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Journal of Advanced Research in Natural and Applied Sciences

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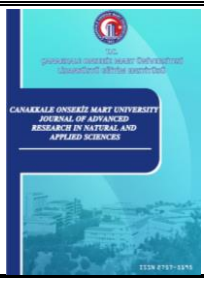

e-ISSN: 2757-5195 2024, Vol. 10, Issue 2, Pages: 426-434 doi.org/10.28979/jarnas.1428996

First Trials of Genome Analyses in Some *Onobrychis* Species using Dot-Blot and Genomic *in situ* Hybridization Techniques

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Article History
Received: 31 Jan 2024
Accepted: 26 Mar 2024
Published: 25 Jun 2024

Research Article

Abstract − The origin and genome composition of tetraploid cultivated *Onobrychis viciifolia* ($2n = 4x = 28$) were analyzed using dot-blot and genomic *in situ* hybridization (GISH) techniques. Dot-blot hybridization was used to find a genomic affinity between *O. viciifolia* and 16 diploid *Onobrychis* species. The hypothesis on the origin of the *O. viciifolia* was tested using GISH. Dot-blot analyses suggested a genomic affinity between *O. viciifolia* and four diploid *Onobrychis* species (*O. kachetica*, *O. supina*, *O. pallasii*, and *O. vaginalis*). Hybridization signals were observed on *O. viciifolia* chromosomes when gDNA of O. *kachetica*, *O. supina*, *O. pallasi*, and *O. hypargyrea* were used as probes. However, the observed chromosomal distribution of hybridization signals did not resemble GISH results. The observed signals colocalized with 35S rDNA or dispearse signals on all chromosomes were observed depending on the probe. Further investigations using more comprehensive and comparative analysis with both coding and repetitive DNA regions may provide a better understanding of the genome composition and evolution of *O. viciifolia*.

Keywords − *Onobrychis, Dot-blot hybridization, GISH, FISH*

1. Introduction

Onobrychis genus is a member of subfamily Faboidae of Fabaceae within the tribe Hedysareae. *Onobrychis* Mill. includes about 170 mostly perennial species distributed through temperate regions of North America, the Middle East and Europe [1-3]. Most *Onobrychis* species exist, especially in Anatolia, Caucasus, and Iran [4]. Based on floral characteristics this genus was classified into two subgenera: (i) *Onobrychis* Mill (consist of four sections *Dendrobrychis* DC., *Onobrychis,* Lophobrychis Hand.-Mazt., *Hemicyclobrychis* (Širj.) Rech.f.) and (ii) *Sisyrosema* Bunge (including sections *Hymenobrychis* DC., *Heliobrychis* Bunge ex Boiss., *Anthyllium* Nab., *Afghanicae* Širj.,) [5].

The most commonly cultivated species of the genus is *O. viciifolia*, used mainly as a forage crop with many valuable agronomic, environmental, and nutritional properties [1, 3, 6]. It is resistant to drought, pests, and diseases, palatability and is an important source for pollinators [1, 7]. Although the genus *Onobrychis* has potential agronomic importance, the genomic structure of *Onobrychis* species remains less studied [1, 8]. Detailed comprehensive analysis of its species in genetic and breeding research are necessary.

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Molecular phylogenetic analyses have shown that within the genus *Onobrychis* two main evolutionary lineages can be distinguished, which correspond to the redefined subgenus *Sisyrosema* and *Onobrychis* [8]. The nuclear genome size of *O. viciifolia* was reported as 2.5 pg/2C [9]. In this genus, two different basic chromosome numbers (x = 7 and 8) were reported with two different ploidy levels, diploids with ($2n = 2x$ = 14 or 16 and tetraploids with $2n = 4x$ = 28 or 32 chromosome number [10-14]. The ancestral basic chromosome number was determined as x = 8 in the nrITS phylogenetic context. Chromosomal organization of rDNA loci was analyzed by fluorescent *in situ* hybridization to provide more comprehensive information on karyotype structure of *Onobrychis* [15]. The genes encoding 5S rRNA and 35S rRNA (18S-5.8S-25S rRNA) consist of evolutionary highly conserved coding sequences [16] which are utilized as chromosomal markers [17, 18]. Several gains and repositioning of rDNA locus repatterning especially among diploids were suggested to explain their organisation in *Onobrychis*. Chromosome number and the organization of rDNA loci showed different patterns in both analysed *Onobrychis* subgenera. Two different basic chromosome numbers (x = 7 and 8) seem to be a result of several events of descending dysploidy (changes in chromosome number due to structural chromosomal rearrangements e.g., translocation or chromosome fusion [15, 19]. Dysploidy and polyploidy are important forces of evolution and diversification in *Onobrychis* genus and many of them are of polyploid origin [15]. Polyploids which derive from a genome doubling which happens in a species are called autopolyploids whereas, allopolyploid genome formation includes an interspecific hybridisation followed by a doubling of genome [20].

