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Contributed Paper

Inter-simple Sequence Repeat Markers Reveal Genetic Relatedness Between Natural *Aquilaria* Populations in Peninsular Malaysia

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ABSTRACT

Aquilaria is a major agarwood-producing genus from the Indomalesian region. The high demand for natural agarwood resources in the market is increasing, directly threatening the survival of the trees in the wild. In order to develop proper conservation strategies for these species, information on their genetic variation is needed. The current levels of genetic variation found in *Aquilaria* populations in Peninsular Malaysia, as well as their genetic relationships, were evaluated using inter-simple sequence repeat (ISSR) markers. A total of 42 individuals, from 21 populations of three different *Aquilaria* species were collected and genotyped using 25 ISSR primers. Twenty four primers amplified bands in all three species, and showed 95%, 82.6%, and 63% polymorphic bands in *A. malaccensis*, *A. hirta*, and *A. rostrata*, respectively. Clustering analyses through the construction of a UPGMA dendrogram and a principal component analysis (PCA) plot revealed three major clusters, grouping samples of each species into their respective clusters. The *A. malaccensis* populations seemed to also be divided into two sub-clades, most probably as a result of isolation by mountain ranges as physical barriers to gene flow between the populations. Findings in this study provide valuable information for the planning of more effective management and conservation of existing *Aquilaria* populations in Peninsular Malaysia.

Keywords: agarwood, ISSR markers, UPGMA, principal component analysis, conservation

1. INTRODUCTION

Aquilaria Lam. (Thymelaeaceae) is an endangered tropical tree that produce a valuable fragrant resin, the agarwood. Species in this genus are currently listed in the Convention on International Trade in Endangered Species (CITES) Appendix II

and categorized as 'Vulnerable' in the International Union for Conservation of Nature (IUCN) Red List [1]. Agarwood is mainly used as an ingredient in high-end perfumes, incense production, and traditional medicines. It is formed inside the tree stem as a defense mechanism to protect itself from open wounds, which can be induced naturally (e.g. breaking of branches due to strong wind, thunder strike, animal or insect attack) or artificially (e.g. by wounding the tree stem) [2]. As natural agarwood formation is random, rare, and reported to be found only on aged trees [3], agarwood harvesters would wound the trees using machetes, nails, or drills, to create open wounds for further pathogenic infections [2]. As agarwood demand is high, harvesters tends to perform indiscriminate felling and unsustainable harvesting in search of agarwood without prior confirmation of the agarwood content. Thus, the heavy exploitation of the trees in the wild directly threatens the survival of the species.

At present, there are 21 *Aquilaria* species recorded in the world and are all endemic to the Indomalesian region [4-5]. In Malaysia, five species have been recorded in the wild, namely *A. beccariana* Teigh, *A. hirta* Ridl., *A. malaccensis* Lam., *A. microcarpa* Baill., and the recently re-discovered *A. rostrata* Ridl. [6]. In Peninsular Malaysia, *A. malaccensis* can be found in most states, predominating over the other *Aquilaria* species; *A. hirta* primarily populates along the east coast region (Johor, Pahang, and Terengganu), while *A. rostrata* can only be found at high elevations in the states of Pahang and Terengganu. On the other hand, in East Malaysia, *A. microcarpa* predominates in the state of Sarawak, while *A. beccariana* predominates in the state of Sabah. There are reports on small and scattered populations of *A. malaccensis* in East Malaysia, and several small populations of *A. microcarpa* and *A. beccariana* in the south of Peninsular Malaysia, mainly within the state of Johor [7]. Being one of the countries with naturally occurring *Aquilaria* trees, Malaysia faces the problem of illegal harvesting that has resulted in natural population size reduction over the years [8]. Conservation efforts are vital to ensure the continued survival of these species in the wild.

While several DNA-based techniques, such as DNA barcoding [9] and real-time PCR [10], were introduced as tools for the identification of agarwood-producing species, genetic diversity estimates on such threatened species are lacking. Such estimates are important information towards conducting a successful conservation plan as the amount of genetic variation present within a population is thought to determine its adaptability for long term survival [11]. Furthermore, understanding the genetic variation within a species and among different natural populations is crucial when developing strategies for both in-situ and ex-situ conservation activities. To date, there have been only a few published reports on the genetic diversity of *Aquilaria* in Malaysia using different genetic markers, and these studies usually had very limited coverage of existing *Aquilaria* populations so that the information generated was not sufficient to practically inform any management plans [12-14]. Therefore, information on the genetic variation and relatedness of *Aquilaria* species in Malaysia, on a wide-scale, is still lacking.

