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Population genetic structure in *Phyla scaberrima* from Mexico and Colombia assessed by AFLP markers and implications for conservation

L.G. Androcioli[1]*, E.A. Ruas[1]*, L.A. Rodrigues[2], C.F. Ruas[1] , H.E.R. Perilla[2] and P.M. Ruas[1]

[1]Departamento de Biologia Geral, Universidade Estadual de Londrina, Londrina, PR, Brasil
[2]Programa de Pós-Graduação em Agronomia, Departamento de Agronomia, Universidade Estadual de Londrina, Londrina, PR, Brasil

*These authors contributed equally to this study.
Corresponding author: E.A. Ruas
E-mail: edu_wicca@yahoo.com.br

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ABSTRACT. *Phyla scaberrima* (Verbenaceae) is a herbaceous perennial species that is distributed from Mexico (center of origin) to Colombia, growing in forest and swamp edges or grasslands from sea level up to an altitude of 1800 m. The chemical properties and uses in popular medicine have drastically affected the population size of this species. In this study, we investigated genetic variability in populations of *P. scaberrima* using AFLP markers. Three AFLP primer combinations rendered a total of 997 markers in a sample of 131 individuals from five populations, including two populations from Mexico and three from Colombia. The average percentage of polymorphic loci, gene diversity and Shannon-Wiener index were 46.62, 0.0695, and 0.119, respectively. Analysis of molecular variance showed that the distribution of the ge-

netic variability within populations (85.41%) was higher than between groups (8.11%) and between populations (6.48%). Principal coordinate analysis and Bayesian analysis for the K number of clusters showed that the individuals were dispersed in five (K= 5) clusters. The low levels of genetic diversity observed in these populations demonstrated that the populations from Mexico and Colombia need urgent management to recover their genetic variability.

Key words: Anthropic effects; Endangered species; Genetic variability; Molecular markers; Population genetics

INTRODUCTION

Phyla scaberrima (Juss. ex Pers.) Moldenke, formerly known as *Phyla dulcis* and *Lippia dulcis* (Plant List, 2013; Tropicos, 2014) is a herbaceous perennial rustic plant that belongs to the family Verbenaceae. It was naturally distributed from southern Mexico to Panama but was also introduced into the region that extends from Honduras to Colombia and also in the Caribbean islands. It grows in forest edges and river banks, open fields and pastures from sea level up to an altitude of 1800 m (Cáceres, 2006). This species has hermaphrodite flowers that are pollinated by small insects, usually bees and flies. Its small seeds are dispersed by the wind, and this species also has vegetative propagation.

P. scaberrima was used by the Aztecs as a medicinal plant in the treatment of respiratory diseases such as catarrh, cough and bronchitis (Cáceres, 1999). Another popular use for this species is in infusions for the treatment of inflammation, stomach pains and diarrhea (Argueta, 1994). Its leaves contain antioxidant constituents such as glycosides, which inhibit the proliferation of gastric adenocarcinoma and uterine carcinoma (Fumiko et al., 2002). Also, this species has sweetener properties due to the presence of sesquiternoids, hermandulcin and epihermandulcin (Compadre et al., 1985), and has been used for this purpose since colonial times. According to Kaneda et al. (1992) and Pérez et al. (2005), *P. scaberrima* can be efficiently used as source of ethanol and hexane.

Due to these many uses, this species has been indiscriminately extracted from nature causing a reduction in its populations, which may, in time, lead to drastic consequences for its genetic variability. The effects of anthropogenic disturbance on the level of genetic variation within plant populations are important issues for species vitality and adaptation to environmental changes (Frankel et al., 1995; Baucom et al., 2005). Decreased genetic diversity could reduce the adaptive potential of populations and their overall fitness (McLaughlin et al., 2002).

Understanding the effects of human activity on genetic diversity is crucial to analyze the patterns of genetic structure in locally disturbed populations (Rossetto et al., 2004) and to develop suitable conservation strategies, especially if we consider, according to Reed and Frankham (2003), that natural populations need to be kept at an effective population size that is large enough to retain genetic diversity and minimize their risk of extinction.

