Genetic diversity and population structure of *Alyssum stapfii* (*Brassicaceae*) in Iran

Saeedeh Sadat MirzadehVaghefi[1], Masoud Sheidai[1] & Mostafa Assadii[2]

[1] Department of Biology, Shahid Beheshti University, Tehran, Iran, e-mail: myalyssum94@gmail.com (corresponding author)

[2] Research Institute of Forests and Rangelands, Tehran, Iran.

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Abstract. *A. stapfii* is growing in different wild regions of Iran and forms several local populations. Genetic study of 15 different Iranian populations of *A. stapfii* using ISSR molecular markers has revealed a high degree of within-population (74%) and among (26%) populations genetic diversity. AMOVA and Gst analyses produced a significant difference. Nm estimation and population assignment test revealed certain degree of gene flow/shared alleles between these populations. Mantel test produced significant correlation between genetic distance and geographical distance of the studied populations ($r = 0.30$, $P \leq 0.01$). STRUCTURE and K-means clustering showed the populations' genetic stratification. Mantel test revealed that the neighboring populations tend to be genetically more similar to each other. NJ tree showed gene flow/shared alleles in the studied populations. A combination of genetic divergence, limited gene flow and local adaptation play an important role in the diversification of *A. stapfii*.

Key words: *Alyssum*, gene flow, population assignment, STRUCTURE analysis

Introduction

Alyssum L., a genus from the *Brassicaceae* family, consists of about 195 species native to Europe, Asia and North Africa. Most of these species grow in the Northern Hemisphere (Al-Shehbaz & al. 2006). The genus comprises annual and perennial herbaceous plants or (seldom) small shrubs, up to 10–100 cm high, with oblong-oval leaves and yellow or white flowers. It contains 28 species and seven varieties introduced to Iran (Rechinger 1968).

Alyssum stapfii Vierh. is an annual plant that reaches 20 cm in height. It has spatulate to oblanceolate leaves (length: 10–25 mm, width: 2–3 mm), attenuate at the base, with entire margins. This species has oblong sepals (2 mm long) and oblong yellow petals (3–4 mm long), with cylindrical inflorescence.

Population genetic study is important to gain data on genetic diversity, population structure, gene flow, and population fragmentation (Sheidai & al. 2012; 2013). These analyses may be of help in developing effective management strategies for endangered and/or invasive species (Hou & Lou 2011).

Different molecular markers have been used to investigate the speciation process and populations' divergence, genetic drift and migration (Cassel-Lundhagen & al. 2009; Heather & Joanna 2011; Pampoulie & al. 2011). ISSR (inter-simple sequence repeats) are extensively used in genetic diversity analysis and populations' genetic structure, these molecular markers are simple to work, uncostly and produce many polymorphic data to be used in the population genetic analysis (Sheidai & al. 2012, 2013; Azizi & al. 2014).

Many species in *Brassicaceae* have been used as medicinal plants. The seeds of *Alyssum* species with their mucilage content are used as a cough relief by the locals (Zargari 1989). Most *Alyssum* species grow on serpentine (ultramafic) soils and are able to hyperaccumulate nickel in their above-ground parts (Ghaderian & al. 2007). *Alyssum stapfii* grows across Iran and forms several geographical populations. This species is used as medicinal plant by locals and is reported to accumulate moderate-high amount of metals, such as Ni, Fe, Cr, Mn, and Mg (Ghaderian & al. 2007). There have been no available reports on the population genetic diversity and population structure of this important medicinal plant in Iran. Therefore, the present study was intended to investigate the genetic and morphological variability in 15 geographical populations of *A. stapfii* in Iran, in order to provide data on the population structure and gene flow among these populations. This information can be used to plan a better management and conservation program for this important medicinal plant species.

Material and methods

Plant material

Ninety-two plants were randomly collected from 15 geographical populations of *A. stapfii* and were used in the molecular and morphological studies. Details of localities are provided in Table 1 and Fig. 1. Voucher specimens are deposited in the Herbarium of Institute of Forests and Rangelands of Iran (TARI).

