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Original article

Determining the Efficiency of ISSR Markers among Wild Prunus spinosa Genotypes Markers

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Abstract

Prunus spinosa L., commonly known as 'Çakal Eriği' or 'Güvem' in Türkiye, is a wild plum species that naturally occurs across many regions of the country, particularly in the Thrace region. Despite its ability to thrive in harsh environmental conditions and its wide habitat range, this species is not cultivated commercially, and scientific studies involving *P. spinosa* remain limited. In this study, the effectiveness of ISSR (Inter Simple Sequence Repeat) markers was evaluated using seven previously unstudied *P. spinosa* genotypes collected from the Thrace region. Of the 27 ISSR primers used, 17 were successfully amplified, yielding a total of 244 bands. Among these, 216 bands were polymorphic, resulting in an average polymorphism rate of 86.49%. The number of bands per primer ranged from 5 (ISSR825) to 24 (ISSR823 and ISSR844), with an average of 14.8 bands per primer. The amplified fragment sizes ranged between 290 and 2100 base pairs. The polymorphism information content (PIC) values varied from 0.313 (ISSR843) to 0.424 (ISSR825), with an average PIC value of 0.365. Genetic similarity coefficients based on Jaccard's similarity index ranged between 0.627 and 0.816, with a mean value of 0.745. These findings demonstrate that ISSR markers are effective molecular tools for assessing genetic diversity among P. spinosa genotypes. In particular, primers ISSR825 (0.424), ISSR841 (0.397), and ISSR808 (0.392) exhibited high levels of discrimination, making them especially useful for detecting genetic differences. The results indicate that ISSR markers can be reliably used to investigate genetic diversity in previously uncharacterized *P. spinosa* genotypes, with ISSR825, ISSR841 and ISSR808 primers standing out in particular due to their high discriminatory potential.

Keywords: Genetic Diversity, ISSR, Prunus spinosa L., Wild Plum

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INTRODUCTION

Türkiye's climate and soil conditions enable the cultivation of a diverse range of fruit species. Fruits such as cherries, apricots, plums, and peaches belong to the group of stone fruits. Türkiye is among the leading countries in fruit production (Duru Fan and Gul, 2022). In particular, plum cultivars are well-suited for cultivation in Türkiye due to their adaptation to cool temperate climatic conditions, and they constitute an important group in terms of fruit production. (Reynders and Salesses, 1989; Yılmaz, Ercişli, Asma, Doğan and Kafkas, 2009). The plum belongs to the subgenus Prunophora within the genus Prunus. It is known that there are approximately 200 plum species belonging to the genus Prunus worldwide. More than 20 species within the subgenus Prunophora play a significant role in the evolutionary development of the genus Prunus, which belongs to the family Rosaceae, due to their morphological diversity (Mirheidari, Khadivi, Moradi and Paryan, 2020; Yaşar, Gülsoy, Aslantaş and Şimşek, 2022). Numerous plum species are capable of growing under a wide range of environmental conditions. Türkiye hosts various plum species, including Prunus domestica L., Prunus cerasifera Ehrh., Prunus spinosa L., Prunus institia L., and Prunus salicina L. While P. domestica and P. salicina species are known as cultivated and marketed species, the other species are found in the environment as wild (Ozzengin, Zannou and Koca, 2023).

P. spinosa L., which belongs to the genus Prunus within the subfamily Prunoideae of the family Rosaceae, is a tetraploid species (2n = 4x = 32) considered to be native to Southern Europe, Türkiye, and Armenia. P. spinosa, commonly known as blackthorn, sloe, or jackal plum, bears fruits that are purple when unripe and turn blackish-purple upon ripening. The fruits are coated with a waxy layer and are abundant in various bioactive compounds, such as minerals, amino acids, vitamins, phytosterols, triterpenes, organic acids, and phenolic compounds. It has been reported to be distributed across Western Asia, Europe, and Northwestern Africa at altitudes ranging from 0 to 1700 m. It is a self-incompatible, insect-pollinated, deciduous wild shrub or small tree (Kırca and Mertoğlu, 2025; Nunes, Santos, Ferreira, Vieira and Vieira 2006; Ozzengin et al., 2023).

Over the past century, local plum cultivars have adapted to prevailing climatic conditions. One of the most effective approaches to preserving plant diversity is to investigate and assess genetic variation through the establishment of germplasm collections (Urrestarazu, Errea, Miranda, Santesteban and Pina, 2018).

