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RESEARCH ARTICLE

Molecular cytogenetic characterization, leaf anatomy and ultrastructure of the medicinal plant *Potentilla alba* L.

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Abstract *Potentilla alba* L. is a valuable medicinal plant widely used in folk and traditional medicine and particularly promising in complex treatment of thyroid pathology. Natural resources of this species are insufficient due to ever-growing use in contemporary medicine. Comprehensive investigations of different *P. alba* populations are essential for the successful extension of *P. alba* plantings. Aiming for a better understanding of karyotype structure, chromosome behaviour in meiosis and developing new diagnostic characters, we performed molecular cytogenetic characterization and leaf structure and ultrastructure analyses of two introduced *P. alba* samples originating from different habitats. Based on chromosome morphology, distribution of 45S/5S rDNA and DAPI-banding patterns, all chromosomes in the karyotypes were identified and the *P. alba* chromosomal idiogram was constructed. Our findings confirmed *P. alba* karyotype stability and also revealed several diagnostic characters of this species: the features of cells of upper and lower leaf epidermis, the presence of calcium oxalate druses and three types of leaf indumentum, essential for evaluation of genetic

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diversity in different populations, validation of raw materials and further selection progress. The meiotic abnormalities were detected probably related to low pollen activity and indicated the advantages of vegetative propagation in the development of a *P. alba* plantation system.

Keywords FISH · Karyotype · Leaf anatomy and ultrastructure · Meiosis · *Potentilla alba* L.

Introduction

The large and polymorphic genus *Potentilla* L. (Rosaceae) consists of more than 500 species including valuable medicinal plants (Heywood 2007). They are annual and perennial herbs or shrubs distributed in arctic and temperate regions of the northern hemisphere (Wolf 1908; Yuzepchuk 1941; Heywood 2007). The phylogenetic relationships within the genus *Potentilla* are rather intricate as its speciation is a continuous process. Inter- and intraspecific hybridization events as well as crossings with various species from other genera of the family *Rosaceae* (e.g., *Fragaria, Sibbaldia*) result in the appearance of polyploids and/or new morphobiotypes with chromosome number polymorphism (Stebbins 1950; Asker and Frost 1970; Asker 1971; Grant 1981; Masterson 1994; Eriksson et al. 2003; Soltis and Soltis 2009). Besides, the capability of *Potentilla* species for different types of propagation (seed and/or vegetative) also complicates the taxonomy of the genus (Dickinson et al. 2007; Dobeš et al. 2015). Currently, for clarification of phylogenetic relationships within the genus and a better understanding of plant adaptation mechanisms to different environmental conditions (Shimono et al. 2009; Ma et al. 2015), the genus *Potentilla* is being intensively investigated at the morphological, biochemical and molecular levels (Eriksson et al. 1998; Kolodziejek and Gabara 2007; Dobes and Paule 2010; Paule et al. 2011; Rani et al. 2012; Faghir et al. 2014; Bogacheva et al. 2016).

Potentilla alba L. is one of the most valuable species of the genus, widely used in folk and traditional medicine. It contains broad range of biologically active compounds (iridoids, saponins, phenolcarbonic acids, quercetin, gallotannin, phytosterols) providing the vast variety of its pharmacologic properties (Smyk and Krivenko 1975; Kovalenko et al. 2004; Matkowski et al. 2006; Ossipov et al. 2017). In pharmaceutical industry, raw material of this medicinal plant is included as a component in various remedies used in the complex treatment of thyroid pathology, hepatotherapy, coronary heart diseases and gastrointestinal tract problems (Kovalenko et al. 2004; Dorman et al. 2011; Kvacheniuk and Kvacheniuk 2012; Kaminskiĭ et al. 2013; Turchaninova 2014).

Nowadays, despite the rather wide distribution area of *P. alba* (Yuzepchuk 1941), it is considered to be a depleted and threatened species, and its poor natural resources are inadequate for the increasing demand for various biopharmaceuticals (Smyk and Krivenko 1975). This requires extensive use of the raw material obtained from introduced *P. alba* populations. Comprehensive morphological and genomic investigations of different *P. alba* populations are essential for successful extension of *P. alba* plantings and selecting new promising varieties with desired characteristics adapted to environments. Morphological and anatomical studies of *P. alba* allowed the establishment of some specific characters (Yuzepchuk 1941; Bogacheva et al. 2016). The genomic peculiarities of *P. alba* are still poorly investigated as only chromosome number and sizes (1–2 μm) have been determined for this species (and for most species of the genus *Potentilla* as well) (Asker 1985a; Iwatsubo and Naruhashi 1991; Delgado et al. 2000; Tomasz and Kołodziejek 2008).