The use of molecular markers provides data that enable the study of genetic variability within and between populations, level of inbreeding, genetic drift and gene flow. Numerous techniques that make use of molecular markers have been used to generate knowledge about the distribution of genetic variability within and between populations of animal and plant species. Among these techniques, amplified fragment length polymorphism (AFLP) stands out as

a molecular marker that can be used to detect a large number of loci per primer combination. Although little information per locus is generated by this molecular marker, due to its dominant characteristic, simulation studies show that the large number of markers offsets this low content of genetic information per locus.

In this study we employed the AFLP markers aiming to determine the genetic variability in populations from Colombia and Mexico and compare the genetic variability between the populations from Mexico (center of origin) and the populations from Colombia.

MATERIAL AND METHODS

Sampling strategy

P. scaberrima was sampled in two Mexican counties, Atzalan and Misantla, and in three regions of Colombia, Cali, Dagua and Pereira (Table 1). The number of individuals sampled from each population varied from 20 to 43 (Table 2), and the individual samples were at least 10 m apart to avoid the collection of close siblings and to sample individuals from the entire area of the population distribution.

Table 1. Localization of populations from *Lippia scaberrima.*

Country	Regions	Populations	Coordinates	Altitude (m)
Mexico	Tomata	Atzalan	19º55'32''N 97º12'36''W	600
Mexico	Santa Cruz Hidalgo	Misantla	19º58'00''N 96º52'48''W	120
Colombia	Cali	Cali	3 º26'7'5''N 76º37'39''W	1525
Colombia	Dagua	Dagua	3 º35'04''N 76º38'25''W	1468
Colombia	Pereira	Pereira	4 º45'00''N 75º 37'67''W	1302

Table 2. Measures of genetic variability from five populations of *Phyla scaberrima* by AFLP markers.

Populations	N	P_A	F_A	P_P	H_S	H'
Cali	24	105	14	48.75	0.0619	0.1111
Dagua	20	65	0	47.44	0.0733	0.1256
Pereira	43	123	2	60.98	0.0774	0.1345
Atzalan	22	24	39	30.49	0.0604	0.0987
Misantla	22	71	6	45.44	0.0743	0.1253

N = number of individuals; P_A = number of private alleles; F_A = number of fixed alleles; P_P = percentage of polymorphic loci; H_S = Nei's gene diversity; H' = Shannon index.

DNA isolation and AFLP reactions

Genomic DNA was isolated from approximately 0.5 g fresh leaves using the CTAB method, as described by Doyle and Doyle (1987). The DNA concentration was estimated using a fluorometer (DyNA Quant 200, Biotech, San Francisco, CA, USA), according to manufacturer instructions. AFLP was carried out as described by Vos et al. (1995). Briefly, 800 to 1000 ng of each DNA sample were subjected to restriction digestion with *Eco*RI/*Mse*I endonucleases (5 U each) and bound to their respective adapters. After incubation for 16 h at

37°C, samples were diluted (1:10) in ultrapure water. Polymerase chain reaction (PCR) amplification was carried out using pre-selected primers complementary to the adapters with addition of one 3'-nucleotide and diluted 1:10. For selective amplification, an initial screening was performed with four individuals from each area using 24 primer combinations. Three primer combinations of 5'-fluorophore-labeled *Eco*RI primers and unlabeled *Mse*I primers were used: *Mse*I-AT with *Eco*RI-GG(NED); *Mse*I-AT with *Eco*RI-CG(HEX), and *Mse*I-AG with *Eco*RI-GC(6-FAM). Genescan-600-LIZ Size Standard v. 2.0 (Applied Biosystems, Foster City, CA, USA) was used as an internal size standard and the AFLP selective reactions including appropriate negative and positive controls were run in the 3500 XL Genetic Analyzer (Applied Biosystems).

