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Comparative Studying of Leaf Trichomes, Teeth and Glands in *Populus nigra* L., *Populus deltoides* W. Bartram ex Marshall and Their Hybrids

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Received: 26 October 2020; Accepted: 25 November 2020; Published: 27 November 2020

Abstract: Poplars from *Aigeiros* Duby section are very widespread in the world. A range of morphological characters were studied in such species of this section as *Populus deltoides* Bartram ex Marshall, *P. nigra* L. and their hybrid *P.* × *canadensis* Moench. However, there is little information about micromorphological characters of their leaves. The aim of this work was to study these characters and understand their species-specific potential. Thus, the morphological features, density and distribution of non-glandular trichoms, marginal glandular trichomes (salicoid teeth or coleters), epiglandular trichomes and basilaminar nectaries-glands were ontogenetically examined by both light and scanning electron microscopy in the certified by molecular markers *P. deltoides*, *P. nigra* and *P.* × *canadensis* samples. Non-glandular trichomes belong to the uni-, multicellular, uniseriate category. Marginal glandular trichomes can be classified as coleter types. Other morphological and anatomical trichome features are discussed with regard to their possible function. In summary, some variations in leaf morphology may be useful for the *P. nigra*, *P. deltoides* and their hybrid *P.* × *canadensis* species identification. These species differ in shape and number of basilaminar glands, as well as non-glandular trichome types and their distribution on the leaf.

Keywords: *Populus deltoides*; *Populus nigra*; *Populus* × *canadensis*; micromorphology; trichomes; glandular trichomes; non-glandular trichomes; basilaminar glands; microscopy; molecular identification

1. Introduction

Populus nigra L. from *Aigeiros* Duby section is the most widespread Eurasian poplar. Primary ranges of *P. nigra* and *P. deltoides* Bartram ex Marshall (from the same section) do not overlap, but *P. deltoides* was introduced to Eurasia, and it produced fertile hybrids with *P. nigra* named *P.* × *canadensis* Sympatric species of the same section may freely cross and produce first-generation hybrids, which tend to last for decades, due to vegetative reproduction via root suckers or shoot layering. In turn, these hybrids may hybridize with one of the parents. At the same time, their genomes alter due to the crossing over during the meiosis. An assessment of hybrids' spread is a challenge. Hybrids are often identified as one of the parent species, as the range of character variability in the species remains undefined.

Major morphological diagnostic characters may be divided into vegetative and reproductive ones. The reproductive characters can be further divided into those of flowers and fruits. The reproductive characters are generally considered indicative and unambiguous. The vegetative ones are available for the most part of the phenological cycle, yet they are less helpful for identification, due to the lack of information about intraspesific variability of poplars and their hybrids. The recommendations for the

representative material collection are based on the study of seasonal and ontogenetic heterophyllia (variations of leaf morphology along abbreviated and long shoots during the growing season) [1–3]. The poplar identification key based on leaf morphology will be more effective if it includes the characters of leaves formed in the autumn inside the buds (previously formed or "early" leaves), as well as those that are newly formed in late autumn, towards the end of the growing season (newly formed, or "late" leaves). However, the shaded branches of old trees often do not form any late leaves, which may distort the estimation of the heterophyllia within the species.

A set of trichome characters associated with leaf morphology is limitedly used for the diagnostics and in the classification of intrageneric groups of *Populus*. Ma et al., (2016) studied the genes related to trichome development in *P. pruinosa* Schrek. and *P. euphratica* Oliv., as well as the trichome location on leaves, stems, petioles and seed coats by scanning electron microscopy (SEM) [4]. The value of trichome parameters for taxonomy was emphasized by many authors [5–9]. In botanical studies, parameters such as type of trichomes [5,9–12], trichome density [12,13] or hair length [11,13] were commonly used. Depending on the investigated plant group, the trichome characters may be informative at the different taxonomic levels [12,14]. The differences in hair length were used to delimit taxa, e.g., *Quercus faginea* ssp. *faginea* and *Q. faginea* ssp. *broteroi* [15] and *Q. canariensis*, which were persistently distinct from other *Quercus* species [9]. On the other hand, Bartlott (1981) formulated the reasons why the features of trichomes were not successfully applied in taxonomy [16]. A number of authors have shown that the lack of data on ontogenetic development and the impact of the environment prevent the use of data on trichomes and hairs for systematic studies [13,16–18]. Consequently, there is a need to study the dependence of characters on the influence of the habitat, as well as to compare with other data to determine the taxonomic value.

In addition to leaf trichomes, salicoid teeth and glands are widely used as important diagnostic and taxonomic characteristics in plant taxonomy and studied in poplars. The first report about salicoid teeth as producing resin was made by Hanstein (1868) on the example of *Populus* [19]. Their secretory function was also described for *P. balsamifera* L. and *P. laurifolia* Ledeb. by Reinke (1876) [20]. Tschirch (1906, cit. by Fehér, 1923) described secretion and teeth in *P. alba* L., *P. balsamifera*, *P.* × *canadensis*, *P. deltoides*, *P.* × *canescens*, *P. nigra* [21,22]. Trelease (1881) reported about the "nectar" release by at *P. balsamifera*, *P. candicans* Aiton, *P. grandidentata* Michx., *P. monilifera* Ait., *P. tremuloides* Michx. and *P. tremula* L. from salicoid teeth [23].