In the present study, aiming for a better understanding of karyotype structure, chromosome behaviour in meiosis and developing new diagnostic characters essential for evaluation of genetic diversity in populations of *P. alba*, we performed its molecular cytogenetic characterization and studied the leaf structure and ultrastructure of two introduced samples originating from different habitats.

Materials and methods

Plant material

In the present study, we investigated two introduced *P. alba* samples (К 127-02) and (К 328-02) which were derived from promising wild morphotypes in Ivanovo (56°59′48″N, 40°58′55″E) and Penza (53°12′00″N, 45°00′00″E) regions (Russian Federation),

respectively. Since 2016, these samples have been cultivated in the Botanic Gardens of the All-Russian Institute of Medicinal and Aromatic Plants (VILAR), Moscow region, Russian Federation with a view to developing a new cultivar.

Chromosome spread preparation

In FISH assays, mitotic chromosome spreads were prepared from plant root meristem according to the technique developed previously for plant species with small chromosomes (Muravenko et al. 2009; Amosova et al. 2014).

For meiotic chromosome preparation, young floral buds were fixed in Carnoy's solution for 30 min at 4 °C and then chromosome spreads were prepared as previously described (Samatadze et al. 2014). The slides were stored in 96% ethanol at − 20 °C until used.

FISH procedure

Following probes were used for FISH:

pTa71 containing a 9 kb long repeated DNA sequence of common wheat encoding 18S, 5.8S and 26S rRNA genes including spacers (Gerlach and Bedbrook 1979);
pTa794 containing a 420 bp long repeated DNA sequence of wheat containing the 5S rRNA gene and the intergenic spacer (Gerlach and Dyer 1980);
The rDNA probes were labelled directly with SpectrumAqua (45S rDNA) and SpectrumRed (5S rDNA) fluorochromes (Abbott Molecular, Wiesbaden, Germany) according to the manufacturer's protocol. FISH procedure was carried out according to Muravenko et al. (2009). After overnight hybridization, the slides were washed as described previously (Amosova et al. 2017).

DAPI-banding

After FISH procedure chromosome slides were stained with 0.1 μg/mL DAPI (4′,6-diamidino-2-phenylindole) (Serva, Heidelberg, Germany) dissolved in Vectashield medium (Vector laboratories, Peterborough, UK).

Chromosome analysis

Chromosome slides were examined using an Olympus BX61 epifluorescence microscope (Olympus, Tokyo, Japan) combined with a monochrome CCD camera (Cool Snap, Roper Scientific Inc., Tucson, USA). The images were captured in grayscale channels. Then they were pseudocoloured and processed with Adobe Photoshop 10.0 (Adobe Systems Inc., Birmingham, USA) and VideoTest-Kario 1.5 (Ista-VideoTest, St Petersburg, Russia) software. Based on the results of measurements of 15 metaphase plates from 10 individual plants of each sample, total chromosome length, length of the short arm and the centromeric index (100 × length of short arm/total chromosome length) were calculated. Identification of *P. alba* mitotic chromosomes was based on their morphology and distribution of chromosome markers. The cytological numerical designation of *P. alba* chromosomes was by the decreasing order of size rather than by centromeric index (Levan et al. 1964). For meiosis analysis, at least 100 cells (10 plants) of each sample were analyzed.

Phytotomy and ultrastructure of leaves

Sample preparation for light and scanning electron microscopy (SEM) was performed according to the standard techniques described earlier (Dolgova and Ladygina 2003).

The study of the ultrastructure of *P. alba* leaf blades was conducted with the use of JEOL JSM – 6490LV scanning electron microscope (3.0 nm at 30 kV high vacuum mode) (Jeol, Tokyo, Japan). For this, the samples were coated with 20 nm (40 s at 40 mA) platinum film in the JEOL auto fine coater JFC – 160 (Jeol, Tokyo, Japan).

Results

DAPI-banding

Karyotypes of both *P. alba* samples had 14 pairs of rather small (1.5–3 μm) chromosomes. Karyotype formula was $K = 2n = 28 = 2(7\ m + 5sm + 2s^t)$. The analysis of DAPI-banding patterns showed similar distribution of DAPI-bands in chromosomes of the *P. alba* specimens: large DAPI-bands were mostly

located in the pericentromeric regions of chromosomes while polymorphic small and middle-sized bands were detected in the subtelomeric and intercalary regions (Fig. 1).