Data analysis

All individuals were scored for presence or absence of AFLP bands (1 or 0) to construct a binary matrix, and we scored only the bands with molecular sizes between 50 and 500 bp. The number of private alleles (P_A) and fixed alleles (F_A) was determined using the FAMD version 1.2 software (Schlüter and Harris, 2006), while the percentage of polymorphic loci (P_P), Nei's genetic diversity (H_S; Nei, 1978), Shannon index (H'), genetic distance (Nei, 1972), total heterozygosity (H_T) were calculated using POPGENE v. 1.31 (Yeh et al., 2000). Analysis of molecular variance (AMOVA) was performed using Arlequin v. 3.11 (Excoffier et al., 2005) to evaluate the distribution of genetic variation within and between populations and between groups of populations, as well as to estimate F_{ST} and population pairwise F_{ST} indices. Principal coordinate analysis (PCoA) was used to evaluate the distribution of genetic distance in clusters with FAMD version 1.2 (Schlüter and Harris, 2006). STRUCTURE version 2.3.3 (Hubisz et al., 2009) was used to identify the number of similar population clusters (K). The analysis of the number of clusters was performed with the mixture model using a burn-in and run length of 10,000 and 100,000 interactions, respectively. The number of clusters was determined following the guidelines of Pritchard and Wen (2004) and Evanno et al. (2005).

RESULTS AND DISCUSSION

Three selective AFLP primer combinations (NED-*Eco*RI-GG/*Mse*I-AT, HEX-*Eco*RI-CG/*Mse*I-AT, and 6-FAM-*Eco*RI-GC/*Mse*I-AG) generated a total of 997 markers, of which 99.9% were polymorphic.

The population of Atzalan (Mexico) showed the lowest P_P = 30.49%, H_S = 0.0604 and I = 0.0987, while the highest genetic variability was observed in the Colombian population of Pereira (P_P = 60.98%, H_S = 0.0774, I = 0.1345). It was expected that Mexican populations would exhibit higher genetic variability since it is considered the center of origin for this species. However, we can see in Table 2 that the populations from Mexico (Atzalan and Misantla) showed a genetic diversity similar to that of the populations from Colombia (Table 2) and that the population from Pereira had the highest genetic variability among all populations, probably as a direct result of its origin, which is composed of individuals from several Colombian populations.

Because there are no studies on genetic variability in other species of the genus *Phyla*, we made comparisons with plants of other genera with similar growing behavior. In *L. originoides*, a native species of Colombia, average values of P_P = 87.30, H_S = 0.2736 and I = 0.4150 were observed (Vela, 2011), and in *Astragalus ampullarioides*, a herbaceous

endemic species in the American state of Utah, Breinhold et al. (2009) found values of P_P = 77.22% and I varying from 0.1537 to 0.4203. *P. scaberrima* showed a genetic variability much lower than that of *L. originoides* and *A. ampularioides*, which is considered to be at serious risk of extinction. With regard to *Dendrobium offinale* (Orchidaceae), its habitats are highly fragmented and discontinuous due to logging, and there is overexploitation of its stems, which are used as a traditional Chinese medicine (Li et al., 2008). In *D. officinale*, the P_P was 40.82%, which is very similar to the results found in the populations of *P. scaberrima* from Cali (48.75), Dagua (47.44) and Misantla (45.44) (Table 2). Only the population of Atzalan showed s smaller percentage of polymorphic loci compared to *D. offinacile*. The Shannon index of *D. offinacile* was 0.2203, a value higher than that observed for the five populations of *P. scaberrima*. The low genetic diversity found in *P. scaberrima* is probably due the fact that this species was and continues to be indiscriminately used in folk medicine, causing a decrease in the effective size of its populations. Such reduction in population size favors the occurrence of genetic drift, which may be directly responsible for the low levels of P_P, H_S, and I in these populations (Table 2). Xiao et al. (2006) demonstrated that habitat fragmentation and population deterioration will increase mating opportunity between closely related individuals and finally result in loss of genetic diversity, as we observed in *P. scaberrima*. According to Hartl and Clark (2007), the occurrence of fixed alleles is probably a direct effect of genetic drift, which may have occurred in these populations of *P. scaberrima* (Table 2). Also, we can see in Table 2 a variation in the number of private alleles in these populations of *P. scaberrima*. Slatkin (1985) and De Knijff et al. (2001) showed that populations, with a high level of gene flow always have a low number of private alleles. In our study, we observed that the populations of *P. scaberrima* were geographically isolated and probably had no gene flow between the populations, which may have caused the high number of private alleles (24 to 105) observed in these populations. The loss of genetic diversity within populations decreases adaptability to environmental changes and exponentially increases the risk of population extinction, removing any unique biological characteristics that it may possess. This process may influence overall species biodiversity and ultimately lead to the disappearance of the species (Izawa et al., 2007). Maintenance of genetic diversity is therefore an important factor for the survival of an endangered species such as *P. scaberrima*.