To date, molecular markers such as RAPD (Random Amplified Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism), ISSR (Inter Simple Sequence Repeat), and SSR (Simple Sequence Repeat or microsatellites) have been employed to elucidate the genetic structure of plum species (Yu, Chu, Ma, Re, Shen and Fang, 2013; Urrestarazu et al., 2018; Hamdani et al., 2022; Antanynienė, Šikšnianienė, Stanys and Frercks, 2023; Sümbül et al., 2023).

The ISSR marker, introduced in 1994, is recognized as a powerful, rapid, simple, reproducible, and cost-effective tool for evaluating genetic diversity across numerous species and for identifying closely related cultivars. ISSR markers are widely employed in various studies, including molecular taxonomy, cultivar identification, genetic mapping, marker-assisted selection, and genetic characterization. ISSR analyses are based on the principle of conducting a PCR reaction using a single primer that corresponds to repeat sequences ranging from 16 to 18 base pairs in length. These markers are widely distributed throughout plant genomes, exhibit high levels of polymorphism, and are characterized by their multi-locus and reproducible nature. ISSR markers generate DNA banding profiles and are regarded as a valuable tool for plants in applications such as phylogenetic relationship analysis, cultivar identification, genetic linkage mapping, and germplasm characterization. (Al-Dulaimy, l-Jaf, Alalwani and Abdulkadhi, 2025; Cekic, Calis and Ozturk Erdem, 2018; Demirel, Pehluvan and Aslantaş 2023; Gupta, Chyi, Romero-Severson and Owen, 1994; Ikhsan, Topçu, Sütyemez and Kafkas, 2016; Pradeep, Sarla and Siddiq, 2002; Wolfe 2005; Zietkiewicz, Rafalski and Labuda, 1994).

This research aimed to assess the utility of ISSR markers and quantify the genetic diversity among wild plum (P. spinosa L.) genotypes, a prevalent Prunus species in the Thrace region. Samples were collected from seven distinct locations that had not been previously analyzed at the molecular level. The genetic differences among the genotypes were elucidated based on the ISSR band profiles obtained, and the polymorphism levels of the utilized primers were also assessed. This study is particularly noteworthy as it represents the first examination of previously uncharacterized P. spinosa genotypes in the Thrace region using ISSR markers, thereby enhancing the literature on molecular genetic diversity within Prunus species.

MATERIAL and METHOD

Plant Materials

In this study, seven distinct genotypes of wild blackthorn (P. spinosa L.) were utilized as plant material, collected from diverse locations in the Thrace region, including Tekirdağ, Istanbul, Çanakkale, Kırklareli, and Edirne. These genotypes had not been previously investigated at the molecular level. Fresh leaves from these individuals, whose geographical location information is provided in Table 1, were collected for DNA isolation. The collected materials were transported under cold chain conditions to the Molecular Genetics Laboratory of the Department of Agricultural Biotechnology, Faculty of Agriculture, Tekirdağ Namık Kemal University, and stored at -80°C until DNA isolation.

DNA Extraction and Quantification

DNA isolation was carried out using the CTAB protocol developed and applied by Doyle and Doyle (1987, 1990) and Kafkas, Ozkan and Sutyemez (2005), with minor modifications as needed. The quantity and purity (OD value) of the extracted DNA samples were determined by measuring absorbance

at 260/280 nm using an LTEK INNO microplate spectrophotometer. The DNA samples were subsequently diluted to a concentration of 5 ng/µl.

Table 1. Coordinate information of wild jackal plum genotypes

Genotypes	Location
Tekirdağ Namık Kemal Üniversitesi	N: 40.992271 E: 27.590133
Tekirdağ Değirmenaltı	N: 40.987289 E: 27.580889
Tekirdağ Yazır	N: 40.895165 E: 27.419706
İstanbul Çatalca	N: 41.089387 E: 28.358815
Çanakkale Gelibolu	N: 40.5228580 E: 26.7391450
Kırklareli Kaynarca	N: 41.6507530 E: 27.4677390
Edirne Havsa Yolageldi	N: 41.2361830 E: 27.1223530

ISSR-PCR Conditions

In this study, ISSR analyses were conducted following the protocol developed by Zietkiewicz et al., (1994), which is widely used in the literature, with minor modifications. ISSR reactions were prepared in a total volume of 12.5 µL containing 1× PCR buffer (10× Green Buffer with $MgCl_2$), 2.5 mM dNTPs, 5 pmol of ISSR primer, 5 U of Taq DNA polymerase, and 10 ng of template DNA. The PCR amplification program consisted of an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 90 seconds, annealing at primer-specific optimized temperatures (Tm, °C) for 60 seconds, and extension at 72°C for 120 seconds. The reaction was finalized with a final extension step at 72°C for 7 minutes. PCR products were electrophoresed on a 2.5% agarose gel using 1× TBE buffer, and the resulting DNA bands were visualized under UV light using a Vilber Lourmat Quantum ST5 Gel Imaging System. A total of 27 different ISSR primers were tested, of which 17 produced successful amplifications. The base sequences of the amplified primers and their corresponding optimized annealing temperatures are presented in Table 2.