Different hybridization methods e.g. dot-blot and genomic *in situ* hybridization (GISH), might be useful in studying the genomic relationship among species. Dot-blot hybridization which is a nucleic acid hybridization technique where complementary single-stranded sequences of DNA probe hybridize with single-stranded sequences of the sample genome on a membrane under suitable conditions [21]. Dot-blot hybridizaiton was successfully used to analyse genome affinity in different genera for example *Arachis* species [22]. On the other hand, GISH is widely used to find the origin of numerous allopolyploids [23]. Molecular phylogenetic and GISH were used to identify the parental taxa of *Chenopodium quinoa* and *C. berlandieri* [24]. In *Passiflora* hybrid species, GISH was successfully used to confirm parental genomes [25].

The specific aims of this study were as: (i) to study genomic affinities of *O. viciifolia* with wild diploid taxa using dot-blot hybridization; (ii) to test the hypothetical parentage by mapping parental genomes in the polyploid using GISH.

2. Materials and Methods

2.1. Plant Material

Cultivated accession of tetraploid *O. viciifolia* and 16 diploid *Onobrychis* species were studied (Table 1). Seeds were grown in a greenhouse facility of University of Silesia in Katowice, Poland.

Table 1. Information of the analyzed taxa

Species name	USDA* Collection Number
O. viciifolia Scop.	PI 170583
O. vaginalis C.A. Mey.	PI 325444
O. megataphros	PI 301107
O. caput- galli (L.) Lam.	PI 205304
O. iberica Grossh.	PI219602
O. kachetica Boiss. & Buhse	PI 314469

*USDA North Central Regional Plant Introduction Station of the US National Plant Germplasm System

Table 1. (Continued) Information of the analyzed taxa

Species name	USDA* Collection Number
O. gaubae Boiss. & Buhse	PI 380931
O. radiata (Desf.) M. Bieb.	W6 24111
O. chorossanica Bunge ex Boiss.	PI 314160
O. sternorhiza D.C	PI 319056
O. vassilczenkoi Grossh.	PI 678913
O. gracilis Besser	W6 19496
O. pallasii	PI 325448
O. alba (Waldst. & Kit.) Desv. subsp. *laconica* (Boiss.) Hayek	W6 19337
O. supina (Vill.) DC.	PI 383721
O. ptolemaica (Delile) DC.	PI 215344
O. grandis Lipsky	PI 440568

*USDA North Central Regional Plant Introduction Station of the US National Plant Germplasm System

2.2. Genomic DNA Isolation

Total genomic DNA (gDNA) was extracted from healthy and fresh leaf tissue following the modified CTAB extraction method [26]. Genomic DNA concentration and quality were checked using Nano drop (ND-1000, peqLab, Erlangen, Germany). The quality of the DNA was verified by electrophoresis in 1% agarose gel.