AMOVA showed that 85.41% of genetic variability was found within populations (Table 3), while 6.48% of genetic variability was found between groups (Group 1: Mexican populations; Group 2: Colombian populations) and 8.11 between populations within groups. We observed that *P. scaberrima* has an outcross breeding system with vegetative propagation by budding. Such AMOVA results permit us to infer that, according to Hamrick and Goldt (1989), *P. scaberrima* has a predominantly outcross mixed breeding system.

Table 3. Analysis of molecular variance using AFLP markers for five populations of *Phyla scaberrima* distributed in Colombia and Mexico.

Source of variation	Sum of squares	Variation components	Percentage of variation
Among groups	351.606	3.55228	6.48**
Among populations within groups	461.546	4.44515	8.11**
Within populations	5386.090	46.83557	85.41
Total	6199.242	54.83299	
Fixation índex	F_{ST} = 0.1459**		

**P < 0.01 (significance test from 1023 permutations).

When comparing the genetic distances between populations, the lowest pairwise F_{ST} was found between the Colombian populations from Cali and Dagua, which have a geographic distance of only 32.25 km (Table 4). Although there is a mountainous terrain separating these two populations, they are at similar altitudes and near rural villages. The other Colombian population, Pereira, is a cultivar of *P. scaberrima*, most likely originating from the crossing of several populations from Colombia. Probably the low pairwise F_{ST} (0.06467) between Pereira and Dagua demonstrated that Pereira should contain genetic variability of this population. The two populations of Mexico (Atzantla and Misantla) showed a moderate genetic differentiation (F_{ST} = 0.1347) because Misantla is a native population from Mexico while Atzantla is a cultivated population used as cover vegetation on coffee and banana plantations. Moreover, these two populations are separated by a valley, river and rainforest, which does not allow the occurrence of gene flow between them. The fact that the Mexican populations have higher pairwise F_{ST} with Pereira (0.18695 and 0.19001; Table 4) than between Colombian populations is possibly due to the fact that Mexican populations contributed little to the genetic constitution of Pereira.

Table 4. Geographical distance, in km (upper diagonal) and pairwise F_{ST} values (lower diagonal) between populations of *Phyla scaberrima*.

Populations	Cali	Dagua	Pereira	Atlazan	Misantla
Cali	****	32.25	179.23	2894.54	2869.84
Dagua	0.04713	****	170.73	2863.04	2838.25
Pereira	0.14279	0.06467	****	2876.68	2850.25
Atlalan	0.29441	0.24296	0.19001	****	36.75
Misantla	0.23071	0.21427	0.18695	0.13467	****

All the values of F_{ST} were significant (P < 0.05) with 1023 permutations.

PCoA showed a clear distinction of the Mexican and Colombian populations (Figure 1), and also, the Bayesian structure analysis for the K number of population clusters (K = 5) revealed a clear distinction between all populations (Figure 2A and B; Evanno et al., 2005). These results were corroborated by the values obtained in the pairwise F_{ST} analysis that showed low to high values, but in all cases, significant differentiation between all populations.

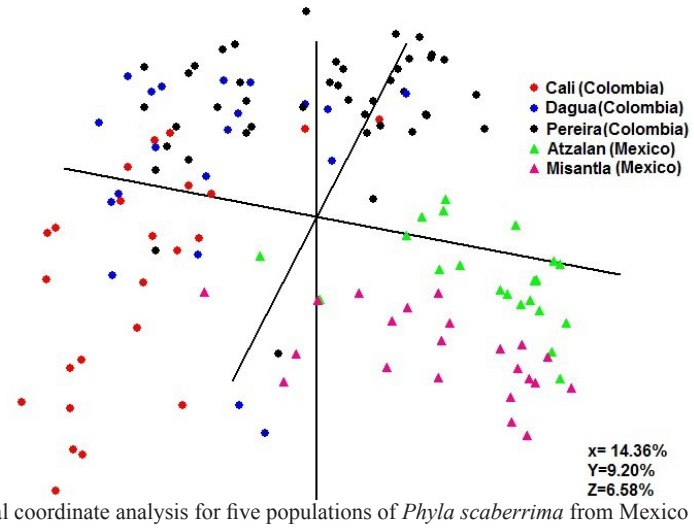

Figure 1. Principal coordinate analysis for five populations of *Phyla scaberrima* from Mexico and Colombia.

