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Molecular characterisation of the genus *Papaver* section *Oxytona* using ISSR markers

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Abstract: *Papaver bracteatum* Lindl., *P. orientale* L., and *P. pseudo-orientale* (Fedde) Medw., species of section *Oxytona* Bernh. in the genus *Papaver* L., are found widely in the native flora of Turkey. They are commercially important for their high alkaloid content. Because genus *Papaver* section *Oxytona* has similar morphological characters, it is not easy to distinguish these species. We used the inter-simple sequence repeat (ISSR) molecular marker system to determine the molecular characterisation of this section. In this study, 20 ISSR primers have been studied in 180 accessions collected from 5 different regions of Turkey. A total of 82 bands were obtained, of which 80 were polymorphic. Average genetic distance was found to be 0.35, the Shannon index was 0.50, and the polymorphism rate was 96.97%. The primer AT10 had the smallest number of bands, which was 3, while the primers AT19 and AT3 had the greatest number of bands, which was 5. In conclusion, the ISSR marker system can be used for the classification of section *Oxytona*.

Key words: *Papaver*, *Oxytona*, ISSR, molecular marker, phylogeny

1. Introduction

Turkey is one of the gene origins for many plants, including *Papaver* L. (Davis et al., 1988; Atalay, 1994; Erik & Tarıkahya, 2004). The species belonging to Papaveraceae are distributed in the northern hemisphere. There are 110 *Papaver* species around the world, and 50 taxa grow naturally in Turkey (Kapoor, 1997; Güner et al., 2000; Parmaksız & Özcan 2011). *Papaver* section *Oxytona* Bernh. comprises perennial herbs, consisting of *Papaver bracteatum* Lindl., *P. orientale* L., and *P. pseudo-orientale* (Fedde) Medw.; they are important for their alkaloid contents such as codeine and thebaine (Sarıyar, 2002; Carolan et al., 2002). *Papaver somniferum* L. is an annual plant, commercially grown because of its morphine and codeine content, while *P. bracteatum* can be grown as a source of the morphinian alkaloid thebaine, which can be converted to some of the opiate analgesics such as codeine, oxymorphone, and oxycodone (Carolan et al., 2002). The species belonging to section *Oxytona* can be differentiated using morphological, cytological, and phytochemical characters, but the distinction is not always clear (Nyman, 1979). Several species from section *Oxytona* are polyploid in structure. *P. bracteatum* is diploid (2n = 14), *P. orientale* is tetraploid (2n = 28), and *P. pseudo-orientale* is allohexaploid (2n = 42) (Goldblatt, 1974). It has been reported that *P. bracteatum* is often illegally cultivated as a garden plant mixed with *P. orientale* and *P. pseudo-orientale* (Hosokawa et al., 2004). It is difficult to distinguish *P. bracteatum*, *P. orientale*, and *P. pseudo-orientale* at their vegetative stage of growth (Hosokawa et al., 2004). These 3 species have also been described previously on the basis of morphological observations and the analysis of chromosome numbers and morphologic characters including flower colour, blackish blotches on petals, bracts, and leaves. However, discrimination in terms of morphological and chemotaxonomic characteristics among *Papaver* species is still somewhat problematic because environmental conditions can affect their various characteristics, and interspecific hybridisation may also occur (Milo et al., 1988; Ojala et al., 1990; Levy & Milo, 1991). Therefore, the correct identification of species is crucial because of the importance of their species-specific alkaloids.

Genetic diversity is very important in plant breeding programs (Xu & Crouch, 2008; Surgun et al., 2012). Knowledge of the genetic relationships between the different accessions supplying this diversity can greatly aid the development of efficient germplasm management and utilisation strategies (Fu et al., 2008). Morphological trait measurements are among the various methods that have

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been employed to estimate the genetic diversity of species. They are commonly used parameters since they provide a simple technique of quantifying genetic variation while simultaneously assessing genotype performance in relevant growing environments (Fufa et al., 2005). However, assessing morphological traits is labour-intensive, and the phenotypic plasticity of plants makes environmental variation a major problem (Van Beuningen and Busch, 1997).

The inter-simple sequence repeat (ISSR) marker system is a polymerase chain reaction (PCR)-based technique that uses a single amplification primer composed of a microsatellite motif to target a subset of simple sequence repeats (SSRs) or microsatellites (Zietkiewicz et al., 1994). SSRs, or microsatellites, and ISSRs have been recognised as useful molecular markers in marker-assisted selection, the analysis of genetic diversity, population genetic analysis, and other purposes in various species (Guptta et al., 2002; Budak et al., 2004; Alam et al., 2009; Bayraktar & Akan, 2011; Hamza et al., 2012). However, this marker system has never been used in *Papaver* section *Oxytona* for genetic characterisation.