The inter-simple sequence repeat (ISSR) marker is a simple and inexpensive genetic marker that could provide rapid screening for DNA polymorphism in organisms for which there is a lack of genetic information [15]. It has been widely applied to population or phylogenetic studies in both cultivated and natural populations, and has been successfully used for the assessment of genetic diversity in *Aquilaria* in other agarwood-producing

countries, such as China [11, 16-19], India [20], and Vietnam [21]. An assessment of the genetic diversity and relationship among *Aquilaria* species in Malaysia is crucial to provide important genetic information to complement conservation and management efforts in the field. This study therefore aims to evaluate the current levels of genetic variation found in natural *Aquilaria* populations in Peninsular Malaysia, as well as to assess their genetic relationship, using ISSR markers.

2. MATERIALS AND METHODS

2.1 Plant Material

Fresh leaves were sampled from 19 natural populations, across 10 different states, in Peninsular Malaysia (Table 1, Figure 1). Each population was represented by at least two individuals; while some populations with small population sizes were represented by only one individual. Permissions to collect the specimens were obtained from the Forestry Department of Peninsular Malaysia and the respective State Forestry Departments. As the studied species are endangered, the exact coordinates/locations of the sampled populations are not revealed in this report. Sampled leaves were kept in laminated aluminum zip lock packets, stored on ice during transportation to the laboratory, and finally stored at -80°C before DNA extraction. Voucher specimens were also prepared for each sample and deposited at the Forest Biotechnology Laboratory, Faculty of Forestry, Universiti Putra Malaysia, Selangor, Malaysia, for future reference.

The 19 *Aquilaria* populations comprised of 15 *A. malaccensis* populations, five *A. hirta* populations, and one *A. rostrata* population. Of the five *Aquilaria* species available in Malaysia, *A. beccariana* and *A. microcarpa* have limited distributions in Peninsular Malaysia and therefore were not included in this study.

Table 1. List of collected samples based on location.

Species	State	Population (Province)	Altitude (m a.s.l.)	Population code	Corresponding code on the map in Figure 1.	Number of individuals analyzed
Aquilaria malaccensis (Am)	Johor	Mersing	34	MERS	1	2
	Melaka	Ayer Keroh	18	AYKH	2	2
		Sungai Udang	44	SGUD	3	2
	Selangor	Puchong	23	HSAH	4	2
	Kuala Lumpur	Bukit Nanas	130	BNNS	5	2
	Perak	Lumut	11	LUMT	6	2
	Pulau Pinang	Bukit Mertajam	54	TKUN	7	2
	Kedah	Langkawi	21	LGKW	8	2
	Pahang	Bentong	149	BTNG	9	2
		Karak	155	KARK	10	2
		Kemasul	130	KEMA	11	2
	Terengganu	Kemaman	28	RUNA	12	2
		Merchang 2	23	ALUR	13	2

Table 1. Continued.

Species	State	Population (Province)	Altitude (m a.s.l.)	Population code	Corresponding code on the map in Figure 1.	Number of individuals analyzed
		Dungun 1	87	DUNG	14	2
	Kelantan	Gua Musang	231	GMSG	15	2
Aquilaria hirta (Ah)	Johor	Mersing	39	MERS	16	2
	Terengganu	Kijal	37	DARA	17	2
		Merchang 1	332	MCHG	18	2
		Merchang 2	241	ALUR	19	1
		Setiu	215	LATA	20	1
Aquilaria rostrata (Ar)	Terengganu	Dungun 2	700	GTBU	21	4

