BIODIVERSITY OF SOME PTERIDOPHYTES SPECIES AND THEIR MORPHOLOGICAL CHARACTERISTICS FROM THE SOUTHWEST OF SAUDI ARABIA

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Abstract. The biodiversity of 11 fern populations representing nine species belonging to three families (*Aspleniaceae*, *Adiantaceae*, and *Pteridaceae*) was evaluated using morphological variations and molecular polymorphism generated by 12 ISSR primers. Substantial variations were scored in 16 morphological characters and reflected in the cluster analysis of the examined accessions based on morphological variation. In the phylogenetic relationships generated based on ISSR polymorphism and morphological variations, the three *Cheilanthes* species clustered with *Asplenium adiantum nigrum* and were delimited from other species, which supports that *A. adiantum nigrum* may be related to *Cheilanthes* species. In the meantime, the two populations of *Asplenium ceterach* and the two populations of *Asplenium aethiopicum* were differentiated as one cluster. These results are congruent with the cluster based on the analysis of ISSR data except for the association of *Adiantum capillus veneris* and *Asplenium trichomanes*, which revealed that *A. trichomanes* may be related to *Adiantum capillus veneris*. Also, these results were confirmed by the tree obtained based on the Elucedine coefficient and with the population grouping based on principal component analysis (PCA) using the PAST program. Also, Online Marker Efficiency Calculator (iMEC) supports the use of ISSR markers for examining the genetic diversity across fern genotypes.
Keywords: *ISSR markers, phylogenetic, iMEC analysis, ferns*

Introduction

Because of its unique environmental conditions, the south-west of Saudi Arabia region has highly diverse vegetation, particularly in Abha province, which is characterized by a high elevation of about 2700 m above sea level, making the area rainy and wet most of the year, and this region holds a variety of plants belonging to the pteridophytes that are rarely found in any other region of the Kingdom of Saudi Arabia (Alshehri and Moustafa, 2018). Ferns have over 90 species and 42 genera and are found worldwide, particularly in subtropical and tropical climates, then moist temperate regions. They are frequently used in landscaping, gardening, traditional medicine, and as vegetables (Ranil and Bussmann, 2021).

Most fern plants have medicinal and economic importance. *Asplenium ceterach* L. (syn. *Ceterach officinarum* Willd.) is a small fern that is commonly found on rocks and walls. Due to its diuretic, anthelminthic, emollient, and expectorant properties, it is commonly used in traditional medicine (Malamas and Marselos, 1992). Moreover, the antimicrobial and antioxidant activities, as well as the potential protection of this fern against DNA damage, have been reported (Berck, 2011; Karadeniz, 2015). Also, Khoja et al. (2022) explained that pteridophytes are a significant plant group in Asian traditional medicine. They were discussed in relation to homeopathy, ayurveda, tribal medicine, and unani medicine. Indian pteridophytes have been extensively studied for their potential ethnomedical use.

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The pteridophytes have an extremely broad range of habitats in addition to their species composition. Therefore, they have been classified as aquatic, terrestrial, and epiphytic. However, most of the species of *Psilotum*, *Adianum*, and *Cheilanthes* grow on rocks (Kessler and Lehnert, 2009). They are vascular plants (with phloem and xylem) with differentiated roots, stems, and leaves on the body of the sporophyte, but they don't produce flowers or seeds (Cantino et al., 2007; Christenhusz et al., 2011). Pteridophytes consist of two classes: Lycopodippsida (lycophytes), which includes three orders, and Polypodiopsida (ferns), which includes 11 orders. According to recent research on land plants, seed plants originated from pteridophytes, which are more closely related to ferns than lycophytes (Kenrick and Crane, 1996; Smith et al., 2006).

Ferns identification can be difficult because many species closely resemble one another. The morphological characteristics of ferns were used as the basis for previous attempts to characterize them in Saudi Arabia (Collenette, 1999; Chaudhary, 2001). Al-Turki (2004) recorded two species of *Selaginella*, namely *Selaginella yemensis* (Swart) and *Selaginella imbricata* (Forssk). Al-Shehri (2002) collected nine species, including *Adiantum capillus veneris, Adiantium nigrium*, *Asplenium ceterach*, *Asplenium viridie*, *Asplenium aethiopicum*, *Asplenium filare*, *Asplenium trichomanes,* and *Cheilanthes pteridioides,* and they were examined for abundance; some of them were discovered to be new records for southwest Saudi Arabia. The use of morphology features in delimiting genetic resources is limited and inadequate as the features used could be influenced by the environment. There is a wide gap in the knowledge of genetic diversity and molecular characterization of ornamental plants, and there is very limited literature on genetic diversity among ferns in Saudi Arabia using molecular markers. Where Alshehri and Moustafa (2018) surveyed molecular biodiversity among only five samples of ferns from Saudi Arabia using RAPD and ISSR markers, the results indicated a close relationship between *Asplenium ceterach* and *Asplenium aethiopicum.*

Recent studies have linked plastid and nuclear genes, frequently with observations of spore size and appearance, to track the origins of hybrids and taxonomic revision. Chang et al. (2018) used a "diploids-first" approach (Beck et al., 2010) within an integrative framework including morphology and plastid and nuclear DNA to clarify the taxonomy of the widespread *Asplenium normale* species complex. Fujiwara et al. (2018) used plastid and cytology, as well as nuclear DNA, to piece together the evolution of *Lepisorus* in Japan. Hori et al. (2014) sorted out the *Dryopteris varia* complex, a collection of several sexual and apogamous taxa, which is a highly diversified group.