The aim of our study was to determine the genetic diversity in section *Oxytona*. The correlations among geographical origin, chromosome numbers, and molecular marker data were evaluated.

2. Materials and methods

2.1. Plant materials

A total of 180 accessions of section *Oxytona* were used in this study. *P. orientale* and *P. pseudo-orientale* accessions were collected from the wild in the provinces of Erzincan, Sivas, Tunceli, and Niğde, the accession numbers being 9, 9, 23, and 135, respectively. Four *Papaver bracteatum* accessions were obtained from the Ankara University Faculty of Agriculture. When all accessions were evaluated according to chromosome numbers, they grouped as 4 *P. bracteatum*, 50 *P. orientale*, 106 *P. pseudo-orientale*, and 20 unknown accessions. The plants were grown in the research field of Gaziosmanpaşa University.

2.2. DNA isolation

Young leaves were freshly harvested from 180 individual plants of each accession. Total DNA was isolated from fresh leaves using the Fermentas Genomic DNA Isolation Kit (Fermentas, USA). DNA preparations were quantified by 1% agarose gel electrophoresis. Isolated DNA with a final concentration of 100 ng mL^{-1} was used in PCR analysis.

2.3. ISSR Analysis

Twenty ISSR primers (AT1–AT20 universal primers) were obtained from İontek (İontek Co., Turkey). Each 25-mL PCR reaction contained 20 ng of genomic DNA template, 10X buffer Mg^{2+}-free (BioBasic, Canada), 20 mM $MgSO_4$, 10 mM dNTPs, 1 unit of Taq DNA polymerase (Promega, USA), and 0.4 μM of each ISSR primer. PCR amplifications were performed in an Apollo Instrumentation ATC401 Gradient Thermocycler. The PCR amplification procedure was performed at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, at 42–63 °C for 60 s, and at 72 °C for 2 min. Finally, the procedure was extended at 72 °C for 7 min. Amplification products were characterised on 1% (w/v) agarose gels (immersed) at 120 V for 2.5 h and visualised with ethidium bromide (0.5 g/mL) under UV light, then photographed using the Gel-Logic 200 image system (Eastman-Kodak, USA). The sizes of ISSR fragments were estimated by using a 1-kb DNA ladder (SibEnzyme M11, USA) as the standard.

2.4. Data analysis

In ISSR analysis, the band patterns were scored as present (1) or absent (0) for each primer pair. Only strong, reproducible, and clearly distinguished bands were used in the analysis. ISSR primers were analysed by using POPGENE32 version 1.32 (Population Genetic Analysis) and MEGA 4.0 (Molecular Evolutionary Genetic Analysis) as previously described (Nei, 1972; Nei & Li, 1979; Kumar et al., 2004). Polymorphism similarities were scored between 0 and 1, with 1 representing 100% similarity.

3. Results

Twenty ISSR primers were analysed against 180 accessions. ISSR primers generated 82 bands with an average of 2.20 bands per primer. Of the 82 bands produced, 80 were polymorphic, and the polymorphism rate was 96.97%. The number of polymorphic bands detected with each primer ranged from 3 (AT10) to 5 (AT3 and AT19). A genetic similarity coefficient matrix was constructed for the primers with the unweighted pair group method with arithmetic mean. The dendrogram showing a relationship among the 180 accessions was constructed (Figure). The Nei's index similarity coefficient values ranged from 0 to 1, with an average of 0.35. Excluding the plants with a 0.00 similarity coefficient, 90 pairs of plants appeared to be closer to each other with a 0.09 similarity coefficient; the farthest genetic distance appeared in 15 pairs of plants, and the Shannon index was 0.50.

The phylogenetic analyses generated 2 major groups, A and B, that comprised 16 and 17 subgroups, respectively (Figure). When the populations were examined according to chromosome numbers, the populations with 14 chromosomes clustered in 4 groups. The populations with 28 chromosomes clustered in only 1 group, and plants with 42 chromosomes clustered in 8 groups. When the populations were examined according to the regions, the plants collected from Niğde clustered in 13 groups by themselves, plants from the Erzincan region clustered in 1 group, and the other regions showed no separation.

