 3.5 (Excoffier and Lischer, 2010). Significant differences were tested using 10,000 permutations. The correlation between census population size and Nei's (1973) gene diversity was examined by correlation analysis using the Pearson Correlation test in SAS 9.4 (SAS Institute, Cary, NC). Population size was based on the most recent census counts of AHTFs (Table 2).

2.4. Population structure

To examine how genetic variation was distributed among and within populations, groups and regions, the non-parametric Analysis of Molecular Variance (AMOVA) was performed based on squared Euclidean distances using ARLEQUIN software version 3.5 (Excoffier and Lischer, 2010). A two-level AMOVA was performed to determine the proportion of genetic variation among and within populations (Table 5A). Two three-level AMOVAs were also performed to determine the distribution of genetic variation when populations were grouped into four and five (Table 5B–C). In both groups, AMOVA values were determined for among groups, among populations/group, and within populations (Table 5B–C). Another three-level AMOVA was performed to determine genetic variation between the two regions (NY and MI), among populations/region, and within populations (Table 5D). Statistical significance of the proportion of variance associated with the fixation index (*F*st) and its *p* value were also determined using ARLEQUIN 3.5. All the above analyses were performed with 10,000 permutations.

The coefficient of genetic differentiation (*G*st) is an analogue of *F*st and both assume Hardy–Weinberg equilibrium, but they are calculated differently. The statistical significance of the proportion of variance associated with *G*st was calculated by POPGENE 1.32 (Yeh et al., 1997) based on the null distribution of alleles. Based on the *G*st value, POPGENE 1.32 generated *Nm*, which is the number of migrants successfully entering a population per generation. *G*st and *Nm* values were determined only at the region and species levels since group level analyses were not possible because of uneven number of populations. Since ISSR loci do not usually provide a true measure of heterozygosity due to their generally dominant inheritance patterns, the