2.3. Dot-Blot Hybridization

The genomic affinity between cultivated *O. viciifolia* and 16 wild diploid *Onobrychis* species (*O. vaginalis*, *O. caput-galli*, *O. iberica*, *O. kachetica*, *O. gaubae*, *O. radiata*, *O. chorossanica*, *O. sternorhiza*, *O. vassilczenkoi*, *O. gracilis*, *O. pallasii*, *O. alba* subp. *laconica*, *O. supina*, *O. ptolemaica*, *O. grandis*) was analyzed using dot-blot hybridization using as a probe gDNA of *O. viciifolia*. The total genomic DNA of tetraploid *O. viciifolia* was labelled with alkali-labile digoxigenin-11-dUTP using DIG Nick Translation Kit (Roche) according to the manufacturer protocol. The 1 µg of genomic DNA isolated from analysed diploid species were denatured at 95 ºC for 10 min and genomic DNA of the species were transferred onto a positively charged nylon membrane using the Dot-Blot 96 System (Biometra, Germany). gDNAs of the analysed samples were fixed to the positively charged nylon membrane by UV treatment (UVP, CL-1000 Ultraviolet Crosslinker), washed with distilled sterile water and then air dried. Hybridization process was performed at 37 ºC using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) following to the manufacturer's protocol with 75% stringency washes. The signal detection process was accomplished using anti-DIG antibody conjugated with alkaline phosphatase and chemiluminescence visualization was used. The hybridisatiton signals were captured and quantified using ChemiDocXRS (BioRad, USA).

2.4. Genomic *in situ* Hybridization and Fluorescence *in situ* Hybridization

Seeds of the species were germinated in Petri dishes on moist filter paper at room temperature. Roots approximately 2.0 cm long were immersed in ice-cold water for approximately 24 h then fixed in methanol/glacial acetic acid (3:1). The fixed materials stored at 4 ºC until use. Fixed roots were washed in 0.01 citrate buffer for approximately 20 min and then digested in a mixture of enzymes, 20 % (v/v) pectinase (Sigma), 1% (w/v) cellulase 'Onozuka R-10' (Serva) and 1% (w/v) cellulase (Calbiochem) for 1.5 h at 37ºC. Dissected meristems from root tips were transferred on a microscope slide into a drop of 45% acetic acid and then squashed. The coverslips were removed from microscope slide following the freezing, the chromosome slides were air dried. The prepared chromosome slides stored at 4˚C [27].

The probe labelling and GISH followed the Kolano et al. [28]. The genomic DNA of *O. kachetica*, *O. supina*, *O. pallasii*, and *O. hypargyrea* were labelled with digoxygenin-11-dUTP using nick translation mix (Roche, Switzerland). gDNA isolated from *O. hypargyerea*, species which seems to be distantly related with *O.viciifolia* [13], was used as a control in GISH. The hybridization mixture containing 10% dextran sulphate, 50% deionised formamide, 0.5% SDS (sodium dodecyl sulphate), 2x SSC (Saline sodium citrate) and labelled gDNA, was denatured approximately 10 minutes at 75 ºC and applied to somatic chromosome slides. The chromosome preparations with the hybridization mixture were denatured together at 72 ºC approximately for 5 min in an *in situ* Thermal Cycler (ThermoHybaid, Franklin, USA). Hybridization was conducted about 48 h at 37 ºC in a humid chamber. FITC-conjugated primary anti-digoxigenin antibody (Roche, Switzerland) was used to detect digoxygenin following post-hybridization washes and signal amplification with FITC-conjugated antisheep secondary antibodies (Jackson ImmunoResearch, USA).

A 2.3-kb fragment of the 35S rDNA coding region of *Arabidopsis thaliana* [29] labelled with tetramethyl-rhodamine5-dUTP (Roche) was used to detect the 35S rDNA loci. The probe labelling process and FISH were performed according to the protocol of the Jenkins and Hasterok [30].

The chromosome slides were stained in Vectashield containing 2.5 ng/µL of DAPI (4′,6-diamidino-2-phenylindole dihydrochloride). All analyzed images were collected using a fluorescent microscope (Zeiss AxioImager.Z.2, Oberkochen, Germany). The somatic chromosome images were processed using ZEN 2. blue edition (Zeiss). Two individuals were analyzed for each species.

3. Results and Discussion

The affinity between the genome of cultivated tetraploid *O. viciifolia* (2*n* = 4*x* = 28) and 16 diploid *Onobrychis* species (2*n* = 2*x* = 14 or 16) was analysed using dot-blot hybridization (Figure 1). After dot-blot hybridization with labelled gDNA of *O. viciifolia*; relatively strong hybridization signals were observed for *O. kachetica, O. supina, O. pallasii* and *O. vaginalis* suggesting a relatively high genomic affinity between the tetraploid and these four diploids. Rest of the analyzed diploid *Onobrychis* species revealed weak or no signals after dot-blot hybridization with genomic DNA of *O. viciifolia* (Figure 1).