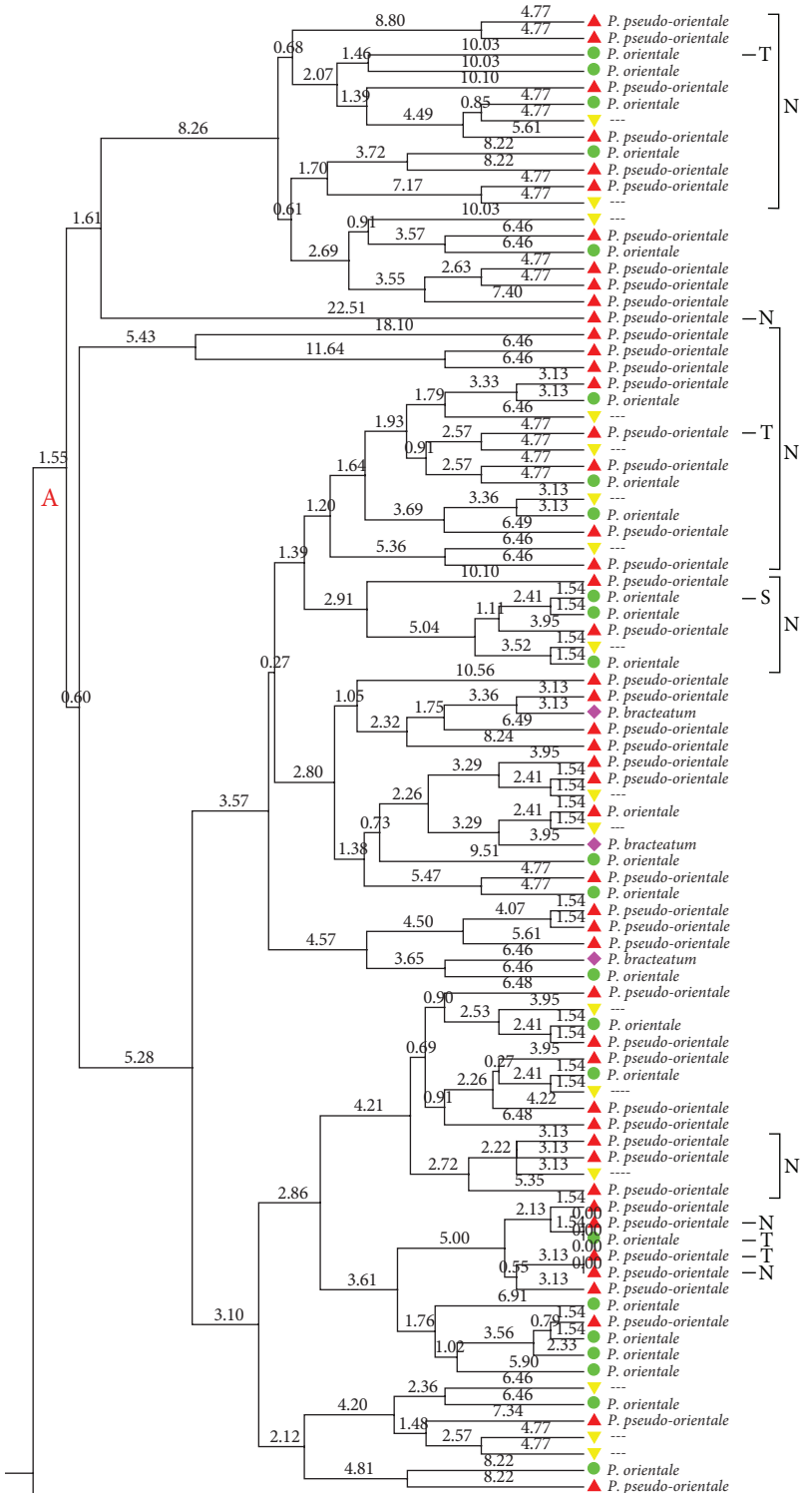

Figure. The phylogenetic tree of *Oxytona* accessions. *Papaver bracteatum* accessions are shown in pink (◆PB), *P. pseudo-orientale* in red (▲PPO), and *P. orientale* in green (●PO). Unknown chromosome numbers are shown in yellow (▼---). E: Erzincan, N: Niğde, S: Sivas, T: Tunceli.