Molecular markers such as random amplified polymorphic DNA (RAPD), and inter-simple sequence repeats (ISSR) are easy to use with a high level of polymorphism and reproducibility. These markers have been reportedly used in genetic diversity studies of wild species in Saudi Arabia as *Solanum* species (Ahmed et al., 2019; El-Shaboury et al., 2020). Therefore, a current study has been undertaken to explore the biodiversity of some pteridophyte species, namely *Cheilanthes pteridiodes* (Reichard) C. Chr, *Cheilanthes vellea* (Aiton) Domin, *Cheilanthes coriacea* Decne., *Asplenium ceterach* L., *Asplenium adiantum nigrum* L., *Adiantum capillus veneris* L., *Asplenium aethiopicum* (Burm. P.) Bech., *Asplenium viride* Huds., and *Asplenium trichomanes* L., growing in the southwestern highlands of Saudi Arabia using morphological variation and ISSR marker profiling its impact on their taxonomic relationships and a detailed description of the morphological characters for each accession/species to be used as a key for researchers to easily identify these plants.

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Materials and Methods

Plant material and morphological measurements

Samples of mature plants from 11 accessions representing 9 fern species (three samples for each accession) were collected in their natural habitats in the Aseer region of Saudi Arabia's southwest highlands. The names and sites of collection of the collected accessions /species are given in *Table 1*. *Fig. 1* shows photographs of the studied species and the arrangement of sori on their fronds. A detailed description of the sixteen morphological characters for each accession/species including quantitative and qualitative characters was performed and the average value of every quantitative character ± standard deviation was calculated, and the state of the qualitative character was recorded based on the species descriptions of Collenette (1999), and Chaudhary (2001) are given in *Table 2*. Voucher specimens of the nine ferns accessions/species have been deposited at the Herbarium of Biological Sciences Department, Faculty of Science and Humanities, Shaqra University, Al-Dawadimi, Saudi Arabia.

Table 1. *The area, GPS location, and elevation of localities from which the examined accessions of fern species were collected*

Se	Collected Species	Family	Code	Area	Elevation	GPS Location
1	*Cheilanthes pteridiodes* (Reichard) C. Chr.	Pteridaceae	P(1)a	Al hashr	1517	17°28'21.5"N 43°02'57.7"E
2	*Cheilanthes vellea* (Aiton) Domin	Pteridaceae	P(1)b	Jabal Fayfa	537	17°14'08.0"N 43°03'16.9"E
3	*Asplenium ceterach* L.	Aspleniaceae	P(2)a	Jabal Fayfa	537	17°14'08.0"N 43°03'16.9"E
4	*Asplenium aethiopicum* (Burm. P.) Bech.	Aspleniaceae	P(3)a	Abha	2261	18°12'44.2"N 42°29'17.2"E
5	*Asplenium aethiopicum* (Burm. P.) Bech.	Aspleniaceae	P(3)b	Elsoda	2845	18°16'05.0"N 42°22'12.7"E
6	*Cheilanthes coriacea* Decne.	Pteridaceae	P(1)c	Al hashr	2048	17°28'21.3"N 43°02'57.7"E
7	*Asplenium adiantum nigrum* L.	Aspleniaceae	P(4)	Jabal Fayfa	1436	17°14'46.9"N 43°05'27.9"E
9	*Asplenium ceterach* L.	Aspleniaceae	P(2)b	Al hashr	2042	17°28'21.5"N 43°02'57.7"E
8	*Adiantum capillus veneris* L.	Adiantaceae	P(5)	Jabal Fayfa	955	17°15'18.8"N 43°04'57.7"E
10	*Asplenium viride* Huds.	Aspleniaceae	P(6)	Elsoda	2985	18°15'58.2"N 42°22'05.9"E
11	*Asplenium trichomanes* L.	Aspleniaceae	P(7)	Elsoda	2957	18°16'05.0"N 42°22'12.7"E

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Figure 1. *Photographs of the nine pteridophytes species and arrangement of sori on their fronds collected from different sites in the south-west of Saudi Arabia* ***A****: C. pteridiodes* ***B****: C. vellea* ***C****: A. ceterach* ***D****: A. aethiopicum* ***E****: C. coriacea* ***F****: A. adiantum nigrum* ***G****: A. capillus veneris* ***H****: A. viride* ***I****: A. trichomanes*

DNA extraction and ISSR fingerprinting

DNA was extracted and purified from the young leaves of the mature plants of the collected samples representing all accessions/species using Qiagen DNeasy™ Plant Minikit following the protocol of the manufacturer (Qiagen Inc, Valencia, CA, and the USA). Twelve ISSR (inter simple sequence repeats) primers have been secured for DNA fingerprinting. In the amplification reactions of genomic DNA, a total of 25 µl reaction mix was prepared (12.5 µl Thermo Scientific Maxima Hot Start PCR Master Mix (2X), 0.5 µl Primer, 0.5 µl Template DNA and 11.5 nuclease-free water-R0581). Amplification conditions were improved using a gradient Biometra Uno thermal cycler, Germany. 20 µl of the PCR-products of each primer and 2 µl of loading buffer were mixed and loaded into the wells of agarose gel 1.7%. A Gel Works 1D advanced gel documentation device (UVP, UK) was used to view and photograph the ISSR fingerprinting. *Fig. 2* shows the ISSR fingerprinting profile produced by three ISSR primers for the examined ferns accessions/species. In the meantime, the unambiguous and clear ISSR bands were scored. For data analysis, each ISSR band was considered a single locus and scored as 1 for presence and 0 for absence. The name, sequence, number of polymorphic bands, and percentage of polymorphism of 12 ISSR primers are given in *Table 3*.