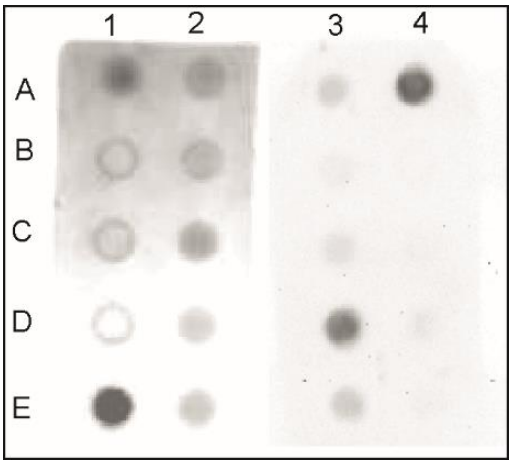

Figure 1. Dot-blot hybridization revealing the genome affinities of *O. viciifolia* with representative diploid *Onobrychis* species from two subgenera which are *Onobryhcis* and *Sisyrosema*: *O. vaginalis*, (A1), *O. megataphros (*B1), *O. caput-galli* (C1), *O. iberica* (D1), *O. kachetica* (E1), *O. gaubae* (A2), *O. radiata* (B2), *O. chorossanica* (C2), *O. sternohiza* (A3), *O. vassilczenkoi* (B3), *O. gracilis* (C3), *O. pallasi* (D3), *O. alba* subp. *laconica* (E3), *O. supina* (A4), *O. ptolemaica* (B4), *O. grandis* (C4).

Because *O. supina* belongs to the subgenus *Onobrychis* while three other diploids (*O. kachetica*, *O. pallasii* and *O. vaginalis*) belong to the subgenus *Sisyrosema* the obtained result suggested that the hybridization between species that belong to two different subgenera might have given rise of *O. viciifolia*. Its allotetraploid origin was also supported by analyses of repetitive sequences showing that two different centromeric satellite repeats interact with CENH3 in *O. viciifolia* also supports the allotetraploid origin of *O. viciifolia* [31, 32]. The origin of *O. viciifolia* and its genome composition was unknown [15, 32-36]. However recently analyses based on EST-SSRs markers and relatively complete chromosome level genome assemble supported rather autotetraploid origin of *O. viciifolia* [37, 38].

GISH is a valuable method to study the origin of polyploid species and enables the identification of two sets of parental chromosomes [24, 39-41]. gDNA of four diploid species, *O. kachetica*, *O. supina*, *O. pallasi* and *O. hypargyrea* were used as a probe in GISH to *O. viciifolia* chromosomes. Recent molecular phylogenetic analyses of nrITS and combined plastid markers (trnL- F, rpl32/rpl32-trnL-F(UAG), ndhFrpl32) have supported the close relationship between *O. viciifolia* and *O. supina* placed them in the same clade within the subgenus *Onobrychis* [8,15]. On the other hand, two other species, *O. kachetica* and *O. pallasi*, were recovered in a different clade and subgenus than *O. viciifolia* [2, 15].

After GISH with gDNA of *O. kachetica* as a probe, intense hybridisation signals were detected on four chromosomes of O. *viciifolia* while the remaining chromosomes showed much weaker and dispersed hybridization signals mainly located at pericentromeric region of *O. viciifolia* chromosomes (Fig. 2. A, C). After GISH using gDNA of *O. supina* as a probe, weak hybridization signals were observed mostly in subtelomeric regions of all chromosomes of the tetraploid (Fig. 2. B, C). The application of GISH with gDNA of *O. pallasi* as a probe allowed to observe four major and several minor hybridization signals on eight chromosomes of *O. viciifolia* (Fig. 2. D). When gDNA of *O. hypargyrea*, was used as a probe, hybridization signals were observed on four chromosomes of *O. viciifolia* (Fig. 2. E). Reprobing of the same metaphase plates with 35S rDNA revealed that four intense hybridization signals observed after GISH with *O. hypargyrea* colocalized with the hybridization signals of 35S rDNA (Fig. 2. F). The coding sequences of 35S rDNA are evolutionary conserved and very similar in all land plant [16]. Additionally, the four major hybridization signals observed with gDNA of *O. pallasii* resemble the results of FISH with 35S rDNA to *O. viciifolia* chromosomes which were obtained earlier by Yucel et al. [15].