Chromosomal localization of 45S and 5S rDNA

In karyotypes of both *P. alba* samples, 45S rDNA sites were localized in the distal region of the short arms of two satellite (SAT) chromosome pairs. Double 5S rDNA sites were detected in the short arm (proximal

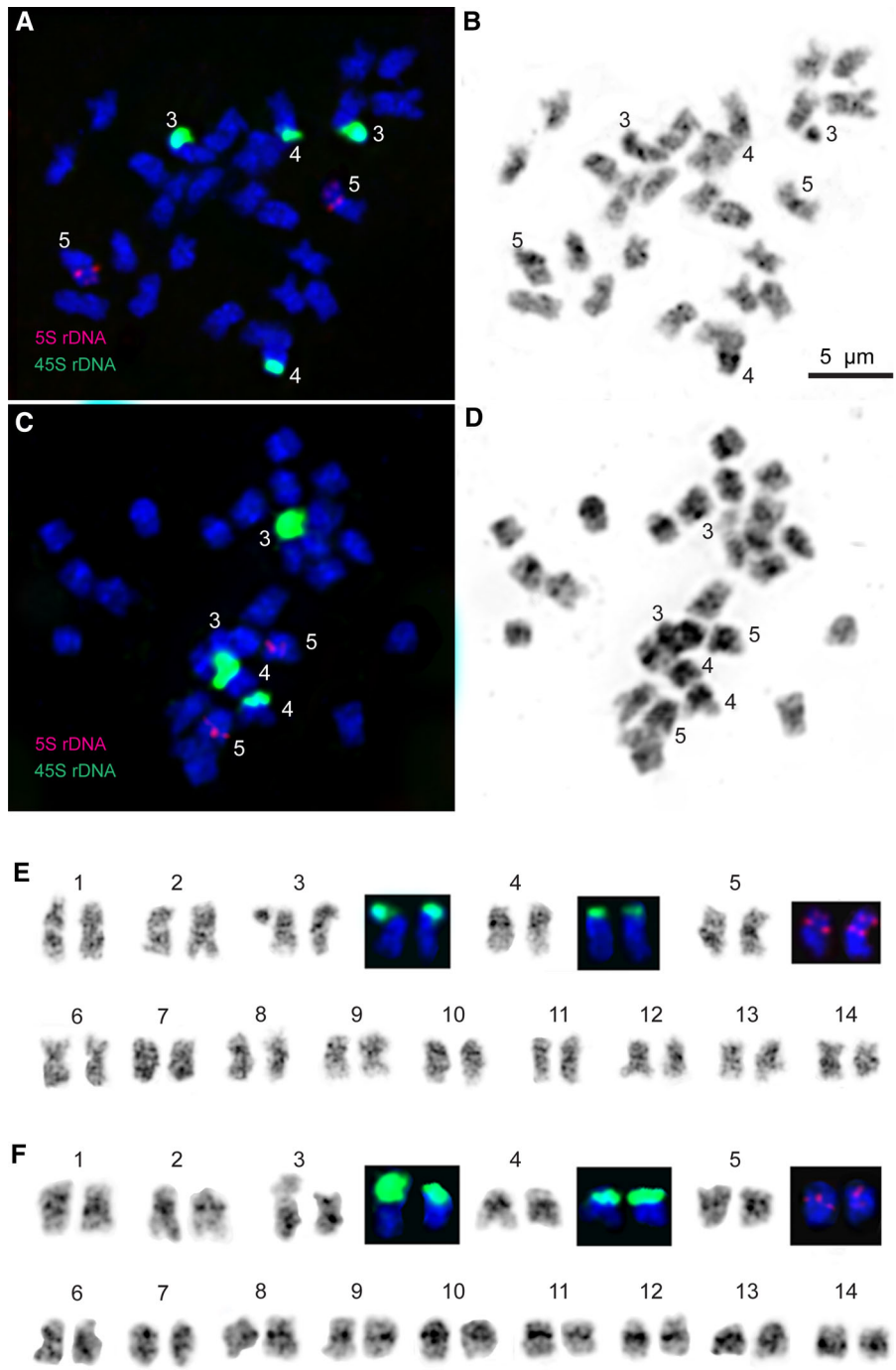

Fig. 1 FISH-based localization of 45S (green) and 5S (red) rDNA and DAPI-banding patterns on *P. alba* chromosomes. Metaphase spreads of the samples from the Penza (**A**, **B**) and Ivanovo (**C**, **D**) regions after FISH and DAPI-banding (inverted images); (**E**, **F**)—Karyograms (the same metaphase plates as in **A**–**D**) of the specimens from the Penza and Ivanovo regions after DAPI-banding (inverted image) and FISH (only chromosomes with the hybridizations sites are presented). (Color figure online)

and telomeric position) of one metacentric chromosome pair (Fig. 1). Based on chromosome morphology, DAPI-banding patterns and distribution of 45S and 5S rDNA sites, all chromosome pairs in the karyotypes were identified (Fig. 1) and chromosome idiograms of *P. alba* were constructed (Fig. 2). In the *P. alba* karyotypes, chromosomal abnormalities were not revealed.

