Palestine Technical University Research Journal, 2014, 2(2), 01-08
(http://www.ptuk.edu.ps)

RAPD for Assessment of Thymes Genetic Diversity in Palestine

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Received 5 April 2014, Accepted 12 June 2014, Published 08 September 2014

Abstract: Several thymes species were grown in Palestine and have socio-economical values. Traditionally, morphological features are used for their taxonomy and discrimination. However, these methods fail to provide an accurate for discrimination and authentication, suggesting the use of other method inevitable. In this study, RAPD method was used for genotypes identification and characterization of five most socio-economical Palestinian thymes: *Thymus syriacus*, *Thymus fruticosus*, *Thymus incanus*, *Thymus majorana*, and *Thymus capitatus*. Eight out of ten decamer primers were tested for their ability to generate polymorphism from selected thyme species using RAPD-PCR, and the obtained data were analyzed. The primers (OPD-19, OPH-02 and OPAN-08) were generated 78.6% average polymorphism across five studied Thyme species. Pairwise similarity analysis revealed banding patterns between the studied plant species ranged from 0.18 to 0.67. By this study, genetic diversities of studied five Palestinian Thyme species were ascertained successfully using RAPD markers, concluding that it could be useful tools for identifying Thymes species in any putative breeding programs that will be carried in the country.

***Keywords*:** Genetic diversity; Thymes; (RAPD); Biotechnology

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Introduction

Palestine considered as one of the world's "hotspots" areas where different plant species diversity are covering its valleys and mountains (Médail and Quézel, 1997). Almost 700 species were cited in ethnobotanical data and named as Palestinian medicinal plants out of 2600 species found in Mediterranean area (Abu-Lafi et al., 2007). Therefore, appropriate measures for the preservation of these plant diversities in Palestine are demanding.

Traditionally, most of plants discriminated on morphological-basis; however, these methods still difficult to apply for an accurate discrimination and authentication use (Arif et al., 2010). Knowledge of genetic diversity within species is necessary for any improvement of cultivars, and biodiversity maintenance and restoration (Karp et al., 1997). DNA-based molecular markers, which are not affected by environmental conditions, have become increasingly important for surveying genetic diversity and genotype identification of medicinal plants (Nybom and Weising, 2007). These markers can also be taxonomically useful, i.e. for phylogenetic studies to distinguish plant species and subspecies (Lynch and Milligan, 1994; Mulcahy et al., 1995; Baigi et al., 2009; and Alamdary et al., 2011).

The genus *Thymus* which belongs to the family Lamiaceae, includes several hundreds of species distributed over world (Akcin, 2006), where Mediterranean basin is considered the main center of this herbal plant (Stahl-Biskup and Seaz, 2002). *Thymus* L.; referred to as Za`ater in Palestine, is an aromatic herb mostly used in food and medicine. Thymes volatile oil constituents are used as antiseptic, antioxidant, insecticidal, preservative and anaesthetic. As in phytotherapy, recent studies showed that *Thymus* species had strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant effects (Stahl-Biskup and Seaz, 2002).

Since Thyme genus taxonomy appears quite complex (Zarzuelo and Crespo, 2002), molecular markers could be promising.

Genetic markers are used to assess genetic diversity in many plants; were could be divided into four types: Single-locus marker; Dominant marker; Co-dominant marker; and Multilocus marker (Lynch and Milligan, 1994). The DNA polymorphism assays

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technologies include restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA markers (RAPD), and amplified fragment length polymorphisms (AFLP) (Powell et al., 1996). Due to its simplicity, the use of RAPD as molecular marker for taxonomic and systematic analyses of plants, as well as in plant breeding and genetic diversity assessments, has considerably increased (Arif et al., 2010). RAPD markers that can be generated by random amplification of DNA segments using arbitrary nucleotide sequence (short primers usually about ten nucleotides length). In many cases, few numbers of primers were necessary to identify polymorphism for a given species (Williams et al. 1990). Indeed, as Mulcahy et al. (1995) reported, a single primer may often be sufficient to distinguish among all of the sampled varieties. In this research study, RAPD (as the most popular molecular tools) was used to assess the genetic diversity among selected Thyme species, grown naturally in Palestine.