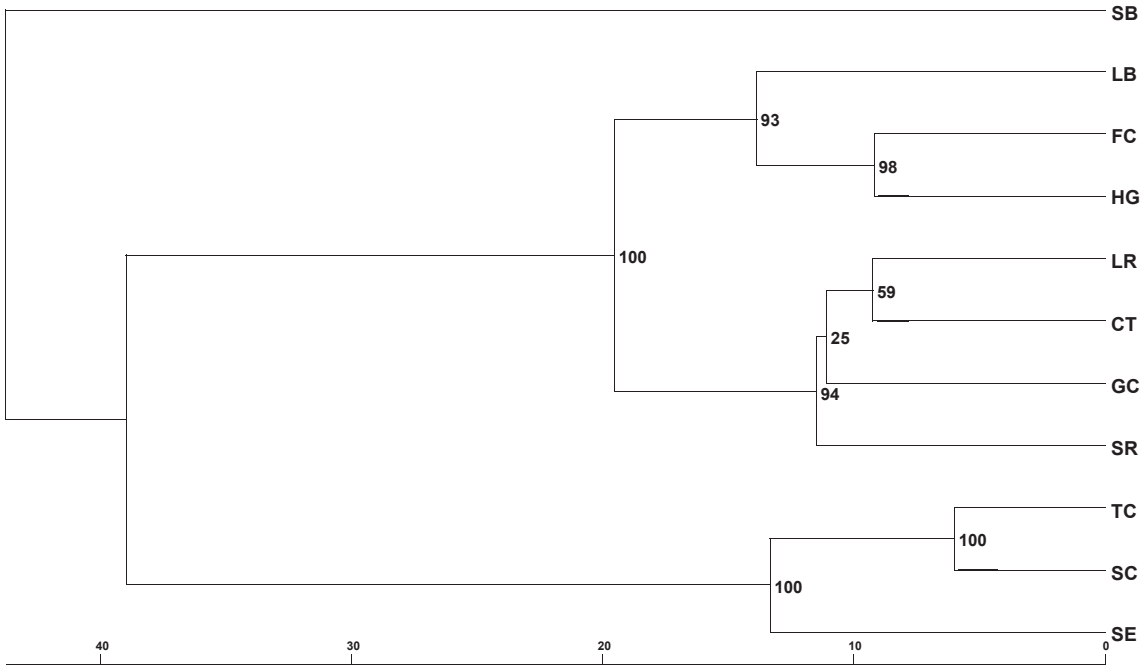

Fig. 2. UPGMA Dendrogram showing the relationships between populations and groups based on Nei's genetic distance. The scale indicates Pearson coefficient values. Numbers on nodes are the percentage bootstrap support values.

Table 5
AMOVA values for *Asplenium scolopendrium* var. *americanum* in eastern United States under various hierarchical levels (A – D).

Source of variation	d.f.	Sum of squares	Variance components	% variation	*Fst*	*p*
A. Populations						
Among populations	10	533.11	6.35 Va	71	0.715	0.001
Within populations	77	195.13	2.53 Vb	29		
Total	87	728.24	8.88	100		
B. Groups (4)						
Among groups	3	320.73	3.67 Va	38	0.738	0.008
Among populations/group	7	212.39	3.48 Vb	36		
Within populations	77	195.13	2.53 Vc	26		
Total	87	728.24	9.68	100		
C. Group (5)						
Among groups	4	429.11	5.38 Va	55	0.740	0.001
Among populations/group	6	104.00	1.85 Vb	19		
Within populations	77	195.24	2.53 Vc	26		
Total	87	728.24	9.76	100		
D. Regions						
Among regions	1	278.41	7.17 Va	55	0.804	0.006
Among populations/region	9	254.71	3.22 Vb	25		
Within populations	77	195.13	2.53 Vc	20		
Total	87	728.24	12.92	100		

values generated for *Nm* should be interpreted as indirect estimates of gene flow (Whitlock and McCauley, 1999; Camacho and Liston, 2001).

3. Results

3.1. Genetic diversity

A total of 108 loci were consistently resolved and these represented DNA fragments that ranged in size from 175 to 1550 bp (Table 3). Of the 108 loci, 87 were polymorphic and this represented 81% fragment polymorphism at the species level (Table 4). The level of polymorphisms within populations ranged from 0% to 60%. SB (60%) represented the population with the highest degree of polymorphism followed by FC (13%) and LB (12%). The three populations from Hiawatha National Forest had the lowest degree of polymorphisms (0–2%). When analyzed as a group of four, CR (73%) showed the highest level of

polymorphism, while SR (6%) had the lowest. When analyzed as a group of five, SB (60%) had the highest degree of polymorphism, while the CRX had 24% and SR had the lowest at 6%. At the region level, NY and MI exhibited 74% and 13% polymorphisms, respectively.

ISSR identified 67 different multilocus genotypes out of the 88 individuals that were examined, which represents 76% genotypic diversity (Table 2). These genotypes were composed of 60 unique multilocus genotypes (exhibited by only one individual out of the 88 that were examined) and 7 shared genotypes (exhibited by two or more individuals from all samples examined). The results indicate that 68% of the AHTFs had unique genotypes while 32% had shared genotypes. Three populations in NY (LB, SB and FC) had individuals that were all genotypically different from each other (Table 2). The other five populations in NY (GC, LR, HG, CT and SR) had two or three individuals that shared exactly the same genotypes (Table 2). The individuals sharing the same genotypes were relatively located close to each other (i.e., about a meter apart). Individuals from the three populations in MI were all (SC) or mostly (TC and SE) genotypically similar (Table 2). However, the genotypes in these three populations were different from each other. Genetic diversity was also analyzed in terms of the presence of a locus that is unique to a specific population, group, or region. No locus was unique to any one population or group from NY, but region level comparison revealed 17 loci were specific to NY. Not all individuals from NY, however, had these region specific loci.