Analysis of meiosis

In both *P. alba* samples, analysis of meiosis indicated fourteen rod bivalents. Besides, at metaphase I (M-I), chromosome associations (trivalents and quadrivalents) were found (Fig. 3A). At anaphase I (A-I) and anaphase II (A-II), most cells had normal chromosome disjunction (14:14) (Fig. 3B) but in 2.07–3.12% of the studied cells, various abnormalities (chromosome lagging, fragments, etc.) were also observed (detailed in Fig. 3C–G). At A-I and A-II, the most frequent aberration was lagging of several chromosomes behind the others resulting in their non-uniform distribution within a cell (Fig. 3C–G) as most lagging chromosomes could not reach the cellular poles and remained in the cell cytoplasm. In both studied *P. alba* samples, at the tetrad stage of meiosis II, normal tetrads (Fig. 3H) as well as few polyads (Fig. 3I) were observed.

Phytotomy and ultrastructure of leaves

In most *P. alba* plants, palmately compound leaf blades with five leaflets were observed though polymorphism in number of leaflets (from 4 to 6) was also detected (Fig. 4).

In both *P. alba* samples, the upper leaf epidermis was represented by cells with straight polygonal or slightly flexuous walls (Fig. 5A). In the mesoderm, calcium oxalate druses located along the leaf veins were detected (Fig. 5A). The cells of the lower leaf epidermis had flexuous walls (Fig. 5B). The glandular hairs were pigmented, and inside the head as well as in the stalk cells, brown secreted material was visible (Fig. 5B).

SEM analysis revealed numerous stomata on the lower leaf epidermis (Fig. 6). The stomata were anomocytic, and they were not detected on the upper epidermis of the leaves. Along the leaf edges, numerous simple trichomes were observed. The lower leaf epidermis was covered with simple unicellular hairs as well as glandular hairs consisting of a one- or two-celled round head continuous with a two- or three-celled stalk, and one basal cell. The simple unicellular hairs observed on the lower leaf epidermis were longer and thinner than the simple hairs on the leaf edges. It was found that the lower leaf epidermis of *P. alba* from the Penza region was more densely pubescent with simple unicellular trichomes than in *P. alba* plants from the Ivanovo region (Fig. 6).

Discussion

Karyotype analysis

For most species of the genus *Potentilla*, chromosome numbers and sizes (1–2 μm) were revealed (Müntzinc

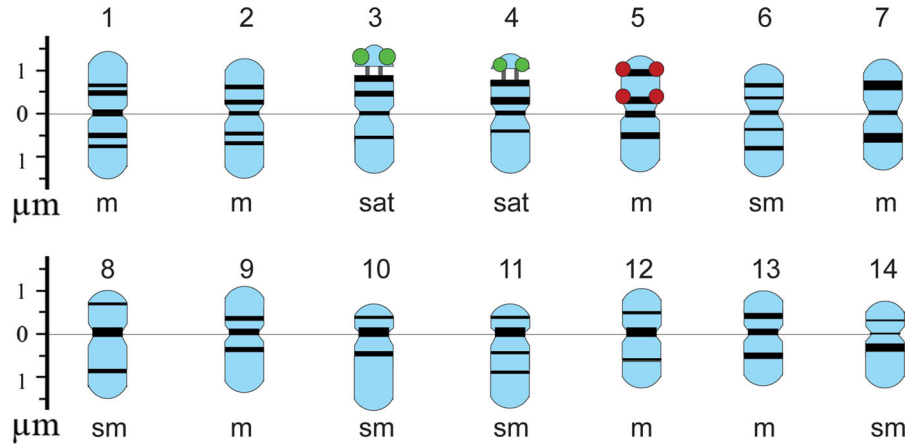

Fig. 2 Idiograms of *P. alba* chromosomes. Idiograms of *P. alba* chromosomes showing relative sizes and positions of DAPI-bands (black segments), 45S (green) and 5S (red) rDNA. (Color figure online)