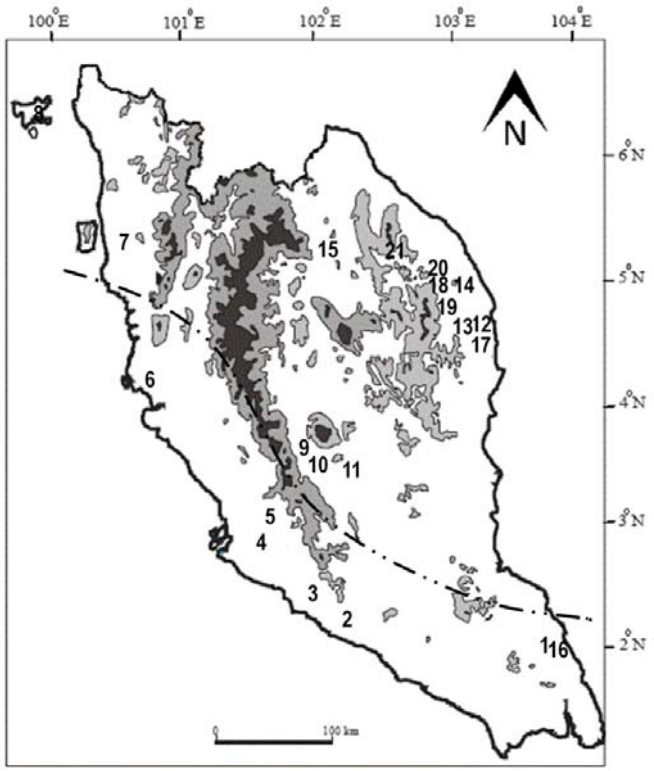

Figure 1. Distribution of the 21 (listed 1 to 21) *Aquilaria* populations from Peninsular Malaysia used in this study. Population names are listed in Table 1. Earth elevations are classified as ▭ below 350 m, ▭ above 350 m, and ▭ above 1000 m. — · · — is the proposed genetic border for natural *Aquilaria malaccensis* populations in Peninsular Malaysia.

2.2 DNA Extraction

Genomic DNA was extracted using FavorPrep™ Plant Genomic DNA Extraction Mini Kit (Favorgen, Taiwan), according to the manufacturer's protocol. The quality and quantity of the extracted DNA were determined by spectrometry (NanoPhotometer™, IMPLEN, Germany).

2.3 ISSR Genotyping

A total of 25 ISSR primers (Table 2) were initially tested on a subset of two DNA samples from each of the three *Aquilaria* species, and only those that generated multiple, clear, and reproducible bands at least in one of the species, were subsequently used to genotype all 42 samples featured in this study. PCR reactions were carried out in final volumes of 15 µl, which consisted of final concentrations of 1× PCRBIO Taq Mix Red (PCRBiosystem, UK), 1 µM ISSR primer, and 25 ng template DNA. PCR amplification was carried out on a MyCycler™ thermal cycler (BioRad, USA), using a touch-down PCR reaction profile, programed for an initial denaturation of 3 min at 95 °C; 13 cycles of 30 s at 95 °C, 30 s at 58-46 °C (-1 °C/cycle), and 90 s at 72 °C; followed by 25 cycles of 30 s at 95 °C, 30 s at 45 °C, and 90 s at 72 °C; and a final extension at 72 °C for 7 min. PCR products were analyzed through electrophoresis on 2% agarose gel, stained with ethidium bromide and photographed under UV light.

Table 2. ISSR primers used in this study.

No.	Primer name	Primer sequence (5'-3')	No. of bands			Polymorphism information content (PIC)	Resolving power (RP)
			A. malaccensis	*A. hirta*	*A. rostrata*		
1.	UBC 834	$(AG)_8YT$	13	9	6	0.44	10.52
2.	UBC 841	$(GA)_8YC$	18	16	12	0.37	9.90
3.	UBC 848	$(CA)_8RG$	16	15	7	0.41	10.38
4.	UBC 855	$(AC)_8YT$	13	8	6	0.45	10.24
5.	UBC 856	$(AC)_8YA$	12	9	7	0.28	5.33
6.	Ng2.01	$(AC)_8B$	18	17	9	0.46	14.05
7.	Ng2.02	$(AG)_8B$	7	5	5	0.40	4.24
8.	Ng2.03	$(TC)_8V$	14	9	1	0.36	9.86
9.	Ng2.04	$(TG)_8V$	9	7	4	0.44	6.52
10.	Ng2.05	$(CA)_8D$	8	7	8	0.45	6.00
11.	Ng2.06	$(CT)_8D$	8	-	5	0.47	6.38
12.	Ng2.07	$(GA)_8H$	11	9	10	0.32	5.43
13.	Ng2.08	$(GT)_8H$	3	3	2	0.37	1.71
14.	Ng2.09	$(AC)_8SS$	11	7	8	0.45	8.29
15.	Ng2.10	$(AG)_8SS$	12	9	6	0.41	7.67
16.	Ng3.01	$(ACA)_5SS$	13	13	9	0.47	10.10
17.	Ng3.02	$(AGA)_5SS$	11	11	8	0.47	8.90
18.	Ng3.03	$(TCA)_5SS$	13	13	-	0.41	8.33
19.	Ng3.04	$(TGA)_5SS$	17	15	9	0.44	12.62
20.	Ng3.05	$(ACT)_5SS$	-	-	-	-	-