Material and methods

Plant material collection and Taxonomical analysis. Survey of the most popular Thyme species grown in Palestine was carried out firstly based on their natural spread, economical importance; and popularity had been carried out during the year 2009-2010. Five of Thyme species [*Thymus syriacus* Bioss synonym of *Majorana syriaca* (L.) Raf.; *Thymus fruticosus* (L.) Link synonym of *Micromeria fruticosa* (L.) Druce; *Thymus incanus* Sm synonym of *Calamintha incana* (Sm.) Boiss; *Thymus majorana* (L.) Kuntze synonym of *Origanum majorana*; *Thymus capitatus* (L.) Hoffmanns & Link synonym of *Coridothymus capitatus* (L.) Rchb.f.] were selected and from different areas in Palestine and potted under suitable growth conditions for assessment of their genetic diversity. The freshly collected Thyme species samples had been classified based on their morphological characters and their botanical properties regarding to botanists taxonomical experts and literatures guides, such as: flora of Palestine (Zohary, 1996), flora of Israel (Danin, 2006), and Traditional Arabic Palestinian Herbal Medicine (Ali-Shtayeh and Jamous, 2008) for ascertained. The classification and the scientific names of Thyme species were also determined and reviewed using plant list web site, WCSP (World Checklist of Selected Plant Families), PFAF (Plant For A future), wild flower of Israel. In addition to Thyme species Salvia officinalis was introduced to this study as a random outgroup. Leaves of each species were used as source of DNA in this research study.

DNA Extraction. Genomic DNAs were extracted from Thyme species young leaves of each as well as for *Salvia officinalis* (an outgroup) using plant DNA miniprepration (Dellaporta et al., 1983) and following the manufacturer instructions. Briefly, 50 mg of plant samples mixed with 500 µl extraction buffer consisting of 500 mM NaCl, 100 mM Tris–HCl pH 8.0, 50 mM ethylendiaminetetraacetic acid (EDTA), and 10 mM 2-mercaptoethanol and ground with mortar and pestle. After adding 33 µl of 20% SDS, the slurries were incubated at 65℃ for 10 min with vigorous shaking. Then, 160 µl of 5 M potassium acetate were added, and centrifuged for 10 minutes at 14000 rpm (MICRO 120, Hettich/Zentrifugen, Germany). 2 µl of RNAase were added to the collected supernatant and incubated at 37℃ for 10 minutes, followed by phenol – chloroform – isoamyl - alcohol (PCIA 25: 24:1) purification step. Then 500 µl PCIA were added to the supernatant and centrifuged for 10 minutes at 14000 rpm. The extracted DNA, then precipitated in 2 volumes of old isopropanol at -20 °C. The purified DNAs were resuspended in 60 µl TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA), and conserved at -20 °C for later use.

Primers selections and RAPD-PCR conditions. Referring to many literatures, ten decamer primers were selected to be tested for their ability to generate DNA polymorphism from Thyme species (Table 1). Primers were 60% - 70% G+C content and were ordered from two biotechnological companies (Metabion Hy.labs, Ltd., Israel) and (Sigma-Aldrich, USA).

Table 1. List of selected RAPD primers for polymorphic DNA generations

Primer Name	*Sequence 5`- 3`*	*Source*
PH-01	AACGCGCAAC	Arif *et al.*, 2010
KFP-6	TCCCGACCTC	Megendi *et al.*, 2010
OPAE-07	GTGTC GTGG	Megendi *et al.*, 2010
OPD-19	CTGGGGACTT	Sudré *et al.*, 2011
OPAG-02	CTGAGGTCCT	Tonk *et al.*, 2010
OPAN-08	AAGGCTGCTG	Tonk *et al.*, 2010
OPB-12	CCTTGACGCA	Tonk *et al.*, 2010
OPJ-06	TCGTTCCGCA	Tonk *et al.*, 2010
OPG-06	GTGCCTAACC	Ben el Hadj Ali, *et al.*, 2012

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Primer Name	*Sequence 5`- 3`*	*Source*
OPH-02	TCGGACGTGA	Chowdhury *et al.*, 2002