DNA extraction and ISSR assay

Fresh leaves were collected randomly from each of the localities and dried in silica gel. Genomic DNA was extracted using CTAB, with activated charcoal protocol (Sheidai & al. 2013). The quality of extracted DNA was examined by running the samples on 0.8 % agarose gel.

Ten ISSR primers – (AGC)5GT, (CA)7GT, (AGC)5GG, (GA)8T, (CA)7AT, (GA)9C, (AG)8T, (GA)8C, (GA)9A, and (GT)7CA – commercialized by UBC (the University of British Columbia) were used. PCR reactions were performed in a 25 µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of single primer; 20 ng genomic DNA, and 3 U of Taq DNA polymerase (Bioron, Germany). Amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation at 94 °C, 30 sec at 94 °C; 1 min at 50 °C and 1min at 72 °C. The reaction was completed by a final extension step of 7 min at 72 °C. The amplification products were visualized by running the samples on 2 % agarose gel, followed by ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Fig. 1. Distribution map of *Alyssum stapfii* Vierh. populations studied in Iran.

Table 1. Locality and voucher number of the studied *A. stapfii* populations.

Populations	Locality	Longitude	Latitude	Altitude (m)	Voucher No.
Pop 1	Hamedan: Hamedan University	34°47'31.20"	48°29'17.64"	1844	TARI_97862
Pop 2	Mazandaran: Damghan to Sari	36°1'2.08"	53°31'41.57"	2100	TARI_29515
Pop 3	Alborz: Sirachal	36° 2'30.38"	51°8'21.26"	2150	TARI_97858
Pop 4	Khorasan Razavi: Sarakhs	36°37'20.06"	61°4'44.73"	250	TARI_4702
Pop 5	Zanjan: 30 km along the road from Zanjan to Dandi, towards Talkhab village	36°37'56.29"	48°2'13.49"	2322	TARI_99708
Pop 6	Zanjan: Qeydar-Lachvan	36° 6'4.52"	48°37'6.05"	1850	TARI_98188
Pop 7	Kordestan, Dehgolan, nead Irankhodro	35°16'39.46"	47°26'0.63"	1810	TARI_97865
Pop 8	Khorasan Razavi: Lotfabad	37°24'53.51"	59°21'32.14"	400	TARI_55696
Pop 9	Tehran: Damavand	35°42'10.93"	52° 2'55.53"	1950	TARI_32117
Pop 10	Markazi: Ashtian, Varsan village	34°30'23.84"	50° 8'3.68"	2140	TARI_101577
Pop 11	Gilan: Loshan to Manjil	36°41'10.98"	49°26'38.60"	400	TARI_101576
Pop 12	Qom: countryside of Ghom, a highway to Tehran	35°32'25.29"	51°21'9.02"	1027	TARI_101578
Pop 13	Tehran: Shahid Beheshti University	35°47'55.78"	51°23'41.88"	1690	TARI_101575
Pop 14	Isfahan: Natanz, Tameh village	33°27'59.98"	51°52'8.34"	2100	TARI_101579
Pop 15	Ardebil: Asalem to Khalkhal	37°38'36.86"	37°38'36.86"	1990	TARI_101580

Data analysis

Genetic diversity and population structure

The obtained ISSR bands were scored as binary characters. Genetic diversity parameters were determined for each population. These parameters were: Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism. Nei's genetic distance was determined among the studied populations and used for clustering (Weising & al. 2005; Freeland & al. 2011).

For grouping of the plant specimens, Neighbor Joining (NJ) clustering method, NeighborNet method of networking, as well as principal coordinate analysis (PCoA) were performed after 100 times bootstrapping/ permutations (Freeland & al. 2011; Huson & Bryant 2006).

The Mantel test was ran to check the correlation between geographical and genetic distance of the studied species (Podani 2000). PAST ver. 2.17 (Hamer & al. 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) programs were applied for these analyses.

A significant genetic difference among the studied populations and provinces was identified by 1) AMOVA (analysis of molecular variance) test (with 1000 permutations) using GenAlex 6.4 (Peakall & Smouse 2006), and 2) Nei's Gst analysis by GenoDive ver.2 (2013) (Meirmans & Van Tienderen 2004). The population genetic differentiation was studied by G'st_est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008). In order to overcome potential problems caused by the dominance of ISSR markers, a Bayesian program, Hickory (ver. 1.0) (Holsinger & Lewise 2003), was used to estimate the parameters related to genetic structure (theta B value). Three runs were conducted with default sampling parameters (burn-in = 50,000, sample=250,000, thin = 50) to ensure consistency of results (Tero & al. 2003).