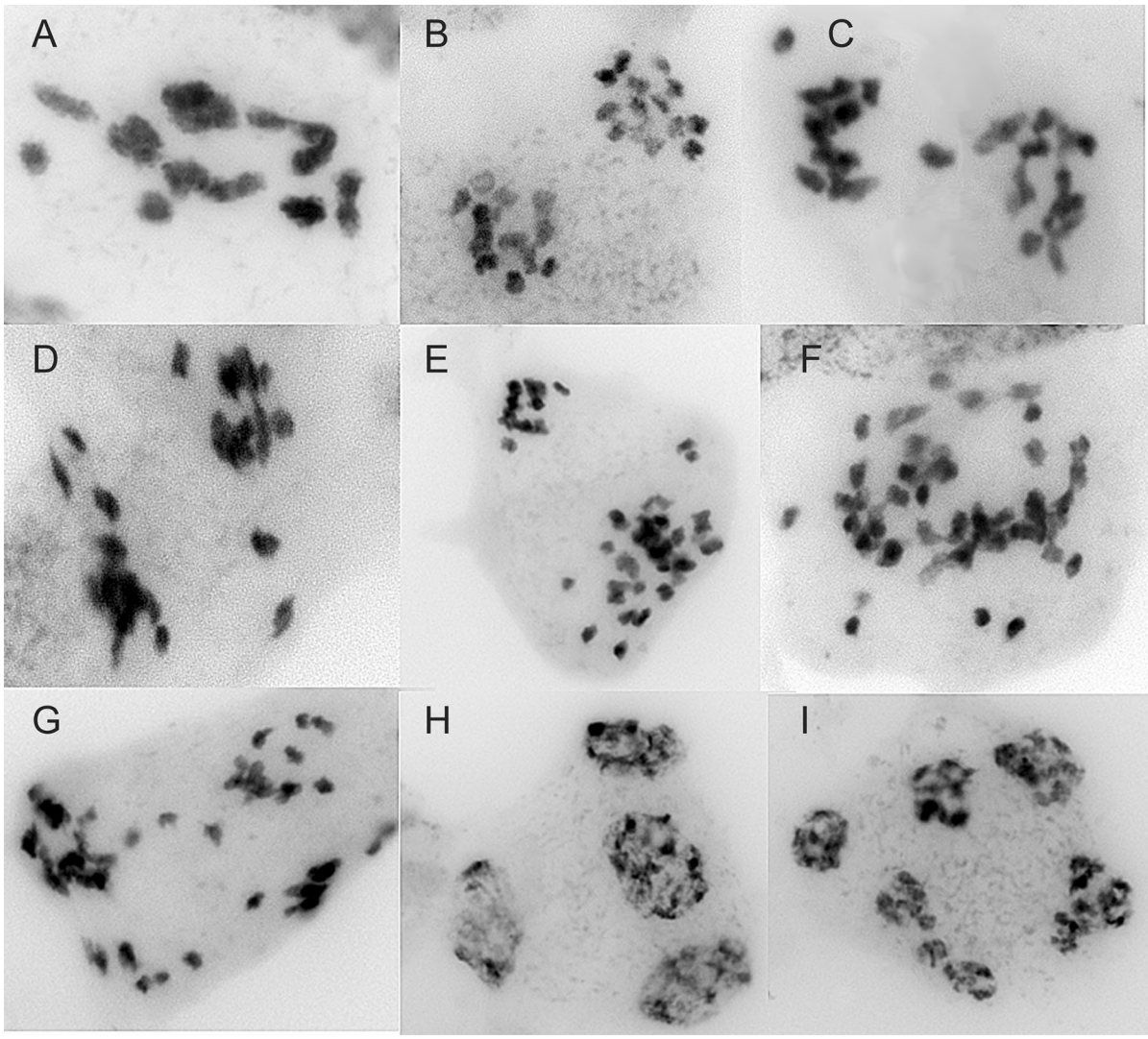

Fig. 3 Meiosis in maternal pollen cells of *P. alba.* **A** M-I: ($n = 10^{II} + 1^{IV}$); **B** A-I: normal distribution of chromosomes in the cell (14:14); **C**, **D** A-I: chromosome lagging; **E** A-I: non-uniform chromosome distribution within the cell; **F** A-II: non-uniform chromosome distribution within a cell; **G** A-II: chromosome lagging; **H** tetrad; **I** hexad

1958; Asker 1985a; Iwatsubo and Naruhashi 1991; Delgado et al. 2000; Tomasz and Kolodziejek 2008). Within the genus, a wide variation in chromosome number ranging from 2n = 14 to 2n = 112 was found; most common is 2n = 28 with the basic (monoploid) chromosome number x = 7 supporting a paleopolyploid origin (Delgado et al. 2000; Tomasz and Kolodziejek 2008; Jeelani et al. 2012). The species within the genus *Potentilla* are usually subdivided into two groups: the species having constant chromosome number and the ones with variable chromosomal numbers which can vary even within the same species (Stebbins 1950; Mesicek and Sojak 1993).