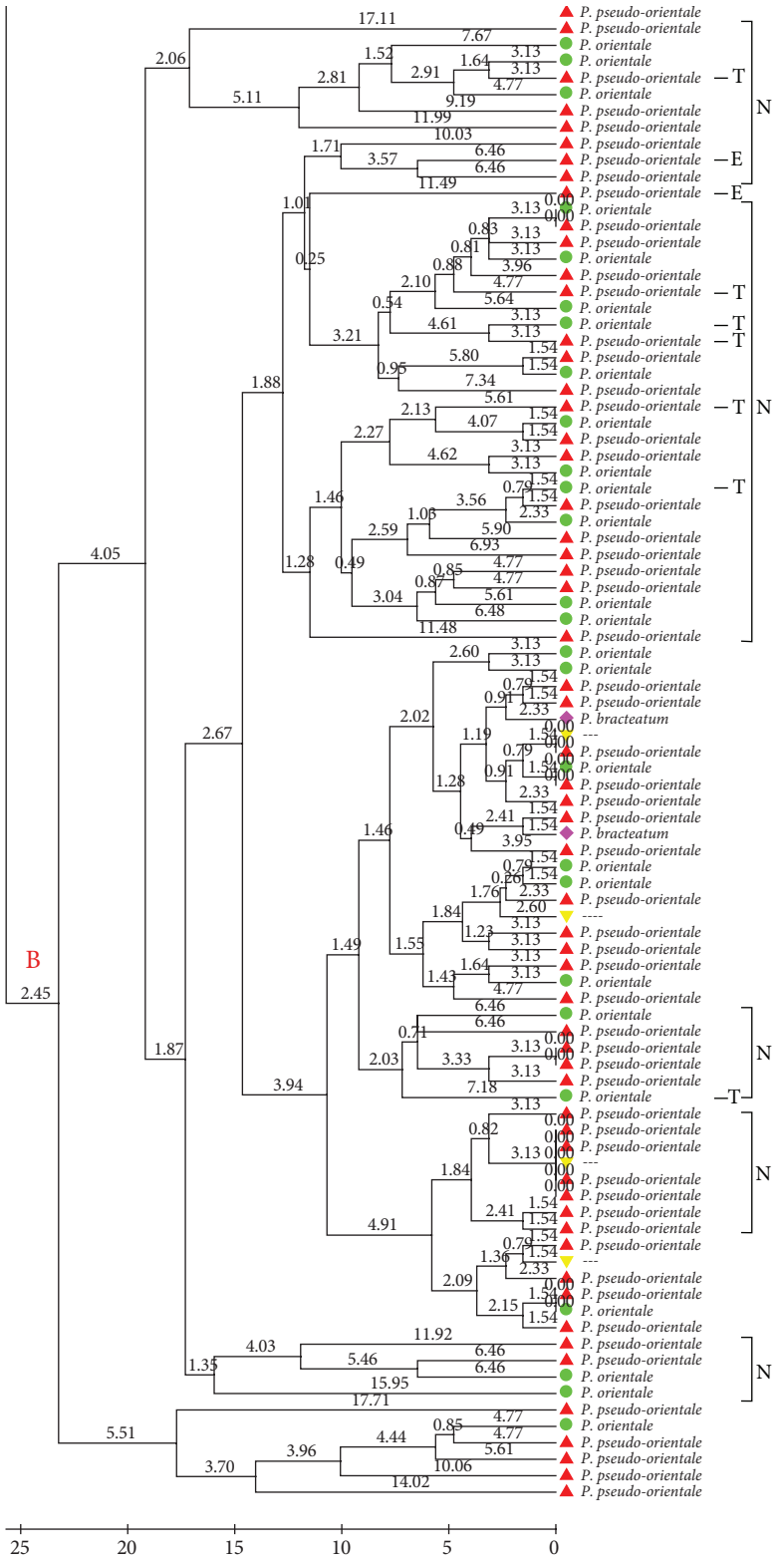

Figure. (Continued).

4. Discussion

The present molecular marker system has been previously used as an effective tool for evaluating the phylogenetic relationships and genetic diversity in *Narcissus* section *Pseudonarcissi* (Jiménez et al., 2009), barley (Wang et al., 2009), radish (Liu et al., 2008), *Trigonella* (Dangi et al., 2004), and *P. somniferum* germplasms (Acharya & Sharma, 2009). These studies have given important clues to aid in better understanding species relationships and developing breeding strategies. Among the molecular marker systems, the ISSR technique is popular because it does not require prior knowledge about the genome and its application is simple and cheap (Alam et al., 2009; Huang et al., 2009; Bayraktar & Akan, 2011; Hamza et al., 2012).

However, there has been no previous report on the genetic diversity of the section *Oxytona* using ISSR molecular markers. In this study, we used ISSR molecular markers to investigate the levels of genetic similarity between different accessions of *Papaver* section *Oxytona*. Random amplification of polymorphic DNA (RAPD) (Parmaksız & Özcan, 2011) and plastid *rpl16* gene and the *rpl16–rpl14* spacer sequences (Hosokawa et al., 2004) have been studied previously for this section.