The genetic structure of populations was studied by two different approaches. First, by using Bayesian based model STRUCTURE analysis (Pritchard & al. 2000), and second, by maximum likelihood-based method of K-means clustering. For STRUCTURE analysis, data were scored as dominant markers (Falush & al. 2007). The Evanno test was run on STRUCTURE result to determine the proper number of *K* by using delta *K* value (Evanno & al. 2005). We did K-means clustering as in GenoDive ver. 2. (2013). Here, the optimal clustering is the one with the smallest amount of variation within clusters. This is calculated by using the within-clusters sum of squares. The minimization of the within-groups sum of squares that is used in K-means clustering is, in the context of a hierarchical AMOVA, equivalent to minimizing the among-populations-within-groups sum of squares, SSDAP/WG. Two summary statistics, 1) pseudo-F, and 2) Bayesian Information Criterion (BIC), provide the best fit for k (Meirmans 2012).

Results

Populations' genetic diversity

We obtained high number of reproducible bands from almost all ISSR primers used. Finally, a data matrix of 92×96 was formed for further analysis. Genetic diversity parameters determined in 15 geographical populations of *A. stapfii* are presented in Table 2. Ardebil population (Population No 15) had the highest values of gene diversity (0.154), Hamedan University (Population No 1) had the highest values of the Shanon's information index (0.241) and genetic diversity of populations (Hs) as estimated in AMOVA (0.24).

Table 2. Genetic diversity parameters in the studied *A. stapfii* populations (population numbers 1–15 are according to Table 1).

Pop	N	Na	Ne	I	He	UHe	HS
Pop 1	8	1.139	1.224	0.241	0.150	0.160	0.24
Pop 2	2	0.111	1.000	0.000	0.000	0.000	—
Pop 3	7	0.861	1.210	0.202	0.130	0.140	0.184
Pop 4	7	0.792	1.175	0.178	0.113	0.122	0.173
Pop 5	4	0.750	1.254	0.202	0.140	0.160	0.19
Pop 6	6	0.833	1.222	0.203	0.133	0.145	0.184
Pop 7	5	0.778	1.198	0.181	0.119	0.132	0.169
Pop 8	6	0.875	1.224	0.207	0.136	0.148	0.205
Pop 9	6	0.931	1.216	0.213	0.136	0.149	0.211
Pop 10	9	0.764	1.166	0.163	0.104	0.110	0.148
Pop 11	8	1.028	1.219	0.224	0.141	0.151	0.217
Pop 12	5	0.861	1.201	0.197	0.126	0.140	0.2
Pop 13	9	1.111	1.239	0.231	0.146	0.155	0.207
Pop 14	5	0.847	1.259	0.227	0.152	0.169	0.222
Pop 15	6	1.000	1.249	0.237	0.154	0.168	0.234

Abbreviations: **N** = number of samples, **Ne** = number of effective alleles, **He** = Nei gene diversity, **UHe** = unbiased Nei gene diversity, **Hs** = genetic diversity due to populations.

AMOVA revealed a significant molecular difference among the studied populations (PhiPT value = 0.264, P = 0.01). It also showed that 26 % of the total genetic variability occurred among the studied populations, while 74 % occurred within these populations. These results indicate a high-level genetic diversity both within and among *A. stapfii* populations. This conclusion was supported by Gst analysis and Hickory test. The Gst value obtained among populations after 999 permutations was 0.239 (p = 0.001), while Hickory test produced theta-II value of 0.50. This is also a very high and significant value.

Populations' differentiation parameters among the studied populations produced high values for Hedrick' standardized fixation index after 999 permutations (G'st = 0.302, P = 0.001) and Jost' differentiation index (D-est = 0.083, P = 0.001). These results indicate that the geographical populations of *A. stapfii* are genetically differentiated from each other.

Pair-wise Fst and Gst analyses produced a significant difference (p = 0.01) for all pair-wise population comparisons.