Genetic diversity is considered as the probability that two individuals taken at random are genetically different. Using Nei's (1973) gene diversity (h) statistics, a value of 0 indicates that all individuals in a population are genetically the same while a value of 1 indicates that all individuals are genetically different (Table 4). SB showed the highest level of Nei's gene diversity (0.251), followed by FC (0.058) and LB (0.051). The GC (0.023) population had the lowest genetic diversity value among NY populations (Table 4). The three MI populations (TC, SE and SC) were the least diverse of all AHTF populations (Table 4). SC showed no genetic diversity among the eight individuals analyzed within it. Results based on Shannon's information index (I) were relatively higher in values than Nei's, but showed exactly the same trend (Table 4), and thus we will only refer to the values from the latter from hereon.

Pairwise comparisons of genetic distances and similarities were examined for all populations. Among the populations in NY, genetic distance was highest (0.426) and similarity was lowest (0.653) between GC and SB. AHTFs from SB exhibited the highest pairwise genetic distances and lowest genetic similarities when compared with any of the other nine populations. These data show that SB is the most genetically divergent population of those sampled from the eastern United States. The AHTFs from the three populations in MI exhibited high genetic distances and low genetic similarities in comparison to any of the populations in NY, indicating that the AHTFs from these two regions are genetically very different. The three most genetically similar populations were LR and CT (0.987), FC and HG (0.986), and LB and HG (0.983), all were from the two groups (Clark Reservation and Chittenango Falls) in NY. These populations were more genetically similar to each other (0.983–0.987) than the three MI populations were to each other (0.899–0.962). The three least genetically similar populations were SB and the three MI populations (TC, SE and SC) with genetic similarity values of 0.640, 0.649, and 0.651, respectively. The genetic similarities between SB and the rest of the populations from NY ranged from 0.653 to 0.684, indicating that AHTF from SB were more similar to those in NY than to those in MI.

The UPGMA dendrogram based at the population level using Nei's genetic distance showed clustering that is similar to the trend in the preceding paragraph. Most of the clusters in the dendrogram had high bootstrap support (93%–100%) indicating strong confidence level on the positions of the associated populations relative to each other (Fig. 2). However, the clustering of three populations (GC, LR and CT) had low bootstrap support (25%–59%) indicating low confidence level regarding their position relative to each other. Since populations from different geographical locations were grouped in the same clusters, this indicates lack of correlation between genetic and geographical distances. The results also indicate that SB and the three MI populations were the most genetically differentiated, followed by LB, FC, HG and SR.

Mantel test that included all 11 populations showed that there was a slight correlation between genetic and geographic distances ($r = 0.416$; $p > 0.05$). However, Mantel test that separately analyzed the populations from NY and MI indicated that there was no correlation between genetic distance and geographic distance for either NY ($r = 0.171$; $p = 0.60$) or MI ($r = 0.456$; $p = 0.67$). This indicates that the slight positive correlation between the combined genetic and geographic distances was due to the obviously large geographic distances between NY and MI. There was also no positive correlation between Nei's gene diversity and population size ($r = 0.3261$; $p = 0.3278$).

3.2. Population structure

Two-level AMOVA compared all of the 11 populations and showed that most (71%) of the genetic variation in AHTF was due to differences among populations and only 29% was due to differences within populations (Table 5A). AMOVA analysis also revealed that the level of genetic differentiation among and within populations was highly significant ($Fst = 0.715$; $p \leq 0.001$). This indicates that there is a very high genetic differentiation among populations as compared to the amount of genetic differentiation within populations. When the populations were grouped into four, three-way AMOVA showed that genetic variation among groups was 38%, among populations per group was 36%, and within populations was 26% (Table 5B). The level of genetic differentiation among groups, among populations per group, and within populations was moderately significant ($Fst = 0.738$; $p < 0.01$). When populations were grouped into five, three-way AMOVA showed that genetic variation among groups was 55%, among populations per group was 19%, and within populations was 26% (Table 5C). The level of

genetic differentiation among groups, among populations per group, and within populations was highly significant ($Fst = 0.740$; $p \leq 0.001$). Three-way AMOVA values for region analysis indicated that most (55%) of the genetic variation was due to differences between regions and only 25% was due to differences among populations per region, and 20% was due to differences within populations. AMOVA analysis also revealed that this level of genetic differentiation was moderately significant ($Fst = 0.804$; $p < 0.01$).