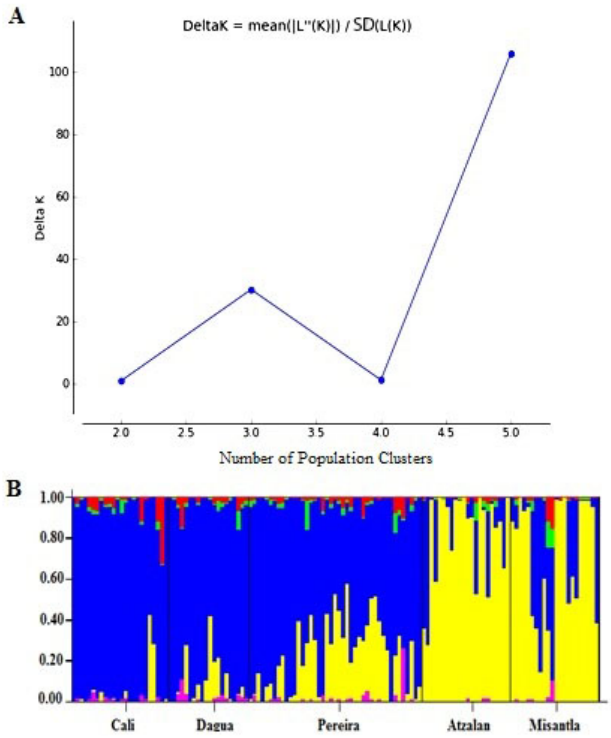

Figure 2. Bayesian analysis for the K number of population clusters **A.** Delta K graphic for the number of population clusters. **B.** Plots of proportional group membership for the individuals of *Phyla scaberrima* (K = 5).

Hence, we may conclude that the populations of *P. scaberrima* have their genetic variability compromised, as demonstrated by the low percentage of polymorphic loci, gene diversity, and Shannon index. Thus, we suggest the construction and maintenance of germplasm banks to preserve the genetic variability still existing in this important species, making a pool of seeds from various locations and then dispersing these seeds in these populations to increase their genetic variability. Also, we suggest that environmental education be implemented in these rural areas to stop the indiscriminate removal of plants from natural populations by local residents.

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REFERENCES

Argueta V (1994). Atlas de las plantas de la medicina tradicional mexicana II. Instituto Nacional Indigenista, Mexico City.

Baucom RS, Estill JC and Cruzan MB (2005). The effect of deforestation on genetic diversity and structure in *Acer saccharum* (Marsh): evidence for loss and restructuring of genetic variation in a natural system. *Conserv. Genet.* 6: 39-50.

Breinhold JW, Van Buren R, Kopp OR and Stephen CL (2009). Population genetic structure of an endangered Utah endemic, *Astragalus ampullarioides* (Fabaceae). *Am. J. Bot.* 96: 661-667.