Statistical Analysis

The data obtained in this study were analyzed using various statistical methods based on a binary (1-0; presence-absence) scoring system. After marker scoring, the polymorphic information content (PIC) for each primer was calculated using the formula suggested for dominant markers by Roldán-Ruiz, Dendauw, Van Bockstaele, Depicker and De Loose (2000). We calculated the frequencies of band presence (p) and absence (q = 1 - p) using GenAlEx v6.5 (Peakall and Smouse, 2012) to aid in assessing allelic variability. Genetic distances among samples were estimated using the Jaccard distance coefficient (Jaccard, 1901) (Table 3). Statistical analyses were performed in Python using the Scikit-

learn library (Pedregosa et al., 2011) and core data-handling functionality from McKinney (2010). The resulting distance matrix was visualized as a heatmap using the Seaborn visualization tool (Waskom, 2021).

Table 2. ISSR primer sequences and annealing temperatures

Primer	Primer Sequence (5' → 3')	Annealing Temperature (Tm, °C)
ISSR808	$(AG)_8C$	52°C
ISSR809	$(AG)_8G$	52°C
ISSR811	$(GA)_8C$	52°C
ISSR812	$(GA)_8AA$	50°C
ISSR813	$(CTC)_6TT$	50°C
ISSR815	$(CT)_8G$	52°C
ISSR823	$(TCT)_6CC$	52°C
ISSR825	$(AC)_8T$	50°C
ISSR827	$(AC)_8G$	52°C
ISSR841	$(GA)_8YC$	54°C
ISSR842	$(GA)_8YG$	54°C
ISSR843	$(CT)_8RA$	52°C
ISSR844	$(CT)_8RC$	54°C
ISSR852	$(TC)_8RA$	52°C
ISSR853	$(TC)_8RT$	52°C
ISSR854	$(TC)_8RG$	52°C
ISSR855	$(AC)_8YT$	52°C

Note: Degenerate bases follow the IUPAC nucleotide code: R = A/G, Y = C/T.

RESULTS

This study aimed to analyze seven wild jackal plum samples collected from different locations in the Thrace region using ISSR markers. The image from the agarose gel electrophoresis, which was created during the screening PCR analysis with the ISSR823 and ISSR827 primers, is shown in Figure 1. The samples shown in the gel image are as follows: TNKU (Tekirdağ Namık Kemal University), TD (Tekirdağ Değirmenaltı), TY (Tekirdağ Yazır), İÇ (İstanbul Çatalca), ÇG (Çanakkale Gelibolu), KN (Kırklareli Kaynarca), and EHY (Edirne Havsa Yolageldi). Amplification products were separated on a 2.5% agarose gel, and the resulting bands were evaluated with reference to a 100 bp DNA ladder.

The data obtained from the evaluation of ISSR banding patterns were scored based on the presence or absence of polymorphic bands observed for each individual following gel electrophoresis. The presence of a band was recorded as (1), its absence as (0), and the absence of amplification as (-9). The results of the analyses were used according to the statistical software used.