Additionally, the basal glands are an important taxonomic character within the *Populus* genus. Studying of the leaf morphology in *P. deltoides* clones from all regions of the distribution area showed that the leaves of northern clones did not have or have few basal glands on the first leaves, whereas the southern clones have four or more basal glands on a leaf [24]. Such conclusions (Marcet's hypothesis), however, are the result of a small number of observations. It is possible that the timing for collecting the leaves was wrong. The leaves of the southern clones were unfurled earlier than the northern clones and therefore were at later stage of development already with several basal glands. Marcet's hypothesis has to be reconsidered with these reasoning.

Unfortunately, it is not easy to identify pure *P. deltoides*, *P. nigra* and *P.* × *canadensis* samples using morphological features only. Therefore, we used the set of morphological characters together with the data of molecular analysis with species-specific markers. The development of such markers was carried out by different authors in poplars [25–27]. Vornam et al., (1994) used some poplar hybrids with *P. deltoides* and *P. nigra* (controlled crosses between reference clones was carried out by Müller-Starck in Germany [28,29]) as a female parent to find the species-specific polymorphism in their chloroplast DNA (cpDNA). As a result, these authors found such polymorphism in one of the cpDNA regions with the *Xba*I enzyme digestion. This polymorphism was used in the article of Holderegger et al., (2005) to the discrimination of *P. nigra* from *P. deltoides* and *P.* × *canadensis* in Switzerland [26]. According to the article, the polymorphic regions was amplified with *trn*L/*trn*F primers and digested by *Rsa*I enzyme. In the case with *P. nigra*, the PCR product was not digested by the enzyme and the 1070 bp fragment was observed. The PCR products of *P. deltoides* and *P.* × *canadensis* were digested to the 370 bp and

700 bp fragments. In the same work, Holderegger et al. also used the nuclear species-specific markers. One of them is *win3*. The primers of this marker were designed by Bradshaw et al., (1994) at the basis of the *win3* gene, which was identified by Hollick and Gordon as a wound-mediated poplar gene having sequence similarity to Kunitz-type trypsin inhibitors [30,31]. Three years ago, Heinz (1997) successfully used the *win3* primers for discernment of alleles in *P. nigra*, *P. deltoides* and their hybrids [25]. It was found that *win3* is an effective codominant marker showed one 265 bp PCR fragment in *P. deltoides*, one or two fragments or smear at approximately. 165–210 bp in *P. nigra* and a combination of both patterns in their hybrids. Thus, the described markers (*trn*L/*trn*F/*Rsa*I and *win3*) are really valuable molecular tools for the species identification in poplars from the *Aigeiros* section, because they was created with the accurately identified plant material (reference clones from leading European collections and hybrids from the controlled cross families). This fact was a reason to use these markers in our work for facilitating the identification of samples by morphological characters. We believe that the plant material verified by the molecular method increased the reliability of the correspondence between the observed micromorphological features of leaves and the studied poplar species.

With the described approach, we were able to achieve the following goals in our work. First, we have reliably documented the types and distribution of trichomes in *P. nigra*, *P. deltoides* and their hydrid *P.* × *canadensis* to provide accessible and validated information for taxonomycal, phylogenetical and evolutional investigations. Second, we studied the anatomical and morphological structure of trichomes at different stages of leaf development in reliably identified poplar species from the *Aigeiros* section. The value of the features discovered in this way is high, since the glandular trichomes (salicoid teeth and basilaminar glands) can necrotize or disappear early, causing confusion in species identification by keys.

2. Materials and Methods

2.1. Plant Materials

Three species of *Populus* genus (*Populus nigra* L., *P. nigra* var. *italica* Münchh. (=*P. nigra* L. ssp. *pyramidalis* Čelak.), *P. deltoides* W. Bartram ex Marshall. ssp. *monilifera* (Ait.) Eckenw., *P. deltoides* W. Bartram ex Marshall. ssp. *deltoides* and *P.* × *canadensis* Moench.) were collected. The latter species is believed to be the result of briding or natural hybridization between *P. nigra* and *P. deltoides*. *P. nigra* is an early-succession tree species common in Euroasia and *P. deltoides* is a tree species common in North America.

Experimental trees of *P. nigra* were collected from their natural habitat of Lower Volga, Kuban' and Moscow region in the field during the field investigations between May and July in 2018–2019 years, and were immediately fixed in ethyl alcohol (70%), in herbarium or grown under greenhouse conditions. Voucher specimens were collected for all taxa examined and are listed in Table S1 and were kept in MW. All fresh material used in the SEM study was collected shoots of known wild origin obtained from other collectors, as well as the cultivation of botanical gardens in greenhouses and experimental sites of the Russian State Agrarian University. All cultivated taxa are listed in the Table S1. Collectors' names and numbers are also indicated in the case of samples provided by individuals or groups.