Populations' genetic structure and genetic affinity

Genetic affinity of the studied populations was determined by NJ tree and Neighbor-Net method. Both produced similar results. Therefore, only NJ tree is presented and discussed (Fig. 2).

NJ tree almost grouped the plants of each population in a separate cluster. This was true particularly for Populations 3, 4, 10, and 15. However, some plants of the studied populations were inter-mixed with plants of the other populations. This result indicated a cer-

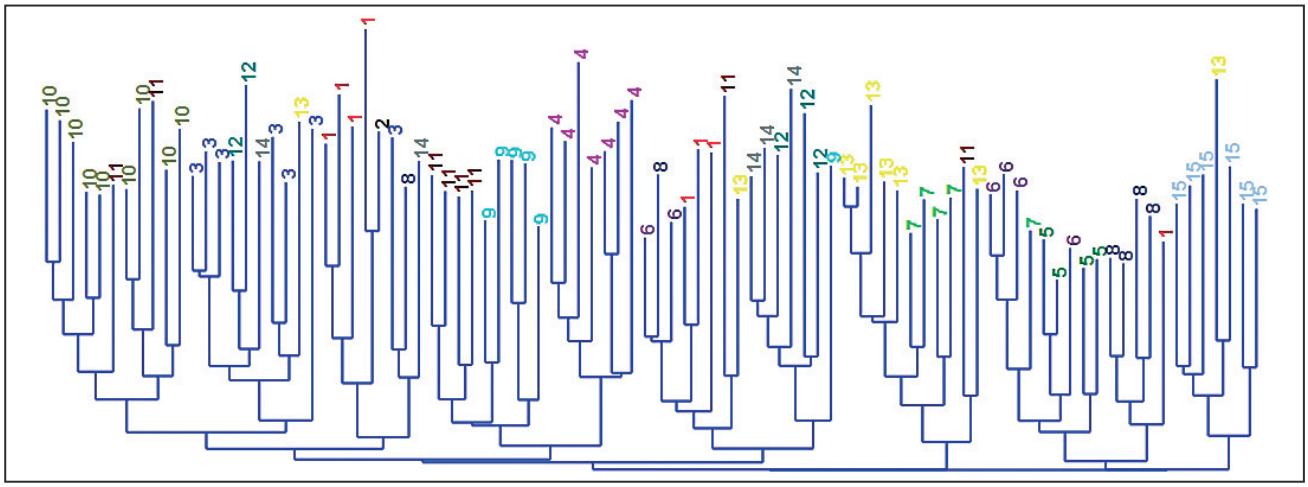

Fig. 2. NJ tree of *A. stapfii* (the population code is according to Table 1).

tain degree of gene flow/shared alleles in the studied populations.

K-means clustering produced the optimum number of k = 2, according to Calinski & Harabasz' pseudo-F (the highest value of pseudo-F = 6.476), and k = 3, according to Bayesian Information Criterion, was 3 (the lowest value of BIC = 191.914). A similar result was produced by the Evanno test run on STRUCTURE analysis by Earl & von Holtd (2012). It produced a major peak at k = 3.

A STRUCTURE plot based on k = 3 is presented in Fig. 3. Populations 1, 6 and 8 showed higher degree of genetic affinity due to their shared alleles (similarly colored segments). The same was true for Populations 5, 7, 13, and 14, as well as for Populations 3, 10 and 11.

Gene flow among populations

We used different approaches to figure out occurrence of the gene flow among *A. stapfii* populations. First, we used the Nm estimation, which is an indirect method and considers the uniform and identical gene flow among populations.

Nm estimation produced Nm value ranging from 0.1 to 3.0, with the mean value of 0.96 for the studied ISSR loci. This indirect estimation of gene flow shows an almost good level of gene flow/shared ancestral alleles among *A. stapfii* populations (Nm = 1 is regarded as a high degree of gene flow).

Second, we used the population assignment test based on the maximum likelihood method. The result is presented in Table 3. Out of 92 studied plants in 15 populations, 25 plants were inferred to be from the other populations (due to gene flow/shared alleles). These plants were members of Populations 1, 3, 6–14. Therefore, the assignment test also revealed a high degree of gene flow/shared alleles in these populations.