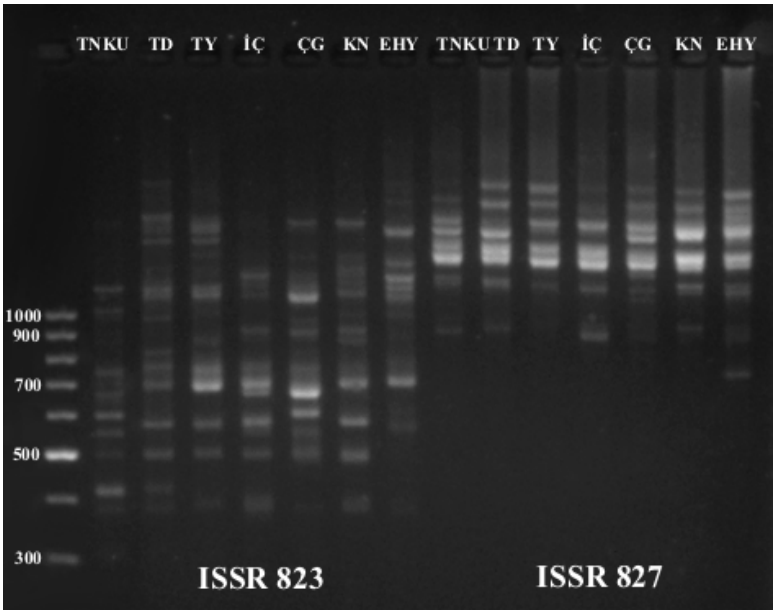

Figure 1. Agarose gel image of ISSR823 and ISSR827 primers

In this study, DNA bands ranging in size from 290 bp to 2100 bp were obtained using 17 ISSR primers, demonstrating their applicability in wild plum genotypes. The total number of bands generated by the primers ranged from 5 to 24, with an average of 14.8 bands per primer. The highest number of bands, 24, was obtained from the ISSR823 and ISSR844 primers, whereas the lowest number of bands, 5, was produced by the ISSR 825 primer. The number of polymorphic bands varied among the primers, with the polymorphism rate ranging from 50% (ISSR815) to 100% (ISSR808, ISSR813, ISSR825, ISSR844, and ISSR855). Most of the primers used exhibited high polymorphism rates, with an overall average of 86.07%. The corresponding findings are presented in Table 3.

PIC values of ISSR primers varied between 0.313 and 0.424. The average PIC value was calculated as 0.365. The highest PIC value belonged to the ISSR825 primer (0.424), while the lowest value was observed with the ISSR843 primer (0.313). In addition, a heatmap was generated to visually compare the informativeness levels of the different primers, with color intensities proportional to the PIC values of each primer being presented (Figure 2). The majority of the primers were clustered within the higher range of PIC values, as indicated by the blue hues in the heatmap. Especially ISSR841 (0.397) and ISSR808 (0.392) primers were among the primers with high PIC values. The band information and PIC values obtained as a result of scoring are presented in Table 4.

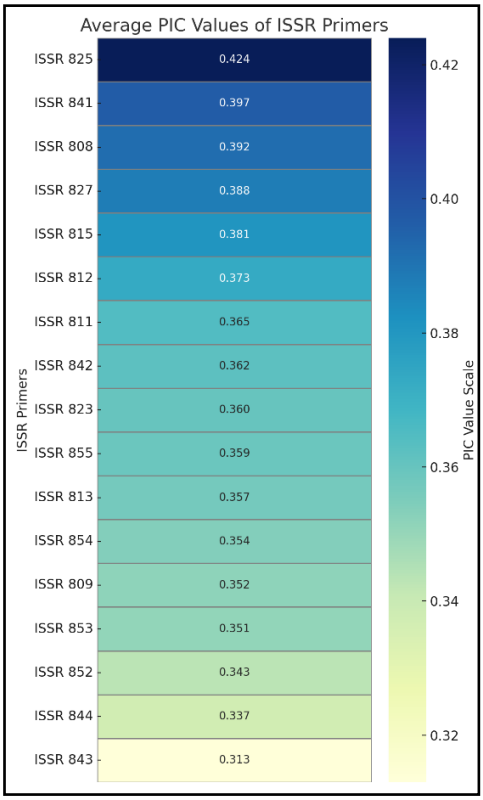

Figure 2. Heat map of PIC values of ISSR primers

Table 3. Band data and PIC values obtained as a result of scoring

ISSR Primer	Band Size (Min/Max, bp)	Number of Bands (NB)	Number of Polymorphic Bands (NPB)	Percentage of Polymorphism (%)	Polymorphic Information Content (PIC)
ISSR808	340/840	15	15	100	0.39
ISSR809	410/900	17	13	76.47	0.35
ISSR811	380/1100	17	15	88.24	0.36
ISSR812	500/1150	15	14	93.33	0.37
ISSR813	440/1700	19	19	100	0.36
ISSR815	770/1100	6	3	50.00	0.38
ISSR823	420/3500	24	22	91.67	0.36
ISSR825	770/1050	5	5	100	0.42
ISSR827	750/2100	13	8	61.54	0.39
ISSR841	450/1300	16	15	93.75	0.40
ISSR842	290/1100	17	14	82.35	0.36
ISSR843	650/1450	13	12	92.31	0.31
ISSR844	370/1150	24	24	100	0.34
ISSR852	1200/1800	7	5	71.43	0.34
ISSR853	610/1300	13	10	76.92	0.35
ISSR854	700/1900	13	12	92.31	0.35
ISSR855	660/1800	10	10	100	0.36
Mean	-	14.35	12.71	86.49	0.37