Table 2. Continued.

No.	Primer name	Primer sequence (5'-3')	No. of bands				Polymorphism information content (PIC)	Resolving power (RP)
			A. malaccensis	*A. hirta*	*A. rostrata*			
21.	Ng3.06	$(AGT)_5SS$	9	4	-		0.32	4.00
22.	Ng3.07	$(TCT)_5SS$	6	-	-		0.33	2.86
23.	Ng3.08	$(TGT)_5SS$	12	11	5		0.41	7.95
24.	Ng3.09	$(ATC)_5SS$	11	9	6		0.39	6.38
25.	Ng3.10	$(ATG)_5SS$	13	13	5		0.39	7.33
	Total		278	219	138	Average per primer	0.40	7.71
	Number of polymorphic bands (% of polymorphic bands)		264 (95%)	181 (82.6%)	87 (63%)			

2.4 Data Analysis

Amplified ISSR bands were scored as present (1) or absent (0) for each individual to produce a binary ISSR data matrix for each species to be used in subsequent analyses. Unclear or non-amplifications were treated as missing data (-1). Basic parameters such as the total number of bands, number of polymorphic bands, and the percentage of polymorphic bands, were manually calculated. The performance of all ISSR markers in evaluating the genetic profiles of *Aquilaria* in this study was analyzed based on two parameters: polymorphism information content (PIC) and resolving power (RP). The PIC value for each locus was calculated based on the formula reported in [22]. The RP of each primer was calculated based on the formula reported in [23]. The resulting binary data matrix was analyzed using GenAlEx version 6.5 [24] to estimate the genetic distance between each individual, and an unweighted pair-group method with arithmetic means (UPGMA) tree was constructed using MEGA 6 [25]. A principal component analysis (PCA) was performed based on the binary character matrix using SIMCA version 13.0 (Umetrics AB, Sweden).

3. RESULTS AND DISCUSSION

3.1 ISSR Genotyping

Of the 25 ISSR primers tested, only one did not produce clear bands in any of the three tested species. The other ISSR primers produced clear and reproducible bands in one or more species, and were subsequently used to genotype all the samples. The 24 primers generated a total of 278, 219, and 138 bands, with 95%, 82.6%, and 63% of the bands being polymorphic, in *A. malaccensis*, *A. hirta*, and *A. rostrata*, respectively. The PIC value for each primer was obtained from the mean of PIC values analyzed for each loci. High PIC value of 0.47 (Ng2.06, Ng3.01, and Ng3.02) and low PIC value of 0.28 (UBC856), with an average value of PIC per primer 0.40 were obtained from this study. The RP value indicates the discriminatory potential of each primer used. In this study, the highest RP value was observed for primer Ng2.01 (14.05) and the lowest for primer Ng2.08 (1.71), with a mean RP value of 7.71 per primer.

The detailed outcome of the genotyping is listed in Table 2.

From the percentage of polymorphic bands, the level of genetic variation found in *A. malaccensis* was higher than *A. hirta*, followed by *A. rostrata*. This is in line with expectation, as the level of genetic variation observed is expected to increase with increasing sample sizes. Samples for this study were also obtained from all the major *Aquilaria* populations in Peninsular Malaysia, and thus reflect the current genetic diversity harbored within these *Aquilaria* populations. In fact, *A. rostrata*, only recently rediscovered in the state of Terengganu, is critically endangered and endemic to Peninsular Malaysia [6]. It was thought to have gone extinct ever since its discovery in the state of Pahang nearly a hundred years ago [26]. *Aquilaria hirta* is known to be a slow-growing species [5]. Their natural populations in Peninsular Malaysia are confined to the east coast region, especially in regions with higher moisture content, in small stands [8]. The current status and life history of the three *Aquilaria* species are therefore in concordance with the genetic diversity estimates reported above.