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Table 2. *A list of morphological traits and their measurements and state of the examined 11 Ferns accessions representing nine species (1 –11); coded as given in Table 1*

Ser.	Character	Fern species/accession						
		C. pteridiodes	***C. vellea***	***A. ceterach*****(1)**	***A. aethiopicum*****(1)**	***A. aethiopicum*****(2)**	***C . coriacea***	***A. adiantum***
1	Plant height (cm)	10.75 ±1.53	7 ±5.86	10 ±1.15	41 ±3.64	58 ±4.93	19 ±3.06	28 ±4.04
2	Plant habit	Herb	Herb	Herb	Herb	Herb	Herb	Herb
3	Stems texture	Hairy	Hairy	Smooth	Hairy	Hairy	Hairy	Hairy
4	Stem color	Brown front and back	Brown front and back	Green	Green front and Brown back	Green front and Brown back	Brown front and back	Green front and Brown back
5	Number of fronds per axis	35 ±5.86	18±3.45	26±4.18	36±2.36	55±6.47	26±3.88	34±4.53
6	Habit of the stem	Herbaceous	Herbaceous	Herbaceous	Herbaceous	Herbaceous	Herbaceous	Herbaceous
7	Rhizoids type	Adventitious	Adventitious	Adventitious	Adventitious	Adventitious	Adventitious	Adventitious
8	Frond width(cm)	5±1.45	3±0.75	3±1.10	7±1.85	8±2.10	5±0.95	6.5±2.05
9	Frond color	Pale green	Pale Green	Green	Pale green	Pale green	Green	Green
10	Pinnule blade	Ovate	Ovate	Ovate	Conical- Lobed	Conical- Lobed	Conical- Lobed	Conical- Lobed
11	Pinnule margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire
12	Frond distribution	Opposite	Alternate	Alternate	Opposite	opposite	opposite	opposite
13	Pinnule apex	Rounded	Rounded	Rounded	Acute	Acute	Rounded	Acute
14	Arrangement of sporangia	Regular	Regular	Irregular	Regular	Regular	Regular	Regular
15	Sporangia distribution on frond	Edges- Back	Edges- Back	Scattered-Back	Frond veins	Scattered-Back	Frond veins	Scattered-Back
16	Sporangia color	Dark brown	Pale brown	Dark brown	Brown	Brown	Brown	Brown

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Ser.	Character	Fern species/accession			
		***A. ceterach*(2)**	***A. capillus veneris***	***A. viride***	***A. trichomanes***
1	Plant height (cm)	17 ±1.15	23 ±4.04	28 ±1.15	40 ±7.64
2	Plant habit	Herb	Herb	Herb	Herb
3	Stems texture	Smooth	Smooth	Smooth	Hairy
4	Stem color	Green	Brown front and back	Green	Brown front and back
5	Number of fronds per axis	32±4.73	18±0.59	33±6.01	25±4.52
6	Habit of the stem	Herbaceous	Herbaceous	Herbaceous	Herbaceous
7	Rhizoids type	Adventitious	Adventitious	Adventitious	Adventitious
8	Frond width(cm)	4±0.85	6.5±1.05	2.5 ±0.22	1.8 ±0.42
9	Frond color	Dark green	Green	Green	Green
10	Pinnule blade	Ovate	Conical	Conical	Conical
11	Pinnule margin	Entire	Entire	Pinnatifid	Entire
12	Frond distribution	Alternate	Alternate	Alternate	Opposite
13	Pinnule apex	Rounded	Rounded	Rounded	Rounded
14	Arrangement of sporangia	Irregular	Regular	Regular	Regular
15	Sporangia distribution	Scattered-Back	Edges- Back	Frond veins	Frond veins
16	Sporangia color	Dark brown	Brown	Brown	Brown