4. Discussion

4.1. Genetic diversity

Our estimate of the genetic diversity of American hart's-tongue fern (AHTF) at the species level is higher than most of the estimates in other rare ferns and other rare plants that used ISSR markers (Table 1) and together with its high percentage polymorphic loci (81%), our results indicate the presence of a relatively high genetic variation at the species level. However, comparison of estimates of genetic diversity at the regional level shows contrasting values for the AHTFs in NY ($h = 0.153$; PPL = 74) and MI ($h = 0.002$ and PPL = 13). These estimates indicate that most of the genetic diversity in AHTF at the species level is due to the populations in NY, while the populations in MI have extremely low levels of genetic diversity. Comparisons between populations show that SB from NY exhibits the highest genetic diversity ($h = 0.251$; PPL = 60) and thus responsible for most of the genetic variation of AHTF at the regional and species levels. Therefore, our analysis indicates that when the estimates of genetic diversity are based at the species level, AHTF may not appear to be at a critically low level as compared to other rare ferns and other rare plants (Table 1). However, comparison of genetic diversity at the region and population levels shows critically low values and identifies the population that is responsible for most of the genetic variations within the species. The population level genetic diversity is more informative, particularly in terms of prioritization for conservation and managements.

Except for SB, all the populations of AHTF examined in our study generally exhibit low levels of genetic diversity. Genetically depauperate populations usually have lower fitness and in combination with inbreeding and small population sizes, they have increased extinction probability particularly under changing environments (Reed and Frankham, 2003; Nybom, 2004). The low genetic diversity and almost uniform genotypes of the populations in MI are distressing since these conditions are not only found in one population but in all the three populations examined. GC is the most genetically depauperate population in NY but it has the largest census population size in the study (Table 2). The low levels of genetic diversity ($h = 0.023$; PPL = 6) and high rate of occurrence of individuals with the same genotypes in GC imply lower effective population size (i.e., the potential number of individuals that undergo cross-fertilization), and the same condition applies to all the other genetically depauperate populations of AHTF. Although several approaches have been used in determining the effective population size in various species, the result is that it is always smaller than census population size (Esselman et al., 1999; Charlesworth, 2009; Chung et al., 2012). Therefore, despite the large census population size of GC, it still requires conservation and management intervention similar to the populations with small census sizes because of lower effective population size. For conservation and management, the condition in GC indicates that rare plants are likely to be at a higher risk of extinction than is currently recognized, particularly if assessment is only based on census population size. On the other hand, the high census population size in GC may serve as a buffer against immediate threats and thus, help buy conservationists some time as they focus first on populations with smaller census sizes.

Pairwise comparisons of genetic distances and similarities and cluster analysis using UPGMA corroborate with the levels of genetic diversity that all 11 populations are genetically differentiated from each other, supporting their designation as populations. There is also strong genetic differentiation between regions, which is supported by the presence of several region-specific loci. Although the three MI populations have very low genetic diversity, the occurrence of several population-specific loci provides support to their genetic differentiation. In comparison, no population-specific locus was observed for NY in spite of their relatively higher genetic diversity. In addition to the slow growth rate of AHTF, the absence of population-specific loci may be due to exposure to generally similar environmental conditions and relatively recent origins of the populations (Ranker, 1994; Schneller, 1996; Chung et al., 2012). Therefore, genetic variation within and among the populations in NY appear to be mainly due to DNA-fragment frequency differences due to sexual reproduction and/or mutations rather than fixation of locally common loci.