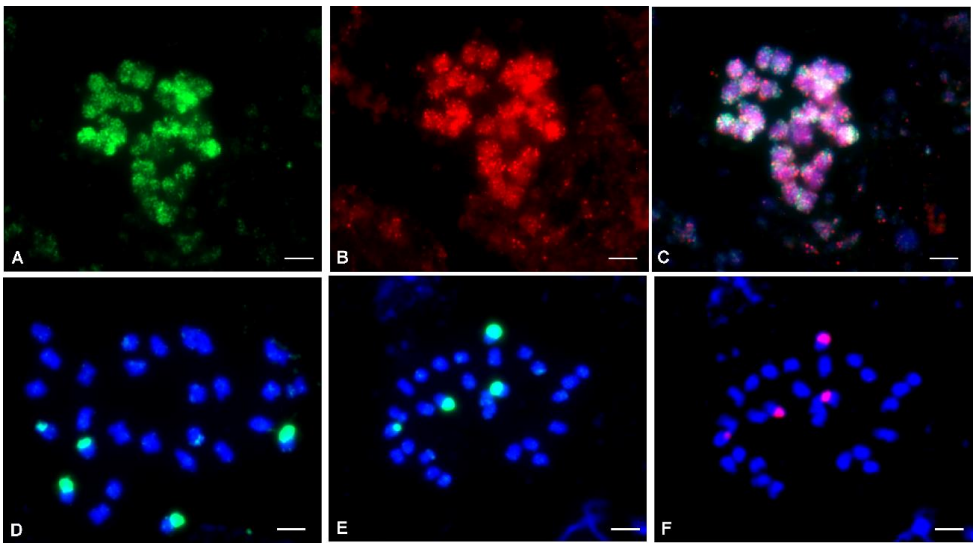

Figure 2. Somatic metaphases of *O. vicifolia* after GISH with gDNA isolated from **A.** *O. kachetica* **B**. *O. supina* gDNA on chromosomes of *O. viciifolia*, **C**. *O. supina* (red) and *O. kachetica* (green) **D**. *O. pallasi*. **E**. *O. hypargyrea* **F.** Reprobing with 35S rDNA on same metaphase plate of *O. viciifolia* chromosome plate.

GISH is a very useful method for the identification of an ancestor genome in hybrids and allopolyploid plants since it usually obtains clear and unambiguous distinction between two parental subgenomes e.g. GISH was successfully used to verify the origin of *Lilium* hybrids [39]. However, after GISH with tested gDNAs to *O. viciifolia* chromosomes observed patterns of hybridization signals did not allow to distinguish the parental subgenome. Obtained results showed that *O. viciifolia* and analysed diploids share similar repetitive sequences but they did not allow to identify parental species of the tetraploid. The coding sequences of 35S rRNA genes are evolutionary highly conserved and very similar in plant genome as it was shown by comparative GISH (cGISH) [42]. Except rDNA, the other hybridization signals were observed mainly in the pericentromeric region of *O. viciifolia* chromosomes (Fig. 2). This heterochromatic region of chromosomes contains many different repeats e.g. retrotransposons which might be similar in related species [43]. The obtained results may suggest several hypotheses: (i) tested diploids are not parental species of *O. viciifolia*; (ii) the parental species of analysed tetraploid were very closely related, and their genomes consist of similar repetitive sequences and (iii) the *O. viciifolia* is a relatively old allotetraploid which underwent extensive diploidization. This post-polyploidyzation evolution, which involve many different mechanisms e.g. homogenization, elimination and gene conversion [44], have made the parental subgenomes extremely difficult to distinguished [44-46].

4. Conclusion

The origin and evolutionary history of *O. viciifolia* is complex, and there is no exact data about progenitor or progenitors. A more global and comparative analyses combining molecular phylogenetic approached and cytogenetic studies should be applied to better understand the evolutionary and origin of *O. viciifolia*. For example, molecular markers such as chloroplast DNA (cpDNA) or methods based on new generation sequencing (NGS) e.g. RAD-seq should be used.