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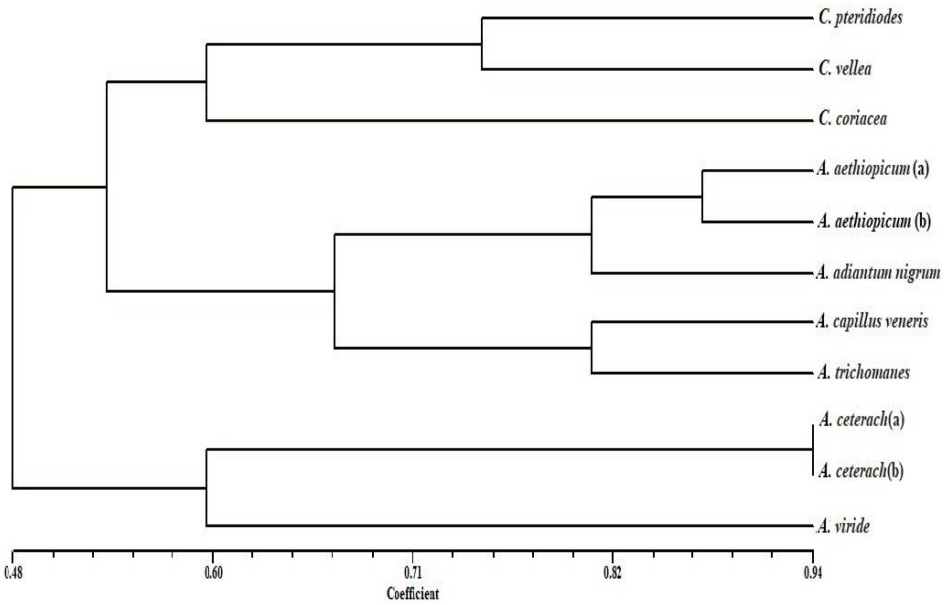

Figure 2. *UPGMA, NTSYS-pc distance tree, based on the analysis of morphological traits, computed with SM coefficient, showing the relationships among the examined ferns accessions*

Table 3. *The number of polymorphic bands and percentage of polymorphism in the ISSR profile of 12 primers in the genome of the collected pteridophytes species/populations*

Serial	Primer code	Sequence (5'→3')	Number of polymorphic bands	Number of Unique bands	Total number of bands	Polymorphism percentage (%)
1	HB-8	$(GA)_6GG$	8	1	9	100
2	HB-9	$(GT)_6GG$	7	4	11	100
3	HB-10	$(GA)_6CC$	6	1	7	100
4	HB-11	$(GT)_6CC$	15	2	17	100
5	HB-12	$(CAC)_3GC$	11	2	13	100
6	HB-13	$(GAG)_3GC$	11	2	13	100
7	HB-14	$(CTC)_3GC$	9	2	11	100
8	HB15	$(GTG)_3GC$	5	2	7	100
9	807	$(AG)_8T$	16	3	19	100
10	808	$(AG)_8C$	9	6	15	100
11	814	$(CT)_8TG$	13	1	15	93.3
12	826	$(AC)_8C$	10	1	11	100
Total			**120**	**27**	**148**	**-**

Data analysis

For numerical analysis of data, the morphological characters were given codes ranging between 0 and 3. The relationships of Ferns accessions/species were assessed based on variations of the morphological characters and molecular fingerprinting separately and combined. The data were analyzed using two software programs; NTSYS-pc software (Rohlf, 2002) was used to construct trees elucidating the relationships and calculate the

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similarity level among the examined ferns population/species using a simple matching coefficient (SM) (Sokal and Michener, 1958). The clustering of the examined accessions/species was also performed, based on squared Euclidean distance to produce a distance tree using the PAST-pc Version 3.22 developed by Hammer et al. (2001). In addition, a Principal Component Analysis (PCA) in the PAST-pc was used to construct a scatter diagram of the examined accessions. The PCA is often applied to assign the variables to genotypes and to classify accession based on their sensitivity or tolerance to drought stress. PCA is sensitive to the relative scaling of the original variables in the PCA scatter plotting visualization (Hammer et al., 2001). All morphological measurements were independently performed in triplicates and are represented as the mean ± standard deviation (SD). The data were subjected to a one-way analysis of variance (ANOVA) Statistica 7.1 (Statsoft, 2007).

iMEC analysis

Twelve ISSR primers with eleven fern genotypes were subjected to analysis using the online iMEC software application. *Table 4* provides a summary of the calculations made as a result. The effectiveness of the primers in distinguishing between different fern genotypes was assessed using the D parameter (discriminating power of primer), which was described by Ahmed et al. (2019). According to Amiryousefi et al. (2018), the Online Marker Efficiency Calculator (iMEC software) is an easy-to-use program that computes seven fundamental polymorphism indices for individual markers, including the heterozygosity index (H), polymorphism information content (PIC), discriminating power (D), effective multiplex ratio (E), marker index (MI), arithmetic mean heterozygosity (H_{avp}), and resolving power (R). The iMEC application is available at https://irscope.shinyapps.io/iMEC/.

Table 4. *Polymorphism statistics estimated using the iMEC tool for 12 ISSR primer types using the data set from the 11 fern genotypes*

Serial	Primer code	Total number of bands	H	PIC	E	Rp	H.av	MI	D
1	HB-8	9	0.4681	0.3648	3.3636	3.6363	0.0047	0.0159	0.8627
2	HB-9	11	0.4111	0.3899	3.1818	5.6363	0.0033	0.0108	0.9180
3	HB-10	7	0.4472	0.3744	2.3636	4.1818	0.0058	0.0137	0.8889
4	HB-11	17	0.4895	0.3546	7.2727	9.4545	0.0026	0.0190	0.8182
5	HB-12	13	0.4504	0.3729	4.4545	7.0909	0.0031	0.0140	0.8841
6	HB-13	13	0.4821	0.3582	5.2727	6.3636	0.0033	0.0177	0.8371
7	HB-14	11	0.4849	0.3568	4.5454	4.0	0.0040	0.0182	0.8312
8	HB15	7	0.4897	0.3545	4.0	2.7272	0.0063	0.0254	0.6766
9	807	19	0.4321	0.3810	6.0	8.9090	0.0020	0.0124	0.9013
10	808	15	0.4021	0.3935	4.1818	5.4545	0.0024	0.0101	0.9235
11	814	15	0.4977	0.3505	7.0	4.5454	0.0030	0.0211	0.7837
12	826	11	0.4996	0.3496	5.6363	4.9090	0.0041	0.0232	0.7395
Average			**0.474**	**0.361**	**4.772**	**5.5757**	**0.00029**	**0.016**	**0.85**