UPGMA analysis shows four major clusters with SB as the most divergent followed by the cluster of the three MI populations, and then by two clusters that are each composed of NY populations from different geographic locations. The lack of clustering of the NY populations from the same geographical locations indicates that they are genetically differentiated from each other. Mantel test of isolation by distance that includes all 11 populations shows a weak but significant relationship between genetic and geographic distances ($r = 0.416$; $p = 0.05$). Since this result is most likely due to the large geographic distances between the NY and MI populations, it was necessary to perform separate Mantel tests for each of the two regions. The results indicate that there was no significant correlation between genetic and geographic distances for neither NY ($r = 0.171$; $p = 0.60$) nor MI ($r = 0.456$; $p = 0.67$), suggesting that difference in the levels of genetic diversity is due to factors other than geographic distance. The lack of isolation-by-distance pattern may be due to the random genetic drift that operated in these populations coupled with minimal spore exchange (Soltis and Soltis, 1990; Chung et al., 2012). There is also no significant correlation between levels of genetic diversity and census population size between and within regions, particularly since the two populations with the largest census sizes (GC and SB) have the most contrasting levels of genetic

diversity (Table 4). Therefore, the prediction by Ellstrand and Elam (1993) that low genetic diversity is tied with small population size is not fully supported by our study. The lack of correlation between census population size and levels of genetic diversity has also been reported in other plants (Pohjamo et al., 2008; Fatemi and Gross, 2009).

4.2. Population structure and mating system

Genetic variability in AHTF is mostly due to variations among populations rather than within populations and this trend is similar to when the populations are analyzed according to groups or regions. Considering that the genetic differentiations among populations, groups or regions are all significant, our results indicate the prevalence of inbreeding within AHTF populations. Inbreeding is a common feature associated with populations that are genetically structured (Soltis and Soltis, 1990; Nybom, 2004; Escaravage et al., 2011). However, since the populations from NY are composed mostly of genotypically different individuals, high rates of inter-gametophytic crossing must be occurring within these populations. On the other hand, the almost uniform genotypes of individuals from each of the three populations in MI indicate the prevalence of intra- and/or inter-gametophytic selfing in these populations. Based on the above, AHTF can be considered to be self-compatible and exhibit a mixed mating system where the gametophytes preferentially undergo inter-gametophytic crossing but in the absence of genetically different mates they undergo selfing. This mating system is similar to what occurs in its close relative, *A. scolopendrium* var. *scolopendrium* (Wubs et al., 2010) and other ferns (Soltis and Soltis, 1990; Ranker, 1992; Ranker et al., 2000). In spite of the occurrence of inter-gametophytic crossing within most of the populations of ATHF, many of the individuals within these populations are genetically closely related and thus inbreeding still occurs through mating between closely related individuals. In addition to selfing, mating between genetically related individuals affects the level and organization of genetic variation through reduction in the effective population size and associated inbreeding and thus, lower fitness of the offspring (Reed and Frankham, 2003; Nybom, 2004). On the other hand, inter-gametophytic crossing in SB with its high level of genetic diversity and heterogenous genotypic composition is unlikely to result in inbreeding depression. This population is also likely to produce more fit offspring, which may help explain the at least 2200% increase in its census population size from 50 individuals in 1920 (Faust, 1960) to 1191 individuals in 2012 (Brumbelow, 2014). SB represents the highest percentage increase from among the populations examined including GC which has 226 individuals in 1920 (Hunter, 1922) and 1797 in 2012 (Brumbelow, 2014) which represents only about 700% increase. Low genetic diversity and high rate of shared genotypes in GC will likely produce less fit progenies, but why this population has been and still with the largest census size is not clear.