Similar to most *Potentilla* species, *P. alba* has 2n = 28 (Delgado et al. 2000; Rani et al. 2012). In the present study, the karyotype analysis of two introduced *P. alba* populations confirmed chromosome number stability as well as a possible tetraploid origin of the genome of this species. It is known that the detailed investigation of karyotypes of the species with small chromosomes needs special approaches (Muravenko and Zelenin 2009). The application of DNA intercalator 9-AMA, which slowed down the

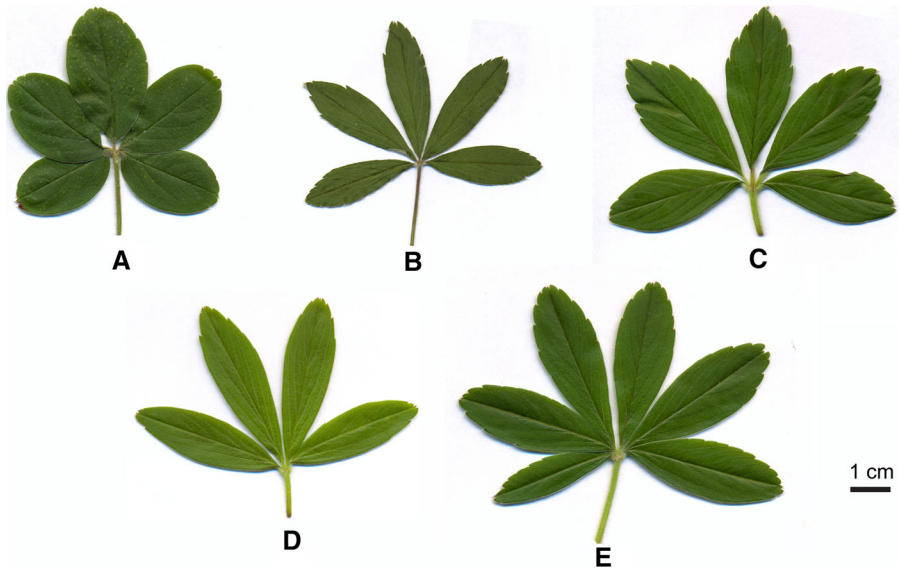

Fig. 4 Morphology of leaf blades in *P. alba*. **A**–**C** Polymorphic palmately compound leaf blades; **D** leaf blade with four leaflets; **F** leaf blade with six leaflets. (Color figure online)

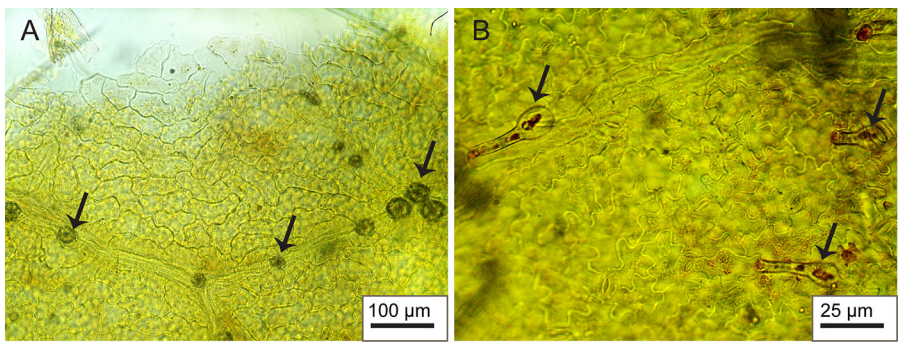

Fig. 5 Optical sections of leaf blades in *P. alba*. **A** Upper leaf epidermis cells with straight polygonal or slightly flexuous walls. Long arrows indicate calcium oxalate druses. **B** Lower leaf epidermis cells with flexuous walls. Long arrows indicate glandular hairs with brown secreted content. (Color figure online)

process of chromosome condensation, allowed us to accumulate prometaphase chromosomes and obtain longer chromosomes in the spreads (1.5–3 μm) resulting in rather informative chromosome DAPI-banding patterns. In *P. alba* karyotypes, we observed large pericentromeric and also small polymorphic telomere and intercalary DAPI bands, and such patterns are typical for plant species having small chromosomes (Guerra 2000; Pinto-Maglio 2006; Muravenko et al. 2009; Samatadze et al. 2012; Yurkevich et al. 2013; Amosova et al. 2014).