In this study, a high percentage of polymorphism (96.97%) was obtained. The high level of genetic variation observed in the ISSR marker system is consistent with the results of RAPD molecular markers that have been used on section *Oxytona*. The polymorphism rate was higher than that of RAPD, with 84.3% polymorphism (Parmaksız & Özcan, 2011). The dendrogram that we obtained with ISSR markers showed a similar topology to that of RAPD markers, though with some differences in the positioning of some genotypes. These results indicated that ISSR markers provide more resolving power than RAPD markers. Goulão et al. (2001) and Huang et al. (2009) also indicated that the ISSR marker system is more effective than RAPD.

It was observed that dinucleotide repeat primers $(TC)_8G$ and $(CT)_8G$, AT3 and AT19, produced the highest number of bands, respectively, and no monomorphic primer was observed. Because they produced the highest number of bands, these primers could be used for discriminating the plants of this section.

The clustering of the accessions was characterised with morphological and chromosomal traits and biogeographic regional data. Our phylogenetic results showed that the section *Oxytona* clustered into 2 main groups (Figure). When we analysed the dendrogram, populations were clustered into 33 different groups. According to chromosome numbers, *P. bracteatum* resided in 4 different groups, *P. pseudo-orientale* clustered in 8 different groups, and *P. orientale* generated 1 group on its own. Twenty-four different groups were formed, where subgroups were mixed in terms of chromosome numbers. Lavania and Srivastava (1999) reported that alterations in chromosome number, size, and structure are common in the genus, the majority having n = 7. The genus had 3 basic chromosome numbers, x = 6, 7, and 11, together with intra- and interspecific polyploidy. Amplified fragment length polymorphism (AFLP) markers were studied to determine the genetic variation among species of *Papaver* (section *Oxytona*). It has been reported that regenerated plants showed different morphological and phytochemical characteristics from those of their source material. In addition, phytochemical chromosome data indicated that the seed used in cultures was of hybrid origin and that the loss in genetic uniformity was not due to somaclonal variation occurring during the in vitro culture process (Carolan et al., 2002). In our study, *P. pseudo-orientale* and *P. orientale* had higher genetic variation than P. *bracteatum*. It could be that they were collected from natural environments and were therefore open to cross-pollination.

The populations collected from the same regions partially clustered together. The accessions collected from Niğde and Erzincan resided in the same clades, while the accessions collected from other regions were positioned in different clades in the constructed phylogenetic dendrogram; however, they grouped in close clades. Therefore, it can be concluded that the pollination rate is apparently high in the Niğde accession. These observations showed that there is significant genetic variability among section *Oxytona* populations. Accessions from different regions or even the same regions may show no similarity (Dangi et al., 2004). In our study, accessions collected from 5 different regions grouped partially among each other, and the regions did not show clear discrimination. Parmaksız and Özcan (2011) indicated that wild types of this section show more diversity. It could be suggested that since their pollens and seeds can easily be spread by wind over long distances, gene flow is facilitated between populations. The fact that they are perennial may also factor in helping the partial maintenance of old genotypes from genetic material.

The analyses of our study indicate higher levels of variability within populations. Eight populations have a 0.00 similarity coefficient and these were found especially among the Niğde and Tunceli populations of *P. orientale* and *P. pseudo-orientale*. Ninety populations appeared to be closer to each other with a 0.09 similarity coefficient in *P. orientale* and *P. pseudo-orientale* populations, and their regions showed no significant separation. The morphological characters like spots on sepals, sepal colour, or sepal numbers in both populations also showed partial or complete similarity. The farthest genetic distance appeared in 15 pairs of plants, although their morphological traits showed partial similarity. Goldblatt (1974) indicated that *P. pseudo-orientale* is intermediate in terms of morphology

between *P. bracteatum* and *P. orientale*. Our observations showed correlation in this regard. As a result, it could be said that either the chromosome numbers of plants and their morphological traits or the number of primers that we used were not enough for differentiation of species in the section *Oxytona*.

These 3 accessions may show interspecific characters or intermediate accessions resulting from natural interspecific hybridisation (Goldblatt, 1974; Shoyama et al., 1998; Carolan et al., 2002, 2006; Coşkun et al., 2010; Dirmenci et al., 2010). Hence, the true naming of these accessions remains under debate. In general, even though molecular analysis is more reliable than morphological analysis, use of parameters from both methods would be highly effective for characterisation of variation in the section *Oxytona* (Parmaksız and Özcan, 2011).

In conclusion, genetic diversity among section *Oxytona* was analysed for the first time with ISSR molecular markers. Optimisation of the ISSR–PCR method was performed. Therefore, for the development of mapping programs for section *Oxytona*, for which this information is still not available, other marker systems must be analysed.

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