Note: Heterozygosity index (H), Polymorphic Information Content (PIC), Effective multiplex ratio (E) Arithmetic mean of H ***(H.av)***, Marker Index (MI), Discriminating power (D), Resolving power (Rp)

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Results

Morphological variation among pteridophytes accessions/species

Sixteen morphological traits were applied for the collected 11 pteridophytes accessions (*Table 2*). The morphological qualitative characters revealed significant inter-specific variation and few differences within species, particularly in stem color, pinnule blade, frond distribution, pinnule apex, and sporangia distribution on frond (*Fig. 1*). As for the quantitative characters, the accessions collected from Abha, El-Soda and Al-hasher mountains at high elevations and moderate temperatures have in general larger plant size compared to accessions at lower elevations in more arid areas. For example, plant height of plants collected from higher elevations have much taller plants for example, *A. ceterach*$_{(2)}$ that was collected from Al hashr mountains at an elevation of 2042 m asl scored a plant height of 17 ±1.15 cm compared with the same species that collected from a more low elevation from Jabal Fayfa at an elevation of 537 m asl scored a plant height of 10 ±1.15 cm (*Table 2*). *Asplenium aethiopicum,* which was collected from El Soda at an elevation of 2845 m asl, has a plant height of 58 ±4.93 cm, while, the other population, which was collected from Abha at an elevation of 2261 m asl, has a plant height of 41 ±3.64 cm. Shorter species e.g. *C. vellea* was collected from Jabal Fayfa (7 ±5.86 cm) at 537 m asl. Some other species have intermediate heights, e.g., *A. capillus veneris*, 23 ±4.04 cm for material collected from Jabal Fayfa (955 m asl).

Diversity based on morphological variations

Based on the simple matching coefficient among the studied species, the UPGMA-NTSYS-pc (*Fig. 2*) separated the eleven examined accessions into three clusters; one cluster separated the two accessions of *A. ceterach* at a high similarity level with the accession of *A. viride* at a low similarity level. The three *Cheilanthes* species were separated in the second cluster, where *C. pteridiodes* and C. *vellea* clustered at a high similarity level where they clustered with *C. coricea* at a relatively low similarity level. The remaining taxa were divided into two clusters; one cluster separated the two accessions of *A. aethiopicum* and the accession of *A. adiantum nigrum*. *Adiantum capillus veneris* separated with *A. trichomanes* in the other cluster at the same similarity level.

ISSR fingerprinting polymorphism in pteridophytes accessions

Data generated by the ISSR fingerprinting pattern of 11 pteridophytes accession/species were analyzed and the ISSR profiles produced by three ISSR primers are shown in *Fig. 3*. ISSR profiles produced 148 bands; 120 bands were polymorphic, one was monomorphic, and 27 were unique. The highest number of bands (19) was produced by primer 807, and the lowest number (7) was produced by the two primers HB-10 and HB-15. The percentage of polymorphism of all primers was calculated and given in *Table 3*.

Polymorphism indices for individual primers were determined using iMEC software as fundamental metrics. *Table 4* provides more information on the fundamental measurement polymorphism indices for the primers. Where the heterozygosity index (H) with an average of 0.474. The polymorphism information content (PIC) for each primer with an average of 0.361. The effective multiplex ratio (E) ranged from 2.3636 recorded with the primer HB-10 to 7.2727 recorded with the primer HB-11 with an average of 4.772. The primer resolving power (R) varied from 2.727 (HB-15) to 9.4545 (HB-11) with an average of 5.575. The arithmetic means H (Havp) ranged from 0.0020 with the

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primer 807 to 0.0063 with the primer HB-15 with an average of 0.00029. The lowest value for marker index (MI) was achieved with primer (HB-15) as 0.0254 to 0.0101(808) with an average of 0.016. The primer discriminating power (D) varied from 0.6766 with (HB-15) to 0.9235 with (808) with an average of 0.85.