3.2 Cluster Analysis

The UPGMA tree (Figure 2), inferred from Nei's genetic distance, resolved the 21 populations into 3 major clusters: clades A, B, and C. Clade A consists of *A. malaccensis* populations, where it further clustered into two sub-clades A1 and A2. Sub-clade A1 consists of *A. malaccensis* populations from Bentong (BTNG), Karak (KARK), Kemasul (KEMA), Bukit Mertajam (TKUN), Pulau Langkawi (LGKW), Dungun (DUNG), Gua Musang (GMSG), Kemaman (RUNA), and Merchang (ALUR), while sub-clade A2 consists of Mersing (MERS), Ayer Keroh (AYKH), Sungai Udang (SGUD), Puchong (HSAH), Bukit Nanas (BNNS), and Lumut (LMUT). Clades B and C consists of *A. rostrata* and *A. hirta* populations, respectively. The three major clades corresponded well with the three *Aquilaria* species, while the two sub-clades A1 and A2 revealed geographical population structuring for *A. malaccensis*. In the PCA plot (Figure 3), individuals were grouped together into three clusters, corresponding to the three *Aquilaria* species included in this study. However, unlike in the UPGMA tree, there was no clear grouping pattern observed within *A. malaccensis*.

There have been few studies on the genetic structure of natural woody tree populations in Peninsular Malaysia, mostly on species within the family Dipterocarpaceae, a sister family to Thymelaeaceae. Examples include the *Dryobalanops aromatica* [27], *Shorea leprosula* [28], *Koompassia malaccensis* [29], and *Neobalanocarpus heimii* [30]. All four species do not show similarities in their population structuring in Peninsular Malaysia. The population structuring of *A. malaccensis* observed in this study also did not show similarities with any of the aforementioned tree species. The border separating the 2 sub-clades of *A. malaccensis* seems to start between the states of Johor and Pahang, stretching along the Titiwangsa Range, then continuing west across the south of the Keledang Range and the Bintang Range (Figure 1). Geographic barriers have been shown to affect the population structure of natural *A. sinensis* populations in China [18]. High elevations of the mountain ranges may have created natural physical barriers that prevent gene flow between populations within these two sub-clades. Other factors such as poor seed dispersal, genetic drift, and local adaptation via phenotypic interaction may have also contributed towards population differentiation within the species [11].

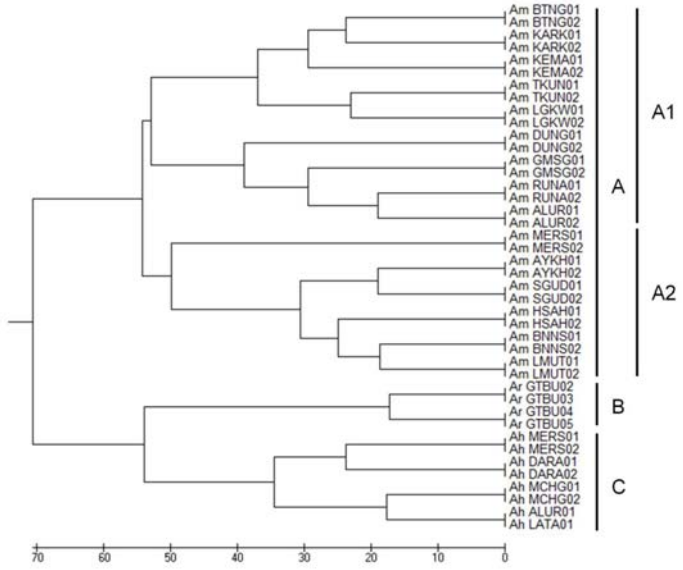

Figure 2. Cluster analysis based on cumulative ISSR data obtained from 21 natural *Aquilaria* population in Peninsular Malaysia. Dendrogram was constructed using UPGMA clustering method. Genetic distance scale as indicated below.