The significantly high among population genetic variation and lack of genotype that is shared between any of the AHTF populations, groups or regions also indicate the lack of or limited gene flow, which corroborates with the low value obtained for gene flow ($Nm = 0.181$) in AHTF. According to Slatkin (1987), a gene flow value of $Nm = 0.167$, as an example, is too low to prevent further differentiation through genetic drift. Also, although fern spores are capable of being dispersed over long distances because of their small size, lightweight and abundance (Soltis and Soltis, 1990; Puentha, 1991), there are many reports on limited gene flow or short-distance spore dispersal in ferns (Richard et al., 2000; Murakami et al., 2005; Wild and Gagnon, 2005; Flinn, 2007; Tájek et al., 2011; Flinn et al., 2014). In fact, some of the reports indicate that spores land within several meters of the parent plant (Peck et al., 1990; Penrod and McCormick, 1996). Studies using genetic markers to assess genetic differentiation of fern populations also show that gene flow between populations on a scale of several to hundreds of kilometers is limited (Holderegger and Schneller, 1994; Stapulionyte et al., 2006; Tájek et al., 2011). Spore release usually requires dry conditions and the spores must be caught by passing airflows to be transported far from the parent plant (Wolf et al., 2001). Extreme long-distance dispersal, for example over 1000 km, requires access of the spores to higher atmospheric winds (Puentha, 1991; Wolf et al., 2001). Long-distance spore dispersal is more likely to occur in taller and epiphytic ferns and those growing in more open habitats than it is for low-growing, terrestrial species under a forest canopy (Peck et al., 1990; Wolf et al., 2001) like AHTF. Also, AHTF commonly grows on steep slopes of glacial plunge basins and ravines and surrounded by thick herbaceous, shrub and tree covers, a type of habitat that generally maintains high humidity and is well protected from strong desiccating wind (Cinquemani-Kuehn and Leopold, 1993). Therefore, although AHTFs are long-lived perennials and produce multiple fertile fronds per year, the vast majority of their spores likely fall in close proximity to their parent plants so that the actual proportion of spores dispersed outside their immediate vicinity is likely very low. Interestingly, Maxon (1900) and Hunter (1922) have already reported that a characteristic of the occurrence of AHTF is the formation of distinct groups or patches with no scattered individuals in between. After more than 100 years, such isolated, colony-like growth pattern of AHTF populations still persist today providing further support to the predominance of local spore dispersal. No new population of AHTF has been discovered in NY for almost a century.

The likelihood that a dispersed spore of AHTF establishes a colony outside of its preferred habitat is also unlikely since it requires a condition that maintains moisture and other factors for spore germination and the rest of the succeeding reproductive stages including the formation of sexually mature gametophytes, fertilization, and growth of the sporelings. According to Wolf et al. (2001), these developmental stages are the hurdles that ferns typically go through in their life history. However, AHTF appears to be more sensitive than most ferns based on its more specific microclimate requirements. In general, mortality of the resulting gametophytes and sporelings may also occur because of soil and substrate erosion particularly in steep slopes where AHTF prefers to grow, which are factors that are not discriminatory with regard to genotype. Therefore, the high mortality rates of gametophytes and sporelings further reduces effective spore dispersal (Peck et al., 1990; Wolf et al., 2001).

Considering the high genetic differentiation among AHTF populations, conservation of one population will be insufficient to preserve all of the genetic variations of the species. Thus, it is necessary to conserve all of the populations of AHTF. Results from our genetic analysis can be used in the prioritization of populations and so on a purely genetics point of view, SB is most likely the population that is able to withstand severe reduction in census population size and still have the highest levels of genetic variability. Therefore, a management recommendation is to focus first on the reinforcements of genetically depauperate populations, particularly those that have small census population sizes because of the higher possibility of losing genetic variants. These populations should be augmented using transplants (e.g., derived from spore culture) from neighboring populations that have higher levels of genetic diversity to allow the capture of a wider array of genotypes that can potentially succeed in the target locations and increase reproductive success and fitness, and thus facilitate natural recruitment.

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