Polymorphic DNA was generated by using 10µl of plant target DNAs in 50µl PCR mixture, containing 4 µl of 5 µM primer (0.4 µM final concentration) (sigma-Aldrich and metabion hy.labs), 25µl one Taq quick load 2x master mix with standard buffer [1x contain: 40 mM Tris–HCl, pH: 8.9, 44 mM KCl, 3.6 mM MgCl2 an, 5% glycerol, 0.4 mM each dNTP, 25 unit/ml of Taq DNA polymerase, 0.06% IGEPAL CA630, 0.05% Tween-20, xylene cyanol FF, and tartrazine)] (New England Biolabs, USA). RAPD-PCR amplifications were performed using thermal cycler (Biometra, An Anaylik Jena Company, Germany) with the following thermal conditions: preheating for 7 min at 94℃, initial DNA denaturation for 3 min at 94℃, followed by DNA amplification for 35 cycles, at these parameters: 94℃/1min, 35℃ / 1 min, and 72℃ / 2 min. Final extension was carried out at 72℃ for 10 min, and the amplified products were stored at 4℃ for later gel electrophoresis analysis. The RAPD-PCR products were analyzed in 1.5% (w/v) agarose by gel electrophoresis with 1xTAE (pH 8), and visualized with ethidium bromide, under UV transilluminater (UVP ltd, USA), and photographed by digital camera (Canon, Japan).

Data analysis. DNA polymorphisms generated RAPD-PCR were calculated based on the presence or absence of amplified bands were scored as present (1) or absent (0) across the 5 Thyme species. The only clear major bands were subjected to scoring. The specific bands useful for identifying species were named with a primer number followed by the approximate size of the amplified fragment in base pairs. To construct a dendrogram describing the genetic relatedness of Thymes species, genetic distance was calculated based on the Jaccard coefficient (Jaccard, 1908) using the correlate module of SPSS software version19, after making a pairwise comparison between them relying on the proportion of shared bands that produced by each used primers. The Jaccard's coefficients, which are common estimator of genetic identity, were calculated as [Jaccard's coefficient = NAB/(NAB+NA+NB)]; where NAB is the number of bands shared by samples, NA represents amplified fragments in sample A, and NB represents fragments in sample B. Similarity matrices based on these indices were calculated. This was used using classify module of SPSS software version19.

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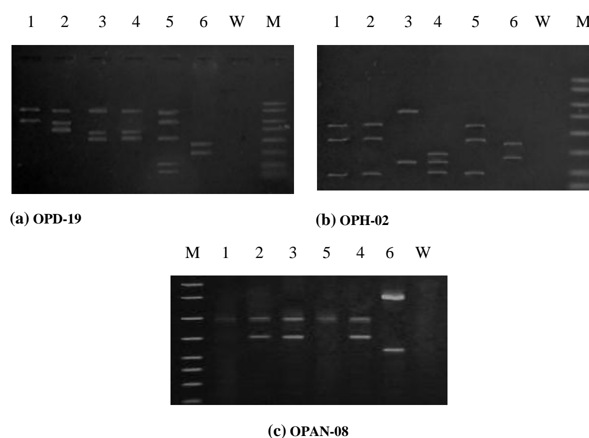

Figure 1. RAPD amplifications profiles patterns obtained using the primers: OPD-19; OPH-02; and OPAN-08, respectively as shown in (a); (b); and (c) analyzed in 1.5% agarose gel electrophoresis. (M) referred to PCR Marker [50-2000 bp]; (W) Water negative control; while the numbers (1) to (5) referred to Thymes species: *Thymus syriacus*; *Thymus majorana; Thymus incanus*; *Thymus capitatus;* and *Thymus fruticosus*. *Salvia officinalis* (6) was used as an outgroup

Results

Generation of Polymorphic DNA. The extracted DNAs from thyme species were screened for generation of polymorphic DNA fragments by RAPD. These primers (OPD-19, OPH-02, OPAN-08, PH-01, KFP-6, OPAE-07, OPJ-06, OPG-06) were found to produce fragments for the studied plant species separately, including an outgroup (*Salvia officinalis*); ensuring their reliability besides their sensitivity and specificity (Table 1). These primers were tested twice to verify their reproducibility and consistency of RAPD banding patterns for each tested species. The RAPD profiles using all samples simultaneously generated amplicons ranging from 50 to 1500 bp (Fig. 1).