Based on the binary (1-0) scoring of the ISSR data, genetic distances among the samples were calculated using the Jaccard distance coefficient. As a result of the analysis, the genetic distance values among the seven samples ranged from 0.627 to 0.816. The lowest genetic distance value (0.627) was calculated between the Istanbul Çatalca and Çanakkale Gelibolu samples, indicating that these samples possess a closer genetic relationship to each other than to the remaining samples. The highest genetic distance value (0.816) was observed between the Tekirdağ Namık Kemal University and Kırklareli Kaynarca samples. The genetic distance matrix generated based on these data is presented in Table 4.

Table 4. Distance matrix between wild jackfruit genotypes

Genotypes	Tekirdağ Namık Kemal Üniversitesi	Tekirdağ Değirmenaltı	Tekirdağ Yazır	İstanbul Çatalca	Çanakkale Gelibolu	Kırklareli Kaynarca	Edirne Havsa Yolageldi
Tekirdağ Namık Kemal Üniversitesi	0						
Tekirdağ Değirmenaltı	0.770	0					
Tekirdağ Yazır	0.805	0.692	0				
İstanbul Çatalca	0.815	0.766	0.682	0			
Çanakkale Gelibolu	0.758	0.774	0.718	0.627	0		
Kırklareli Kaynarca	0.816	0.813	0.723	0.756	0.746	0	
Edirne Havsa Yolageldi	0.808	0.697	0.661	0.744	0.744	0.738	0

DISCUSSION

A total of 252 bands were obtained using the 17 ISSR primers employed in this study, with an average of 14.8 bands per primer. A total of 217 polymorphic bands were obtained, and the overall polymorphism rate was determined to be 86.07%. These data show notable differences when compared with the ISSR results reported by Yılmaz, Paydas-Kargi, Dogan and Kafkas (2012) in their study on *Prunus armeniaca* genotypes. In that study, 164 bands were generated using 20 primers, with an average of 8.2 bands per primer. The polymorphism rate was reported as 88%, and the mean PIC value was calculated to be 0.564. However, the higher number of bands and the broader band length range (290-2100 bp) obtained in the present study indicate that the primers are capable of providing a more comprehensive assessment of genetic variation and exhibit high amplification efficiency. Therefore, the ISSR systems employed in both studies yielded successful results within their respective contexts, and the distinction of the present study lies in its effective demonstration of genetic diversity through high polymorphism and a broad band distribution in a wild species such as *P. spinosa.*

The average polymorphism rate of 86.49% obtained in our study reveals that the genetic diversity on the *P. spinosa* species is high. This finding is consistent when compared with similar studies in the literature. Similarly, Gülay, Sevindik, Sofyalıoglu, Cayır and Filiz, (2023), who conducted a study using ISSR markers on wild jackal plum populations in the Western Black Sea Region, reported a high polymorphism rate of 81.89% and stated that this genetic variation was consistent with the geographical distribution. In this context, the high level of polymorphism detected in the present study supports the notion that *P. spinosa* is a genetically rich species among local populations, possessing a strong ability to adapt to diverse ecological conditions. However, in the study conducted by Erturk, Ercisli, Maghradze, Orhan and Agar (2009) using RAPD markers on 16 *P. spinosa* samples collected from the Çoruh Valley, a polymorphism rate of 65% was reported. Indeed, although high levels of polymorphism were detected in both the present study and other ISSR-based studies employing similar materials, lower levels of polymorphism have been reported in studies utilizing RAPD markers in the same species.

The PIC values obtained in our study ranged from 0.313 to 0.424, with an average of 0.365. Considering other studies conducted on plums using ISSR markers, Ait Bella, Bouda, Khachtib and Haddioui, (2021) reported that PIC values ranged between 0.34 and 0.49 in an ISSR analysis performed on *P. domestica* and *P. salicina* species.