Author Contributions

The first author performed investigation, methodology, experiments, writing original draft preparation. The second, third, and fourth authors planned conceptualization, writing, review, and editing. The second author also planned methodology. All authors read and approved the final version of the paper. This paper is derived from the first author's doctoral dissertation supervised by the third and fourth authors.

Conflicts of Interest

All the authors declare no conflict of interest.

Acknowledgement

The study was supported by the Scientific and Technological Research Council of Turkey, Grant number: 215O526.

References

[1] C. H. Carbonero, I. Mueller-Harvey, T. A. Brown, L. Smith, *Sainfoin (Onobrychis viciifolia): A beneficial forage legume*, Plant Genetic Resources: Characterization and Utilization 9 (1) (2011) 70–85.

[2] C. H. Carbonero, F. Carbonero, L. M. J. Smith, T. A. Brown, *Phylogenetic characterisation of Onobrychis species with special focus on the forage crop Onobrychis viciifolia Scop*, Genetic Resources and Crop Evolution 59 (2012) 1777–1788.

[3] T. Sutcu, B. B. Bilgen, M. Tuna, *Analysis of genetic diversity among Onobrychis accessions with high agronomic performance by simple sequence repeat (SSR) markers*, Molecular Biology Reports 49 (2022) 5659–5668.

[4] B. Yıldız, B. Çıplak, E. Aktoklu, *Fruit morphology of sections of the genus Onobrychis Miller (Fabaceae) and its phylogenetic implications*, Israel Journal of Plant Sciences 47 (4) (1999) 269–282.

[5] G. I. Sirjaev, Onobrychis Generis Revisio Critica, Pars Prima; Faculte' des Sciences de I'Universite Masaryk: Brno, Brno, 1925.

[6] E. C. Yilmaz, B. B. Bilgen, M. Tuna, *Evaluation of the genetic structure of some accessions belonging to Onobrychis spp. using microsatellite DNA markers*, Journal of Tekirdag Agricultural Faculty 20 (2) (2023) 399-409.

[7] M. Mora-Ortiz, L. Smith, *Onobrychis viciifolia; a comprehensive literature review of its history, etymology, taxonomy, genetics, agronomy and botany*, Plant Genetic Resources 16 (5) (2018) 403–418.

[8] A. Amirahmadi, S. Kazempour-Osaloo, A. Kaveh, A. A Maassoumi, R. Naderi, *The phylogeny and new classification of the genus Onobrychis (Fabaceae-Hedysareae): Evidence from molecular data*, Plant Systematics and Evolution 302 (2016). 1445–1456.

[9] C. H. Carbonero, F. Carbonero, L. M. J. Smith, T. A. Brown, *Cytological characterisation of the underutilized forage crop Onobrychis viciifolia Scop. and other members of the Onobrychis genus*, Genetic Resources and Crop Evolution 60 (7) (2013) 1987–1996.

[10] M. M. Abou-El-Enain, *Chromosomal criteria and their phylogenetic implications in the genus Onobrychis Mill. sect. Lophobrychis (Leguminosae), with special reference to Egyptian species*, Botanical Journal of Linnean Society 139 (4) (2002) 409–414.

[11] H. Hoşgören, *Total numbers of chromosome numbers in species of Onobrychis Miller (Fabaceae) in Southeastern Anatolia region*, Biotechnology Biotechnoligal Equipment 20 (2) (2006) 57–61.

[12] H. H. S. Mohsen, M. Z. Nasab, *Cytotaxonomy of some Onobrychis (Fabaceae) species and populations in Iran*, Caryologia 63 (2010) 18–31.

[13] S. E. Akçelik, S. Avcı, S. Uzun, C. Sancak, *Karyotype analysis of some Onobrychis (sainfoin) species in Turkey*, Archieves of Biological Sciences 64 (2012) 567–571.

[14] M. Ranjbar, F. Hajmoradi, R. Karamian, *An overview on cytogenetics of the genus Onobrychis (Fabaceae) with special reference to O. sect. Hymenobrychis from Iran*, Caryologia 65 (2012) 187–198.