The polymorphism generated by the primers (OPD-19, OPH-02, OPAN-08) were used to determine the genetic relationships among the selected five thyme species (Table 2). The percentage of polymorphisms was calculated based on Jaccard's coefficients to be (100%) by OPH-02 primer while for the primers OPD-19 and OPAN-08 were found (85.7% and 50%) respectively. As a total these three primers (OPD-19, OPH-02 and OPAN-08) were polymorphic and generated 78.6% average polymorphism across the studied five Thyme species.

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Table 2. The polymorphism generated by RAPD using three of decamer primers

Primer Code	*Scored Bands*	*Common Bands*	*Polymorphic Bands*
OPD-19	16	7	6
OPH-02	14	6	6
OPAN-08	8	2	1
Total	38	15	13
Mean	12.7	5	4.3

Genetic relationship between Thyme species. Data of RAPD profiles scanned from the five Thyme species with three reproducible primers were used to generate similarity coefficients with Jaccard measure using SPSS software version19 (Table 3). Pairwise similarity of banding patterns between the studied plant species were ranged from 0.18 to 0.67 for the primers (OPD-19, OPH-02, OPAN-08). The maximum pairwise similarity values (0.67) were observed between *Thymus syriacus* versus *Thymus majorana; Thymus syriacus* versus *Thymus capitatus*; and *Thymus fruticosus* versus *Thymus incanus*, suggesting them to be most closely related species. The lowest similarity value was observed between *Thymus syriacus* and *Thymus incanus* (0.18) indicating them as genetically most diverse. *Thymus majorana* showed closest relationship with *Thymus capitatus*, *Thymus fruticosus*, and *Thymus incanus* having similarity values of 0.50, 0.42 and 0.33, respectively. *Salvia officinalis* as an outgroup, confirmed the reliability of the RAPD method and similarity analysis tests to show quite divergent from other thymes species with similarity coefficient range from 0.00- 0.08 as shown in the similarity matrix Table 3.

Table 3. Jaccard's coefficient based similarity matrix for the five *Thymus* species and *Salvia officinalis* as an outgroup by RAPD pattern generated by the primers OPD-19, OPH-02, and OPAN-08

Thymus species	*T. syr.*	*T. maj.*	*T. inc.*	*T. fru.*	*T. cap.*	*S. off.*
T. syriacus	1.00					
T. majorana	0.67	1.00				
T. incanus	0.18	0.33	1.00			
T. fruticosus	0.27	0.42	0.67	1.00		
T. capitatus	0.67	0.50	0.23	0.31	1.00	
S. officinalis	0.08	0.07	0.00	0.07	0.06	1.00

Based on the Jaccard coefficient, the obtained distance coefficients were used to construct a dendrogram using classify module of SPSS software version19 (Figure 2). The cluster tree analysis showed that thyme species' were divided into two main groups: first group including *Thymus syriacus*, *Thymus majorana* , and *Thymus capitatus*, and the second group including *Thymus fruticosus*, and *Thymus incanus*. However, *Salvia officinalis* was found to be quite divergent and did not fall in any of these two clusters (Figure 2) with similarity coefficient of 0.08.

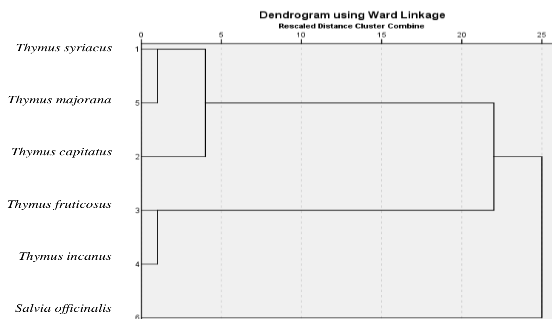

Figure 2. The dendrogram cluster tree was generated by using classify module of SPSS software version19, for five Thyme species where *Salvia officinalis* was used as an outgroup.