Similarly, in a study conducted by Hamdani et al., (2022) using 20 ISSR primers, the average PIC value was reported as 0.34 in *P. domestica* and *P. salicina* genotypes. In line with these findings, the high PIC values obtained in the present study support the conclusion that ISSR markers represent a reliable and powerful molecular tool for assessing genetic diversity not only in cultivated varieties but also in wild species such as *P. spinosa*. When evaluated for primer performance, the ISSR825 (100% polymorphism, PIC = 0.424), ISSR841 (PIC = 0.397), and ISSR808 (PIC = 0.392) primers were found to exhibit high informativeness.

These results are consistent with the ISSR analyses conducted by Wu, Chen, Yeh and Chen (2019) on *P. salicina* genotypes cultivated in southern China, where an average polymorphism rate of 89% and an approximate PIC value of 0.45 were reported. This parallelism demonstrates that ISSR markers are reliable and effective for determining genetic diversity across different *Prunus* species and geographical regions, thereby supporting the high discriminatory power and reliability of the primers used in the present study.

Upon examining the Jaccard genetic distance values, it was found that the distances among the genotypes ranged from 0.627 to 0.816. The minimal distance was recorded between the genotypes from Çatalca, Istanbul, and Gelibolu, Çanakkale (0.627), identifying them as the genetically closest pair. Conversely, the maximal distance was observed between the genotypes from Namık Kemal University, Tekirdağ, and Kaynarca, Kırklareli (0.816), indicating these genotypes as the most genetically distant samples. Notably, relatively low distance values were also observed between the genotypes from Yazır,

Tekirdağ and Yolageldi, Edirne (0.661), as well as between Değirmenaltı, Tekirdağ and Yazır, Tekirdağ (0.692). Overall, these results indicate a certain degree of genetic differentiation among the examined P. spinosa genotypes. This suggests that while some genotypes may exhibit partial similarities due to geographical proximity, the genetic distance does not consistently align with geographical distance across all samples.

The results obtained from the application of 17 ISSR primers in this study revealed a notable level of genetic diversity among the P. spinosa genotypes and confirmed the distinctiveness of the primers used. The analysis of genotypes sourced from the Thrace region, which had not been previously examined at the molecular level, yielded essential insights into the genetic structure of wild populations. These findings offer significant information for the conservation of natural genetic resources and inform future breeding initiatives. Additionally, the effectiveness of ISSR markers in detecting intraspecific variation provides foundational data for subsequent molecular investigations of Prunus species.

However, due to the restricted number of genotypes analyzed in this study, the findings should be regarded as preliminary. Nevertheless, the band profiles obtained from the 17 ISSR primers facilitated the identification of overarching trends in genetic diversity, despite the limited sample size. This research serves as an initial framework for the molecular investigation of P. spinosa genotypes in the Thrace region and can provide a foundation for future studies involving larger sample populations.

Conclusion

In this study, ISSR-based molecular analyses conducted on seven different P. spinosa genotypes effectively revealed the genetic diversity within the species. The 17 ISSR primers employed exhibited high polymorphism information content (average polymorphism rate: 86.07%; mean PIC value: 0.365), supporting the presence of substantial genetic variation among P. spinosa genotypes. The most informative primer, ISSR825 (PIC = 0.424), clearly demonstrated the effectiveness of ISSR markers in this species. Analyses based on the Jaccard distance coefficient revealed the presence of genetic differences among the samples, which partially corresponded with their geographical distribution. This suggests that local variations may have been shaped by regional adaptation, isolation, or historical dispersal. Considering the analysis performed, ISSR primers are thought to be a suitable molecular marker system by providing high variation detection on the wild type of P. spinosa. This finding indicates that it is both feasible and appropriate to conduct genetic characterization of a broader range of P. spinosa materials collected from different ecological regions of Türkiye using ISSR markers in future studies. In the future, increasing the number of samples and conducting analyses based on materials collected from different geographical regions, supported by various marker systems, will enable a more comprehensive understanding of the population structure of this species. Furthermore, this study demonstrates that ISSR markers constitute an effective tool for the conservation of local genetic resources and the assessment of biodiversity.

Additional Declaration

Author Contributions

In this study, Conceptualisation, ÖEA, HT and BBB; Data Collection, ÖEA and HT; Interpreted data; ÖEA, Writing original draft preparation, ÖEA and HT, Writing, review, and editing, HT and BBB. All authors have read and agreed to the published version of the manuscript

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Responsible Artificial Intelligence Statement

No artificial intelligence support was received in any part of this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest related to the publication of this study.

Ethics Approval

In all processes of this study, the principles of Pen Academic Publishing Research Ethics Policy were followed.

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