It is common knowledge that eukaryotic ribosomal DNA is highly conserved and consists of tandem repeat units with thousands of copies which are clustered in one or several chromosome pairs (Pedersen and Linde Laursen 1994). For this reason, sites of rDNA are easily mapped on chromosomes by FISH and used as chromosomal markers essential for genomic investigations as well as clarification of phylogenetic relationships among species (Liu et al. 2006; Moraes et al. 2007; Las Peñas et al. 2008; Amosova et al. 2015; Bolsheva et al. 2016). In the present study, for the first time, we performed localization of 45S and 5S rDNA loci in chromosomes of *P. alba* and found 45S rDNA hybridization signals in two chromosome pairs whereas double 5S rDNA sites were detected in only one chromosome pair. These findings agreed with the hypothesis on a possible tetraploid origin of the *P. alba* genome, and localization of two 5S rDNA loci in one chromosome pair could indicate chromosome reorganization occurred during speciation.

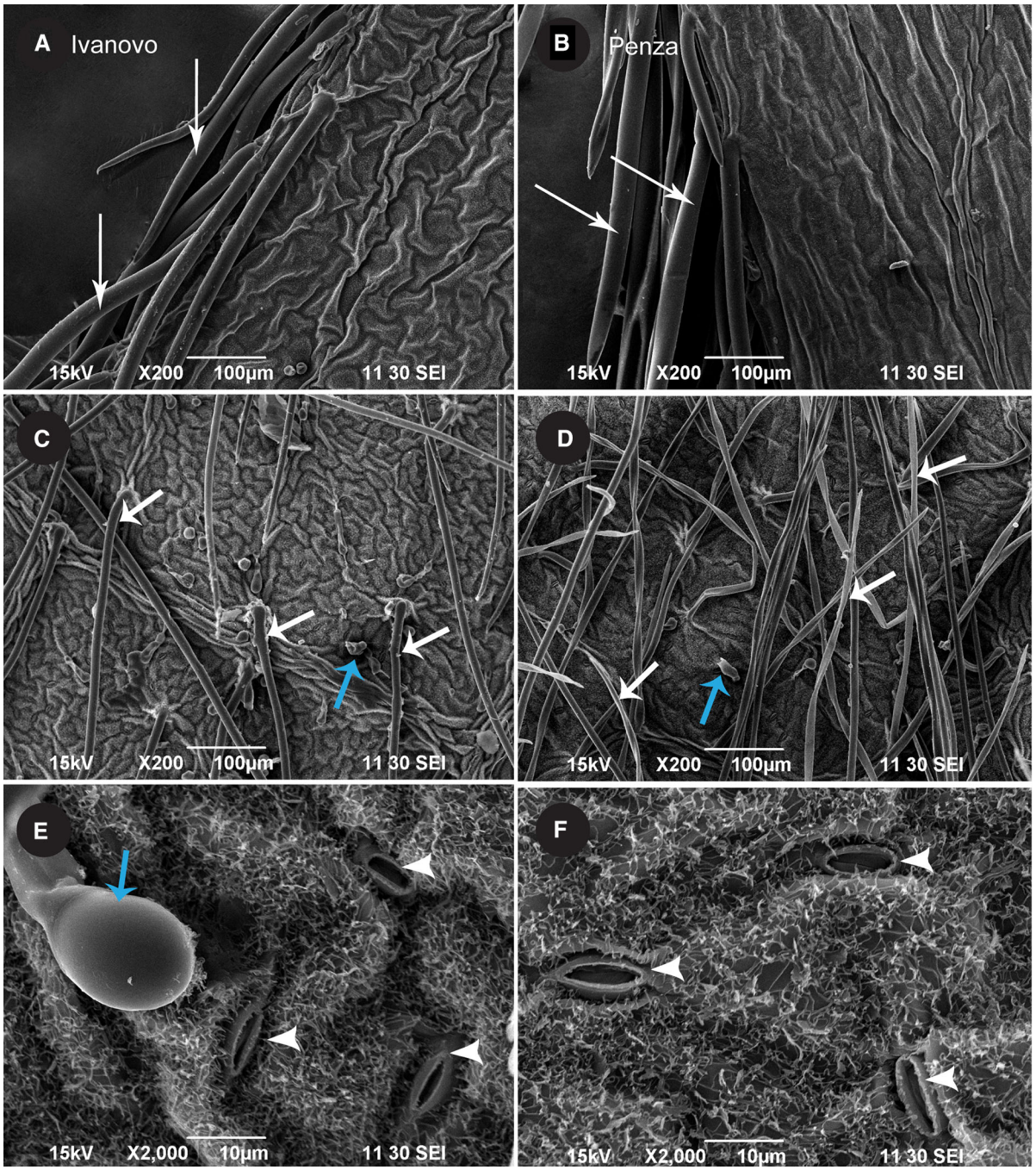

Fig. 6 Leaf ultrastructure in *P. alba*. SEM images of leaf blades of the specimens from the Ivanovo region (**A**, **C**, **E**) and Penza region (**B**, **D**, **F**). Long white arrows indicate simple trichomes; short white arrows indicate simple unicellular hairs; arrow heads indicate stomata; blue arrows indicate glandular hairs. (Color figure online)