[15] G. Yücel, A. Betekhtin, E. Cabi, M. Tuna, R Hasterok, B. Kolano, *The chromosome number and rDNA loci evolution in Onobrychis (Fabaceae)*, International Journal of Molecular Sciences 23 (19) (2022) 11033 17 pages.

[16] R. Volkov, F. Medina, U. Zentgraf, V. Hemleben, Molecular Cell Biology: Organization and molecular evolution of rDNA, nucleolar dominance and nucleolus structure, Progress in Botany, Springer-Verlag, Berlin, 2004, Ch. 4 pp. 106–146.

[17] I. Alvarez, J. F. Wendel, *Ribosomal ITS sequences and plant phylogenetic inference*, Molecular Phylogenetics and Evolution 29 (3) (2003) 417–434.

[18] G. N. Feliner, J. A. Rossello, *Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants*, Molecular Phylogenetics and Evolution 44 (2) (2007) 911–919.

[19] M. A. Lysak, I. Schubert, Mechanisms of chromosome rearrangements, Plant Genome Diversity Volume 2, Springer, Vienna, 2013, Ch. 9, pp. 137–147.

[20] M. Te Beest, J. J. Le Roux, D. M. Richardson, A. K. Brysting, J. Suda, M. Kubesova, P. Pysek, *The more*

the better? The role of polyploidy in facilitating plant invasions, Annals of Botany 109 (1) (2012) 19–45.

[21] A. I. Bhat, G. P. Rao, Dot-Blot hybridization technique, Characterization of Plant Viruses, Springer Protocols Handbooks, New York, 2020, Ch. 34, pp. 303–321.

[22] G. Robledo, G. Seijo, *Characterization of the Arachis (Leguminosae) D genome using Fluorescence in situ Hybridization (FISH) chromosome markers and total genome DNA hybridization*, Genetics and Molecular Biology 31(3) (2008) 717–724.

[23] M. Markova, B. Vyskot, *New horizons of genomic in situ hybridization*, Cytogenetic and Genome Research 126 (4) (2009) 368–375.

[24] B. Kolano, J. McCann, M. Orzechowska, D. Siwinska, E. Temsch, H. Weiss-Schneeweiss, *Molecular and cytogenetic evidence for an allotetraploid origin of Chenopodium quinoa and C. berlandieri (Amaranthaceae)*, Molecular Phylogenetics and Evolution 100 (2016) 109–123.

[25] C. A. Melo, G. S. Silva, M. M. *Souza Establishment of the genomic in situ hybridization (GISH) technique for analysis in interspecific hybrids of Passiflora*, Genetics and Molecular Research: GMR 14 (1) (2015) 2176–2188.

[26] K. Emadzade, T.-S. Jang, J. Macas, A. Kovařík, P. Novák, J. Parker, H. Weiss-Schneeweiss, *Differential amplification of satellite PaB6 in chromosomally hypervariable Prospero autumnale complex (Hyacinthaceae)*, Annal of Botany 114 (8) (2014) 1597–1608.

[27] R. Hasterok, J. Draper, G. Jenkins, *Laying the cytotaxonomic foundations of a new model grass, Brachypodium distachyon (L.) Beauv*, Chromosome Research, 12 (2004) 397–403.

[28] B. Kolano, J. McCann, M. Oskedra, M. Chrapek, M. Rojek, A. Nobis, H. Weiss-Schneeweiss, *Parental origin and genome evolution of several Eurasian hexaploid species of Chenopodium (Chenopodiaceae)*, Phytotaxa 392 (3) (2019) 163–185.

[29] I. Unfried, P. Gruendler, *Nucleotide sequence of the 5.8S and 25S rRNA genes and of the internal transcribed spacers from Arabidopsis thaliana*, Nucleic Acids Researches 18 (13) (1990) 4011 1 pages.

[30] G. Jenkins, R. Hasterok, *BAC "landing" on chromosomes of Brachypodium distachyon for comparative genome alignment*, Nature Protocols 2 (2007) 88–98.