Discussion and Conclusion

Medicinal plants have broad popularity usage in Palestinian culture as sometimes alternative medicines for many chemically synthesized drugs (Azaizeh et al., 2006). Although, medicinal plants contain powerful natural and bioactive chemical constituents that are available to cheaply compared with those available at the pharmacy (Halberstein, 2005). However, most of these medicinal plants are subjected to extensive harvesting, habitat destruction due to buildings and agricultural land expansions, which altogether threatened their existence as their relative abundance decreased annually (Abou Auda, 2010; Alkowni and Sawalha, 2012). Thus most of these plants including thymes species were considered as threatened species. Many tentative trials to conserve these species were carried out in the recent years (unpublished data) where thymes species had been included.

Conventional methods for discrimination among Thymes species were usually used which sometimes confusing imprecise. In this study, molecular tools could be much reliable for measuring the genetic relationship among thyme species'. To our best knowledge, no molecular tool had been reported in

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studying Thymes grown in Palestinian territories. Five of thyme species were identified and subjected to RAPD as the simplest molecular markers that able to recognize the genetic relationships among them.
Nearly 78.6% of the bands were found as DNA polymorphic, confirmed the results obtained from different research studies from different countries on thymes species with reported polymorphism percentage varies from 62-92% (Bagherzadeh, 2009; Alamdary et al., 2011; Ben el Hadj Ali et al., 2012; Pluhár et al., 2012; and Khalil et al., 2012).
Using three of universal primers ((OPD-19, OPH-02 and OPAN-08) out of eight were found to generate relatively high level of DNA polymorphism among five thyme species (78.6%) in this study. These results obtained in this study, might suggest that these chosen primers which screened in RAPD tests were able to generate polymorphic bands on most genetic diverse loci among the thymes species as proved byTonk et al., (2010). The primers OPD-19, OPH-02, and OPAN-08 were able to produce 7, 6, and 2 bands in accordance with other RAPD research studies. For examples, the KFP-6 primer produces 6 bands when it was used in studying genetic diversity among cultivated and non-cultivated Moringa oleífera Lam. provenances and assessed by RAPD markers (Megendi et al., 2010), while the OPD- 19 primer was produced 2 bands when used in molecular characterization of 27 wild genotypes of hawthorn (Crataegus azarolus L.) (Dumireih et al., 2010). Using RAPD Technique in genetic variability study of selected Turkish oregano (Origanum onites L.) clones, these primers OPAG-18, OPAG-06, OPAC-12, OPAG-02, and OPG-07 were able to produce 3, 5, 5, 6, and 6 polymorphic bands (Tonk et al., 2010) suggesting that limited numbers of polymorphic bands was common for that. The same was obtained in other RAPD analysis of *Thymus* species growing in eastern Anatolia region of Turkey, using primers OPA-2, OPH- 16, and OPH- 18 with totally 8 polymorphic bands were produced (Sunar et al., 2009).
In Syria, a research study for determination of genetic variation among *Thymus vulgaris* populations by RAPD markers, almost 27 primers were used which were able to produce 198 total bands, with a mean of 7.3 bands for each primer (Khalil et al., 2012).
The mean score of 12.7 bands, obtained using the three primers in this research study was good enough to reveal the genetic relationships among the selected Thymes species' subjected to RAPD tests, compared with previous studies. This was in accordance with the reported RAPD studies for 13 accessions of Thymes species that amplified by primers (OPP3) and (DPA 9) (Alamdary et al., 2011). Due to significant similarity found between their DNA sequences in the amplified region, sequence-based analysis were failed to distinguish between thymes species in other studies. So far, RAPD primers (OPD-19 and OPH-02) were able to distinguish clearly between these five different Thyme species that subjected to this study. In addition to that, RAPD primers were able to distinguish taxa below the species level (Choo et al., 2009), since RAPD analysis reflects both coding and non-coding regions of the genome (Vanijajiva et al., 2005). Thus, it would not be surprisingly that RAPD was able to detect the genetic diversity among different plant species in Palestinian figs, *Faba beans*, wheat, …etc, using the same procedure with same or different primers that used in these studies (Al-Fares and Abu-Qaoud, 2012; Basheer salimia et al., 2012)
In this study, the optimized RAPD procedure was able to distinguish clearly among the five different Thyme species where Primer OPD-19 was found to differentiate between any two Thyme species except *Thymus incanus* and *Thymus capitatus*. It was also observed that OPD-19 primer gave 2 unique bands for *Thymus fruticosus*. The primer OPH-02 was found to distinguish *Thymus incanus* from any of four Thyme species subjected to this study with a single specific band. In the other hand, primer OPH-02 generated one specific band for *Thymus capitatus*, which was absent in all other species; thus enabling it to distinguish between *Thymus capitatus* and of other Thyme species used in this study. Even though, primer OPAN-08 did not show any discriminatory bands for all species, as similar banding patterns were observed for *Thymus majorana*, *Thymus incanus*, and *Thymus capitatus*, and also similar band for *Thymus capitatus*, and *Thymus syriacus*.
Unexpectedly, Primers (PH-01, KFP-6, OPAE-07, OPJ-06, and OPG-66) were able to generate a single band for *Thymus incanus*, *Thymus capitatus*, *Thymus syriacus*, *Thymus fruticosus*, and *Thymus majorana* respectively. These results were considered to be promising for future specific DNA fingerprinting studies on thyme species variants; suggesting to researchers to use them for specific detection of these species or expanding that work on other medicinal plants. This discovery was ascertained by the reported studies on genetic divergence among *Dimorphandra* spp. accessions where the primer OPAE-09 produced only a single polymorphic band (Sudré et al., 2011), as well as primer OPC-6 which generated only one specific band to *Tinospora cordifolia*; *Emblica officinalis*, and *Tribulus terestris* (Shinde et al., 2007).
The coefficient value of 0.67 that was revealed between *Thymus syriacus* versus *Thymus majorana*;