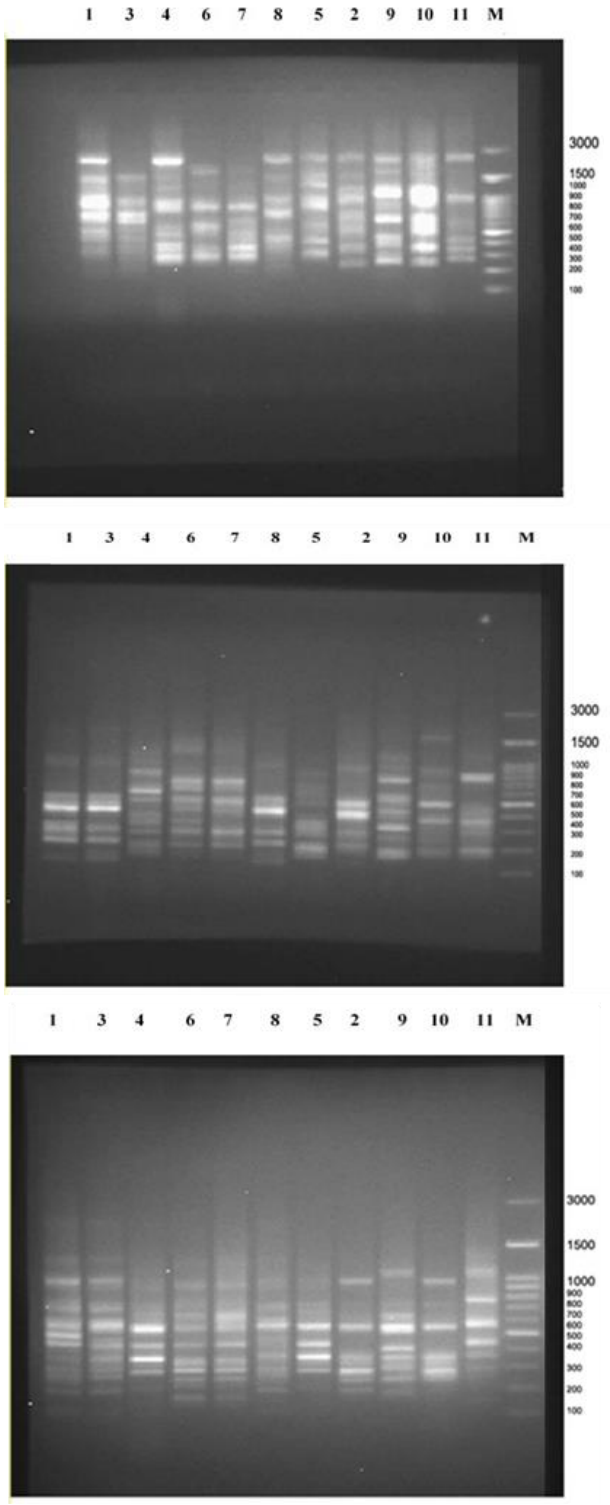

Figure 3. *ISSR fingerprinting profile produced by three ISSR primers for ferns accessions as coded in Table 1. *M: 100 bp marker DNA ladder*

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Diversity based on ISSR markers

The tree based on the ISSR fingerprinting analysis (*Fig. 4*) revealed that the pteridophytes accessions/species were divided into three groups, where *A. viride* was differentiated as a separate identity at a relatively high distance from the examined accessions. *Adiantum capillus veneris* separated with *A. trichomanes* at a relatively high distance in one group. The second group contained the two accessions of *A. aethiopicum*, which clustered at a low genetic distance, and the two accessions of *A. ceterach*, which had a relatively low genetic distance. The three *Cheilanthes* species were clustered with *A. adiantum nigrum* in the other group.

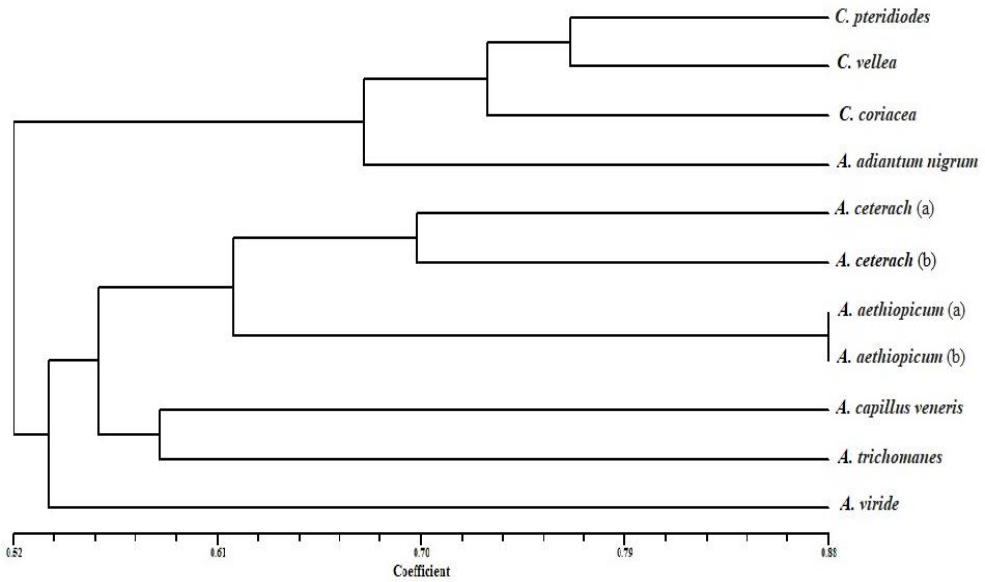

Figure 4. *UPGMA distance tree computed using the NTSYS-pc, based on the analysis of ISSR data, showing the relationships among the examined ferns accessions*

Relationships of species based on morphological variations in conjunction with ISSR markers