Table 3. Results of the population assignment test run on 92 plants in 15 populations of *A. stapfii*.

Individual	Current	Inferred	Lik_max	Lik_home	Lik_ratio
2	Pop 001	Pop 011	-28.544	-31.601	6.114
8	Pop 001	Pop 013	-53.383	-58.639	10.512
10	Pop 003	Pop 008	-29.886	-32.952	6.133
15	Pop 003	Pop 011	-25.63	-29.798	8.335
32	Pop 006	Pop 001	-27.207	-36.808	19.201
33	Pop 006	Pop 001	-29.741	-32.832	6.181
35	Pop 007	Pop 013	-26.174	-27.778	3.207
38	Pop 007	Pop 013	-25.452	-34.175	17.445
40	Pop 008	Pop 006	-21.672	-37.837	32.33
46	Pop 009	Pop 001	-33.676	-39.055	10.756
50	Pop 009	Pop 011	-35.706	-40.153	8.894
51	Pop 010	Pop 011	-18.217	-20.624	4.814
57	Pop 010	Pop 008	-34.263	-35.243	1.96
64	Pop 011	Pop 010	-21.583	-31.672	20.178
66	Pop 011	Pop 010	-26.278	-31.51	10.465
68	Pop 012	Pop 011	-32.358	-41.004	17.291
69	Pop 012	Pop 014	-35.771	-41.004	10.466
70	Pop 012	Pop 014	-25.184	-26.665	2.962
72	Pop 012	Pop 014	-23.798	-46.015	44.433
78	Pop 013	Pop 001	-25.164	-29.296	8.264
80	Pop 013	Pop 003	-35.101	-37.967	5.733
81	Pop 013	Pop 001	-39.524	-70.278	61.508
85	Pop 014	Pop 012	-39.151	-43.231	8.16
86	Pop 014	Pop 013	-42.529	-43.231	1.404

Third, we used STRUCTURE, analysis based on admixture model to investigate the possible genetic admixture among the studied populations. A detailed analysis of alleles (their frequency and combination as evidenced by colored segments in STRUCTURE plot) (Fig. 4) revealed high degree of genetic variability within the studied populations. These populations had shared alleles with the other studied populations due to gene flow/shared alleles (Populations 8, 9, 12–15).

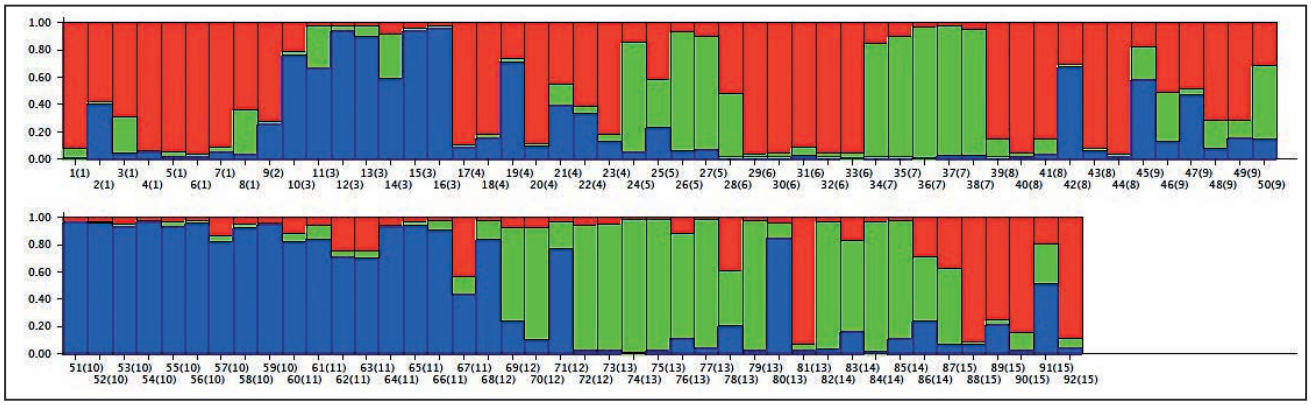

Fig. 3. STRUCTURE plot of *A. stapfii* plants based on k = 3.