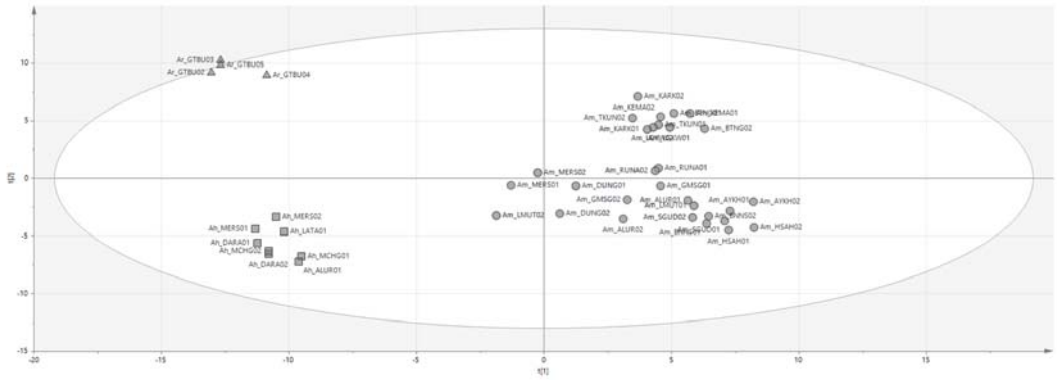

Figure 3. Principal component analysis using Nei's (1972) genetic distance for all 21 natural *Aquilaria* populations in Peninsular Malaysia.

3.3 Implication for Conservation

Based on a nationwide surveys conducted between 2002 and 2004, the number of wild *Aquilaria* trees in the natural forests of Peninsular Malaysia was reported to be more than 3 million individuals [31]. However, this number is expected to decline steeply after a decade as a result of diminishing natural habitat due to agricultural land conversion and illegal agarwood harvesting activities [32]. *Aquilaria beccariana* and *A. microcarpa*, for example, were previously recorded in Mersing and Kota Tinggi, and Mersing, respectively, but the records have not been updated. A recent effort to confirm the current status of both ended up in vain as the latter species was recorded with a single herbarium specimen, and the reported site is

at present, next to an agriculture plantation. The search for *A. beccariana* populations in Kota Tinggi, Johor, was not carried out, although further inventory should be conducted to ascertain its status [6]. Habitat lost and inaccessibility to their surviving populations probably are the reasons for failing to identifying these two species. The latest conservation status for *Aquilaria* species in Peninsular Malaysia listed *A. hirta* and *A. malaccensis* as 'Vulnerable', and *A. beccariana*, *A. microcarpa*, and *A. rostrata* as 'Data Deficient' [33], calling for the need for more effort to document and conserve these endangered species in Malaysia.

The effort to cultivate agarwood-producing trees with the intention to sustainably produce agarwood was first introduced when *A. malaccensis* was listed in the CITES Appendix II in 1995. The wild trees of this species were being threatened because of the search for agarwood to fulfill the demand in the market [32]. The demand is exacerbated by the hype in agarwood value, which promoted *Aquilaria* cultivation at the beginning of the 20th century. While *A. malaccensis* is a native species in Malaysia, foreign species *A. crassna* and *A. subintegra*, claimed as being relatively fast-growing, were introduced into the country and planted in both large scale and home gardens. Although the claim for being fast-growing has not been scientifically proven, locals brought in seedlings without prior knowledge on the species, in high hopes to gain profits after few years of cultivation. In our previous expeditions in search of natural populations, we found *Aquilaria* plantations of non-native species (*A. crassna* and *A. subintegra*) situated nearby natural populations. As *Aquilaria* is considered a highly out-crossing species [33], such observation during our expedition raises concern on the possibility of hybridization between the native and non-native trees, directly threatening the genetic integrity of native *Aquilaria* populations. Similar incidents have been reported on other tree species, such as in eucalyptus trees in Australia [34]. Hybridization involving small populations typical of endangered species is of great concern in plant conservation genetics, as it may cause extinction of local populations through outbreeding depression or genetic assimilation [35]. We therefore urge the authorities to conduct measures to prevent further contamination of the local *Aquilaria* gene pool.

4. CONCLUSION

In this study, ISSR markers were successfully used to elucidate the levels and patterns of genetic variation among natural *Aquilaria* populations in Peninsular Malaysia. Such information is important to guide conservation and management efforts of this threatened species.

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