The diversity of the examined accessions/species based on morphological variations and ISSR marker polymorphism was assessed using a clustering analysis, based on the Euclidean equation using the software PAST (*Fig. 5a*) and a PCA scatter plot (*Fig. 5b*). The cluster tree indicated more differentiation between the three species, *A. capillus veneris*, *A. trichomanes*, and *A. viride*. Whereas the three *Cheilanthes* species were clustered with *A. adiantum nigrum*. The two *A. aethiopicum accessions* clustered with the two *A. ceterach accessions.* The examined accessions/species were clearly differentiated into three groups by the PCA scatter plot (*Fig. 5b*), which agreed with their separation in the cluster tree. The three groups are 1. *C. pteridiodes*, C. *vellea*, *C. coricea*, and *A. adiantum nigrum.* 2. Two accessions of *A. aethiopicum* and two accessions of *A. ceterach*; 3. *A. capillus veneris*, *A. trichomanes*, and A. *viride.*

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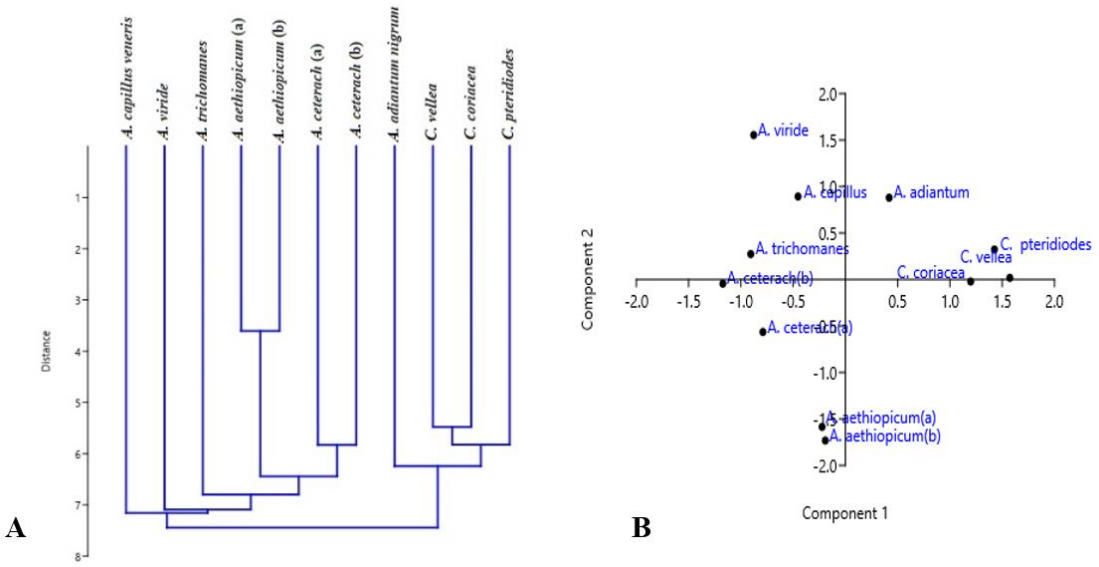

Figure 5. *UPGMA distance tree (A) and a PCA scatter diagram of the examined species (B), constructed using the PAST-pc software showing the relationships among the examined ferns accessions based on the analysis of variation in the morphological traits and ISSR fingerprinting polymorphism*

Discussion

Pteridophytes are vascular plants (with phloem and xylem) that produce neither flowers nor seeds (Cantino et al., 2007; Christenhusz et al., 2011). The body of the sporophyte is differentiated into roots, stems, and leaves, so they do not have as many morphological characters as angiosperms. Ferns' identification is difficult because many species often look very similar. In Saudi Arabia, previous efforts to classify ferns have relied on morphological characteristics (Migahid, 1978; Collenette, 1999; Chaudhary, 2001). Little literature deals with ferns biodiversity in Saudi Arabia.

In our study, most qualitative traits such as the pinnule blade, frond distribution, pinnule apex, sporangia distribution on the frond, and sporangia color varied between species. For example, the pinnule apex was acute in *A. aethiopicum* and *A. adiantum nigrum* but rounded in the other collected samples. Also, sporangia are distributed on frond edges in C. *pteridiodes*, *C. vellea,* and *A. capillus veneris.* However, it is found on the frond veins of *C. coriacea, A. aethiopicum*$_{(1)}$, *A. viride, and A. trichomanes.* Sporangia have scattered distribution on frond in other collected samples (*Table 2* and *Fig. 1*). This may be correlated with the wide morphological variation in *Ptrediophytes* species in different geographical regions under different environmental conditions (Al-Shehri, 2002). Phenotypic variation in some morphological characters, like plant heights, could be related to variations in the elevation and landscape topography of the area.