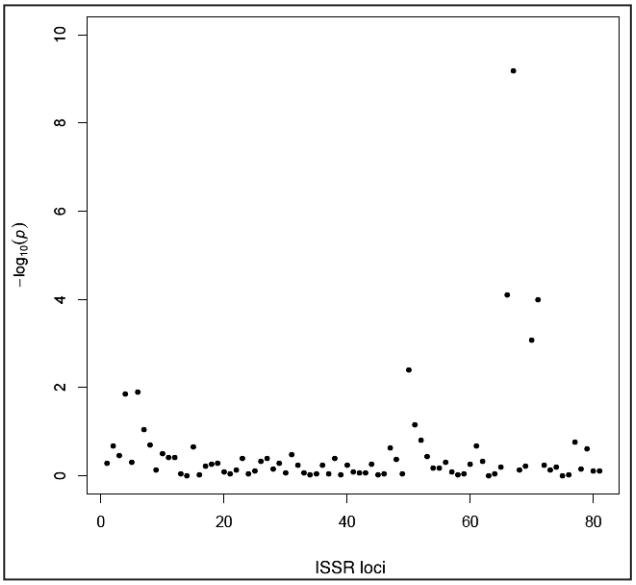

Fig. 4. Manhattan plot of ISSR loci in the studied populations.

Mantel test produced significant correlation between the genetic and geographical distance of the studied populations ($r = 0.30$, $P \leq 0.01$). Therefore, in spite of genetic differentiation of the studied *A. stapfii* populations, they are not totally isolated and still some amount of gene flow has occurred between them.

LFMM result showed that 6 ISSR loci had -log10 (p-value) of equal or greater than 1.50 and were significantly correlated with the studied environmental parameters (longitude, latitude and altitude) ($P \leq 0.05$). These results are summarized in the Manhattan plot which is presented in Fig. 4. Out of these loci, only ISSR loci 66 had Nm value = 2.21, while the other five ISSR loci had $Nm \leq 0.30$. Therefore, mostly ISSR loci with lower Nm value (private alleles) were adaptive.

Discussion

A. stapfii is medicinally important in Iran and is extensively used by locals. Although these plants grow in different geographical regions of the country, they form small populations of 30–50 individuals in each location. Extensive use of natural resources to meet the needs of the expanding human populations, deforestation and habitat fragmentation lead to reductions in the rate of gene flow among populations. This in turn increases the genetic differentiation among populations due to genetic drift (Setsuko & al. 2007; Hou & Lou 2011). Therefore, monitoring of genetic diversity and obtaining data on population genetic structure is important for future conservation of *A. stapfii* in Iran.

Genetic diversity parameters showed a good degree of within-population genetic variability in the studied populations. Moreover, population assignment and STRUCTURE analysis revealed a good level of gene flow among the geographical populations. These results show that the populations achieve a new combination of alleles through limited gene flow.

Genetic diversity is of fundamental importance in the continuity of species as it ensures the necessary adaptation to the prevailing biotic and abiotic environmental conditions, and makes possible a change in the genetic composition so as to cope with changes in the environment (Çalişkan 2012; Sheidai & al. 2012, 2013). A high genetic variability within population and among populations is due to the outcrossing nature of *A. stapfii*. The reproductive system of *Alyssum* usually involves insect pollination and seed dispersal by gravity, wind and water flow and transportation by birds (Adamidis & al. 2014: Li & al. 2014). These mechanisms together can support the gene flow among different populations in *Alyssum*.

Mantel test revealed a pattern of isolation-by-distance across the distribution range of the studied *A. stapfii* populations. This pattern suggested that dispersal of the populations might be constrained by distance and gene flow is most likely to occur between neighboring populations. In the result, more closely situated populations tend to be genetically more similar to each another (Medrano & Herrera 2008).

LFMM analysis revealed that some of the genetic loci were adaptive, mostly of private alleles and possibly used by local populations to adapt to their environment. Therefore, a combination of genetic divergence, limited gene flow and local adaptation has influenced diversification of *A. stapfii*. In conclusion, the present study may provide some useful information about the population genetic structure and genetic variability of *A. stapfii* that could be applied in the conservation of these important species.

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