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Thymus syriacus versus *Thymus capitatus*; and *Thymus fruticosus* versus *Thymus incanus*, was within the range of those obtained in previous studies: among different thymes species accessions (Ben el hadj ali et al., 2012: for *Thymus capitatus* and *Thymus algeriensis*); (Echeverrigaray et al., 2001: *Thymus vulgaris* cultivares), and (Alamdary et al., 2011: for 13 accessions of *Thymus migricus* and *Thymus daenensis*).

Since the information on genetic relatedness and diversity of available breeding genotypes increase the success of any plant-breeding programs, this study, revealed successfully the genetic diversity of some selected Palestinian Thyme species that could be highly valuable for any putative Thyme-breeding program could be carried in Palestine.

In general, RAPD procedure was able to distinguish clearly the selected different Thyme species subjected to this study; and also could be potentially used for identifying Thyme species from any mixed populations; a similar approach had been successfully used for molecular diagnosis of several species and cultivars by many other researchers (Yamamoto and Duich, 1994; Sosinski and Doucher, 1996). Also these markers could be used as a method of choice for identifying components for herbal medicine complex since RAPD technique had been used for determination of different components presented in herbal formulation (Cheng et al., 1987; Cheng et al., 1997). So, these will contribute significantly in quality control.

Beside it gives information about genomic variability below the species level (Williams et al., 1990), RAPD provides relatively quick results, less time-consuming and low expensive (Arif and Khan, 2009). It is worth to mention that the RAPD bands pattern obtained in this research were reproducible under our lab and experimental conditions. Nevertheless, further studies for finer molecular analysis of medicinal plants genotyping's to overcome the discrepancies left unresolved by RAPDs were advisable.

The use of RAPD markers had enabled to discriminate of 5 Thyme species grown in Palestine and can be used successfully for any clonal selection and improvement of such species cultivars in any future studies. These results can be further used to manipulate genetic determinants of horticulturally important traits and to characterize the basis of productivity of Thymes. RAPD markers were proved to be a useful tool in germplasm characterization and diversity analysis of thymes, and can be used beside other molecular markers as AFLP, ISSR and SSRs. Therefore, our findings provide guidance for identification of Thyme species, and help in their subsequent management and utilization in sustainable ways to combat human and natural pressures on these valuable natural resources.

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