According to Ahmed et al. (2019) and Amiryousefi et al. (2018), a calculation of the polymorphic information content (PIC, a measure of discriminatory ability) was made using the outcomes of molecular marker investigations. Heterozygosity (H) and polymorphic information content are two measures of the efficacy or informativeness of polymorphism as a genetic marker (PIC). Due to the assumption of two alleles per locus, the maximum value of H and PIC for binary data is 0.5, and both are affected by the number and frequency of alleles; for codominant markers, these values range between 0

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and 1. Our results showed that the PIC value provides an estimate of a locus' discriminatory power by considering not only the number of alleles but also their relative frequencies. PIC values range from 0 (monomorphic) to 1 (extremely discriminative, with many alleles occurring in equal frequency). Polymorphism indices for individual primers were calculated using iMEC software as basic measures. *Table 4* contains more information on the primers' basic measurements and polymorphism indices (Ahmed et al., 2019)

In other words, PIC refers to the probability that polymorphism would be detected by a primer/primer combination between two randomly chosen individuals and depends on both the quantity and distribution of detectable alleles. These findings pointed to reliable diversity sources that will aid breeders in assessing genetic diversity and interactions between various genotypes. The findings demonstrate that ISSR is an effective, convenient, and affordable method for examining the genetic diversity and interactions across flax genotypes. In order to analyze genetic variation, DNA analysis using ISSR-PCR has proven to be a great marker. It has also been used successfully to establish genetic similarities for many other plants.

The clustering tree based on morphological characters (*Fig. 2*) resulted in the separation of the three *Cheilanthes* species, where *C. pteridiodes* and *C. vellea* clustered at ahigh similarity level because they were relatively similar morphologically, whereas the ISSR analysis (*Fig. 4*) could not differentiate between the two species, indicating close genetic affinity between them. The clustering of *C. coriacea* with the two later species at a relatively low similarity level based on morphological characters, which are also supported by ISSR analysis, indicates that this species is genetically different from *C. pteridiodes* and *C. vellea.* This finding is consistent with the findings of Ebihara (2011), who studied the RbcL phylogeny of pteridophyte flora in Japan and determined that the four *Cheilanthes* species under study separated into one group, with *C. brandtii* and *C. kramrn* separating into one cluster and the two other species delaminating from them.

The presence of *A. adiantum nigrum* with the three *Cheilanthes* species based on ISSR data analysis (*Fig. 4*) confirms that *A. adiantum nigrum* may be genetically related to *Cheilanthes* species, where this grouping is supported by the tree generated based on the analysis combination of morphological characters and ISSR data (*Fig. 5A*) and not supported based on morphological characters alone. The resemblance between the two species does not agree with Alshehri and Moustafa (2018), their results confirmed the clustering of *C. pteridiodes* with *Adiantum capillus veneris* in one cluster separated from three *Asplenium* species based on data analysis of five ISSR primers.

The clustering of the examined accessions/species based on the analysis of ISSR data (*Fig. 4*) resulted in the separation of the two accessions of *A. aethiopicum* and the two accessions of *A. ceterach* into one cluster, which confirms the relationship between the two species. This result is not supported by the tree generated based on the analysis of morphological characters. This result agrees with Alshehri and Moustafa (2018), who confirmed that the morphological characteristics of ferns have limitations in sorting them and revealed a close relationship between *A. aethiopicum* and *A. ceterach* based on ISSR data analysis. This result is also confirmed by the tree generated based on the analysis of morphological and ISSR data. Based on ISSR data analysis, the two *A. ceterach* accessions have relatively low similarity. The genetic differences between the two accessions could be attributed to the elevation of the collection sites, with *A. ceterach*$_{(a)}$ collected from the Fyafa mountains at 537 m asl and *A. ceterach*$_{(b)}$ collected from the Al

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Hashr mountains at 2042 m asl (*Table 1*). Ellstrand et al. (1993); Coates et al. (1988); and Krauss et al. (2000) explained that many rare and endangered plant species may have reduced their genetic diversity because of their small population size, and the plant species probably differentiated into genetically unique populations adapted to local environmental conditions for growth and survival.

On the other hand, the analysis of ISSR data (*Fig. 4*) indicated a relationship between *A. capillus veneris* and *A. trichomanes*, where the two species separated into one group. The investigation of morphological features indicated this relationship. In contrast, *A. viride* was separated individually from the other examined samples based on the analysis of ISSR data. This finding is consistent with the findings of Chang et al. (2013), who studied the diversity and evolution of the *Asplenium normale* complex and reported that the taxonomy of the group under study is unclear, and the presence of diploids and tetraploids in this group may indicate reticulate evolution; they identified three diploids and four tetraploid individuals. The separation of *A. viride* is not supported based on the analysis of morphological characters, where this species clustered with the two accessions of *A. ceterach*, which may suggest a connection between the two species. The PCA scatter plot, which was generated using the Elucedine coefficient based on the analysis of morphological trait variations and ISSR fingerprinting polymorphism (*Fig. 5B*), confirmed the grouping of the collected ptrediophytes species and accessions.

Conclusion

The three *Cheilanthes* species were separated into one group with *A. adiantum nigrum*. The accessions of *A. ceterach* and *A. aethiopicum*, were clearly distinguished, whereas *A. capillus veneris* and A. *trichomanes* were related to each other. The presence of all examined *Asplenium* species in one group based on the analysis of ISSR data except, *A. adiantum nigrum* and *A. viride* these results may be of interest for further studies on the genetic and taxonomic differentiation of this species. Our findings support the use of ISSR marker as a quick, easy, and affordable method for examining the genetic diversity and interactions across fern genotypes. Also, the separation of the above species clusters justifies further research on closely related species as important genetic resources for potential economic and medicinal uses of pteridophytes species based on their affinity.

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Conflict of Interests. The author declares that he does not have any conflict of interests.

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