[31] A. L. Tek, S. D. Kara Öztürk, *High allelic diversity of the centromere-specific histone H3 (CENH3) in the legume sainfoin (Onobrychis viciifolia)*, Molecular Biology Reports 47 (2020) 8789–8795.

[32] S. D. Kara Öztürk, *Analysis of centromeric heterochromatin components by bioinformatics, molecular and cytogenetics methods in sainfoin (Onobrychis viciifolia scop.)*, Master Thesis Niğde Ömer Halisdemir University (2022) Niğde.

[33] M. D. Sacristan, Estudios citotaxonómicos sobre el género Onobrychis (L). Adanson conreferencia especial a la citogenética de la esparceta (*O. viciifolia* Scop.), Zaragoza, Spain, 1965.

[34] M. C. De Vicente, P. Arus, *Tetrasomic inheritance of isozymes in sainfoin (Onobrychis viciaefolia Scop.)*, Journal of Heredity 87 (1996) 54–62.

[35] M. Zarrabian, M. M. Majidi, M. H. Ehtemam, *Genetic diversity in a worldwide collection of sainfoin using morphological, anatomical, and molecular markers*, Crop Science 53 (6) (2013) 2483–2496.

[36] K. Kempf, *Self-fertilization and marker-trait associations in Sainfoin (Onobrychis viciifolia)*, Doctoral Dissertation ETH (2016) Zurich.

[37] S. Shen, X. Chai, Q. Zhou, D. Luo, Y. Wang, Z. Liu, *Development of polymorphic EST-SSR markers and characterization of the autotetraploid genome of sainfoin (Onobrychis viciifolia)*, PeerJ, 7:e6542 19 pages.

[38] J. He, D. Tian, X. Li, X. Wang, T. Wang, Z. Wang, H. Zang, X. He, T. Zhang, Q. Yun, R. Zhang, R. Jiang., S. Jia, Y. Zhang, *A chromosome-level genome assembly for Onobrychis viciifolia reveals gene copy number gain underlying enhanced proanthocyanidin biosynthesis*, Communicaitions Biology 7 19 (2024).

[39] A. Marasek, R. Hasterok, K. Wiejacha, T. Orlikowska, *Determination by GISH and FISH of hybrid status in Lilium*, Hereditas 140 (1) (2004) 1–7.

[40] G. Seijo, G.I. Lavia, A. Fernandez, A. Krapovickas, D.A. Ducasse, D. J. Bertioli, E. A. Moscone, *Genomic relationships between the cultivated peanut (Arachis hypogaea, Leguminosae) and its close relatives revealed by double GISH*, American Journal of Botany 94 (2007) 1963–1971.

[41] Z. Fredotovic, I. Samanic, H. Weiss-Schneeweiss, J. Kamenjarin, T. S. Jang, J. Puizina, *Triparental origin of triploid onion, Allium x cornutum (Clementi exVisiani, 1842), as evidenced by molecular, phylogenetic and cytogenetic analyses*, BMC Plant Biology 14 (2014) Article Number 24 14 pages.

[42] J. F. Zoller, Y. Yang, R. G. Herrmann, U. Hohmann, *Comparative genomic in situ hybridization (cGISH) analysis on plant chromosomes revealed by labelled Arabidopsis DNA*, Chromosome Research 9 (2001) 357–375.

[43] H. B. Ali, M. A. Lysak, I. Schubert, *Genomic in situ hybridization in plants with small genomes is feasible and elucidates the chromosomal parentage in interspecific Arabidopsis hybrids*, Genome 47 (5) (2004) 954–960.

[44] K. Wolfe, *Yesterday's polyploids and the mystery of diploidization*, Nature Reviews Genetics 2 (2001) 333–341.

[45] I. J. Leitch, L. Hanson, K. Y. Lim, A. Kovarik, M. W. Chase, J. J. Clarkson, A. R. Leitch, *The ups and downs of genome size evolution in polyploid species of Nicotiana (Solanaceae)*, Annals of Botany 101 (6) (2008) 805–814.

[46] T. S. Jang, H. Weiss-Schneeweiss, *Formamide-free genomic in situ hybridization allows unambiguous discrimination of highly similar parental genomes in diploid hybrids and allopolyploids*, Cytogenetic and Genome Research 146 (4) (2015) 325–331.