Cáceres A (1999). Plantas de uso medicinal en Guatemala. Editorial Universitaria, Guatemala.
Cáceres A (2006). Propuesta de monografia farmacopeicas de 10 plantas medicinales Centroamericanas. OEA-AID (Organización de Estados Americanos OEA, Agencia Interamericana de Cooperación para El Desarrollo AICD). Guatemala.
Compadre C, Pezzuto J, Kinghorn A and Kamath S (1985). Hermandulcin: an intensely sweet compound discovered by review of ancient literature. *Science* 227: 417-419.
De Knijff P, Denkers F, van Swelm ND, Kuiper M, et al. (2001). Genetic affinities within the Herring Gull *Larus argentus* assemblage revealed by AFLP genotyping. *J. Mol. Evol.* 52: 85-93.
Doyle JJ and Doyle JL (1987). A rapid DNA isolation from small quantities of leaf tissue. *Phytochem. Bull.* 19: 11-15.
Evanno G, Regnaut S and Goudet J (2005). Detecting the number of clusters of individuals using the software Structure: a simulation study. *Mol. Ecol.* 14: 2611-2620.
Excoffier L, Laval G and Schneider S (2005). Arlequin (Version 3.1): an integrated software package for population genetics data analysis. *Evol. Bioinform. Online* 1:47-50.
Frankel OH, Brown AHD and Burdon JJ (1995). The conservation of plant biodiversity. Cambridge University Press, Cambridge.
Fumiko A, Tsuneatsu N and Hikaru O (2002). Antiproliferative constituents in plants 1) Aerial parts of *Lippia dulcis* and *Lippia canescens*. Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka University, Japan, 25: 920-922.
Hamrick JL and Godt MJW (1989). Allozyme diversity in plant species. In: Plant population genetics, breeding and genetic resources (Brown AHD, Clegg MT, Kahler AL and Weir BS, eds.). Sinauer Associates, Sunderland.
Hartl DL and Clark AG (2007). Principle of population genetics. 4th edn. Sinauer Associates, Inc. Publishers, Sunderland.
Hubisz JM, Falush D, Stephens M and Pritchard JK (2009). Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Resour.* 9: 1322-1332.
Izawa T, Kawahara T and Takahashi H (2007). Genetic diversity of an endangered plant, *Cypripedium macranthus* var. *rubunense* (Orchidaceae): background genetic research for future conservation. *Conserv. Genet.* 8: 1369-1376.
Kaneda N, Lee I, Gupta M, Soejarto D, et al. (1992). (+) -4 beta-hydroxyhernandulcin, a new sweet sesquiterpene from the leaves and flowers of *Lippia dulcis*. *J. Nat. Products* 55: 1136-1141.
Li X, Ding X, Chu B, Zhou Q, et al. (2008). Genetic diversity analysis and conservation of the endangered Chinese endemic herb *Dendrobium officinale* Kimura et Migo (Orchidaceae) based on AFLP. *Genetica* 133: 159-166.
McLaughlin JF, Hellman JJ, Boggs CL and Ehrlich PR (2002). Climate change hastens population extinctions. *Proc. Nat. Acad. Sci. U. S. A.* 99: 6070-6074.
Nei M (1972). Analysis of gene diversity in subdivided populations. *Proc. Nat. Acad. Sci. U. S. A.* 70: 3321-3323.
Nei M (1978). Estimation of average heterozygosity and genetic distance from small number of individuals. *Genetics* 89: 583-590.
Pérez S, Meckles M, Pérez M, Susunaga A, et al. (2005). Antiinflammatory activity of *Lippia dulcis*. *J. Ethnopharmacol.* 102: 1-4.
Plant List (2013). Available at [http://www.theplantlist.org/tpl1.1/record/kew-153231]. Accessed March 21, 2014.
Pritchard JK and Wen W (2004). Documentation for structure software, version 2. University of Chicago, Chicago.
Reed DH and Frankham R (2003). Correlation between fitness and genetic diversity. *Conserv. Biol.* 17: 230-237.
Rossetto M, Jones R and Hunter J (2004). Genetic effects of rainforest fragmentation in an early successional tree (*Elaeocarpus grandis*). *Heredity* 93: 610-618.
Schlüter PM and Harris SA (2006). Analysis of multilocus fingerprinting data sets containing missing data. *Mol. Ecol. Notes* 6: 569-572.
Slatkin M (1985). Rare alleles as indicators of gene flow. *Evolution* 39: 53-65.
Vela NEW (2011). Estrutura poblacional de *Lippia origanoides* HBK em el Cañón del rio Chicamocha (Boyacá y Santander, Colombia). Master's Science Dissertation, Universidad Nacional de Colombia, Facultad de Agronomia, Bogotá.
Tropicos (2014). Available at [http://www.tropicos.org/Name/33700802]. Accessed March 21, 2014.
Vos P, Hogers R, Bleeker M, Reijans M, et al. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407-4414.
Xiao M, Li Q, Guo L, Luo T, et al. (2006). AFLP analysis of genetic diversity of the endangered species *Sinopopiphyllum hexandrum* in the Tibetan region of Sichuan province. *China Biochem. Genet.* 44: 47-60.
Yeh FC, Yang R, Boyle TJ and Xiyan JM (2000). Popgene 32. Microsoft Window-based freeware for population genetic analysis, version 1.32. Molecular Biology and Biotechnology Center, University of Alberta, Edmonton.