Analysis of meiosis

Meiotic abnormalities (cytomixis, chromatin stickiness, the presence of unoriented bivalents, chromatin bridges, etc.) were early revealed in some *Potentilla* species (Rani et al. 2012). These abnormalities were described in other plant species and can be related to the intraspecific genetic variability (Baptista-

Giacomelli et al. 2000; Sheidai et al. 2003). They are considered to be the result of some genetic factors (Bellucci et al. 2003; Ghaffari 2006; Fadaei et al. 2010), environmental factors (Nirmala and Rao 1996) as well as genomic responses to environmental variation (Baptista-Giacomelli et al. 2000). It is known that in tetraploid, allo- and autopolyploids, meiotic abnormalities can lead to pollen sterility and decreasing seed production (Stebbins 1950).

Serious problems during the introduction of different *P. alba* populations are the lack of planting material and also lower seed production which slow efforts on plantation system development. *P. alba* seeds are characterized by a low germination ability and a long period of seedling emergence. Besides, the seedlings grow very slowly. Consequently, vegetative propagation is considered to be more effective (compared to seed propagation) for increasing plantation areas of this valuable medicinal plant (Smyk and Krivenko 1975; Kosman et al. 2013). The analysis of meiosis is a rather effective tool for clarification of the cause of low seed production as it enables to reveal abnormalities in early ontogenesis, which can lead to pollen sterility and, thus, decreasing seed production.

In meiosis of the *P. alba* samples, normal chromosome disjunction (14:14) typical for both diploids and paleopolyploids was observed. Besides, small number cells with various abnormalities were also detected. Chromosomal lagging represented considerable proportion among the revealed aberrations, and this is illustrative of the spindle assembly defects. As the result of such chromosomal aberrations, gametes with unbalanced chromosome number were formed (Sybenga 1996). Misorient tetrads and polyads observed at the tetrad stage of meiosis II were probably caused by defects in achromatic spindle orientation.

Phytotomy and ultrastructural characterization of leaves

The identification and quality of the raw material of *P. alba* are especially important for its medicinal use. Morphological analysis is one of the most universal and available approach. For specification within the genus *Potentilla*, the features of the leaf blades are important diagnostic characters (Faghir et al. 2010). The type of leaf is usually characteristic of a species (monomorphic), although some species produce more than one type of leaf (dimorphic or polymorphic). Polymorphic leaves were early observed in several *Potentilla* species (Müntzinc 1958; Chitwood and Sinha 2016). In *P. alba*, palmately compound leaves with five leaflets are considered to be a characteristic feature (Yuzepchuk 1941). In the present study, *P. alba* plants with polymorphic leaf blades were found in both studied samples. As leaf shape polymorphism can be related to ontogenetic factors and/or environmental plasticity (Chitwood and Sinha 2016), it cannot be considered as a constant diagnostic character for the species, and accordingly, other diagnostic features (epidermis structure, stomata types, trichome characters, the presence and forms of crystal inclusions, etc.) are needed for species characterization (Faghir et al. 2010).

Our study of the leaf structure and ultrastructure of the *P. alba* samples revealed several common characters in the *P. alba* samples: the structure of cells of upper and lower leaf epidermis, the presence of calcium oxalate druses as well as three types of leaf indumentum. Similar features were earlier detected in the cells of *P. alba* rhizomes and roots (Bogacheva et al. 2016). Moreover, the distinctive differences in leaf indumentum ultrastructure between the *P. alba* samples were also observed. This finding could be related to the species environmental plasticity as ontogenetic polyvariety, which is considered to be a species adaptation mechanism, was early detected within representatives of the genus *Potentilla* (Müntzinc 1958; Asker 1985b).

Conclusion

In this study, for the first time, we performed molecular cytogenetic characterization and analyzed leaf structure and ultrastructure of two introduced *P. alba* samples from different habitats and distant regions. Based on chromosome morphology and distribution of chromosomal markers, all chromosomes in karyotypes were identified and the chromosomal idiogram of *P. alba* was constructed. Our findings confirm *P. alba* karyotype stability and also reveal several diagnostic characters of this species essential for evaluation of genetic diversity in different populations, validation of raw materials as well as further progress in selection. The various meiotic abnormalities together with the low seed production of most *P. alba* populations, hindering efforts on

development of a plantation system, suggest the advantages of vegetative propagation for the successful extension of plantings for this valuable medicinal plant.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests in this work.

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