2.2. Light Microscopy (LM)

Morphological and light methods were used for the study of salicoid teeth of fresh materials in ontogenesis, basal glands and pubescens features and trichomes types of *P. nigra*, *P. deltoides* and possible hybrid *P.* × *canadensis*. Two–three perennial long shoot (auxiblast with 5–7 perennial short shoots) and brachyblasts were collected from each threes. All young leaves (1 cm in length) were used for indicating activity of marginal and basilaminar trichomes. For the micromorphological studies, there were cut out three small 55 mm^2 squares from the central part of the adaxial and abaxial leaf surfaces, including the midrib, and from the leaf edge. The exact density of trichomes was not

calculated, because of their uneven distribution within a leaf and the high variation within and between leaves. The trichomes and indumentum features were classified using the terminology of Payne (1978) to describe the trichomes of *Populus* in this study [32]. Indumentum types were identified according to those recognized by Hewson (2019) [33]. All observations of indumentum presence, abundance and type were confined to the surface of the lamina (including margins). Leaves were considered to be glabrous when no sign of any trichomes could be seen (including on the midrib) with a light microscope or SEM.

The observations were carried out on a Zeiss microscope. In order to examine the trichome types, the paradermal sections of fresh material were cut out with a razor blade and explored with a Zeiss microscope. In solitary acicular and ribbon trichomes investigations, the length and width (at the widest point) were measured and the number of cells per trichome was determined on SEM and LM photographs. The additional observations were made with a light microscope on herbarium specimens, as well as on potted plants. In the latter case, both young and mature leaves were analyzed.

2.3. Scanning Electron Microscopy (SEM)

The samples grown under nature and greenhouse conditions, as well as the specimens fixed in herbarium in the field or fresh were dehydrated in a graded ethanol or acetone series, critical point-dried with CO_2, mounted on aluminum stubs using double-sided sticky tape and coated with a thin layer of gold-platinum under vacuum. The types, morphology of trichomes and indumentums were examined with SEM (scanning electron microscope JEOL JSM-840) using secondary-electron detection and an acceleration voltage of 4–5 kV (Laboratory of Electron Microscopy of MSU, East Lansing, MI, USA).

2.4. PCR Analysis and Electrophoresis

DNA extraction from all samples (Table S1) was conducted according to the protocol of Doyle and Doyle (1990), with some modifications [34,35]. The concentration of DNA in samples was equalized. DNA quality was tested by preliminary PCR with a universal primer pair based on 5S rRNA gene (see Table S2) by Falistocco et al., (2007) [36].

The species-specific PCR tests were conducted with nuclear *win3* and plastid *trn*L/*trn*F markers. The primers are presented in Table S2. The primers were synthesized by ZAO "Synthol" (Moscow, Russia). The PCR conditions in experiments with the *win3* marker were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 4 min. The PCR conditions in experiments with the *trn*L/*trn*F marker (the used in these experiments primers for amplification of the *trn*L/*trn*F cpDNA region were designed by Taberlet et al., (1991) [37]) were as follows: 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min, with a final extension of 72 °C for 10 min. The PCR mix volume is 25 µL. It contained approximately 10 ng of genomic DNA, 2.5 U Taq-polymerase (ZAO "Sibenzyme", Novosibirsk, Russia), 1× SE-buffer, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 0.25 µM of forward and reverse primer and ddH2O. The digestion of the *trn*L/*trn*F PCR products was carried out in 20 µL reaction volume with 20 U of *Rsa*I restriction enzyme in 1 × restriction buffer at 37 °C overnight. PCR and restriction results were detected by electrophoresis on 2.5% agarose gel at 10 V/cm in 0.5 M TBE buffer using a Sub-Cell Model 192 camera (Bio-Rad, Hercules, CA, USA) and photographed using the gel documentation system GelDoc XR Plus (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. PCR Analysis with Molecular Markers

The *win3* and *trn*L/*trn*F markers showed the presence of the amplification in all studied samples The picture of the win3 amplification results corresponded to Heinz's discription [25]. The *P. nigra* samples had patterns with one fragment 170 bp in length. The differences between *P. nigra* and *P.nigra* var. *italica* were not found. The samples of *P. deltoides* ssp. *monilifera* and *P. deltoides* ssp. *deltoides*

had patterns with one 260 bp fragment. All *P.* × *canadensis* samples showed both 170 bp and 260 bp fragments in their patterns (Figure 1a). The *trn*L/*trn*F marker were not present the differences between *P. nigra* and *P.nigra* var. *italica* samples. The expected 1070 bp fragments were observed in these samples after the PCR product digestion by *Rsa*I enzyme. In contrast to this case, the *trn*L/*trn*F marker identified diffences between *P. deltoides* ssp. *monilifera* and *P. deltoides* ssp. *deltoides* samples. The *P. deltoides* ssp. *deltoides* products were as it was described by Holderegger et al., (2005) for *P. deltoides* trees [26]. The 370 bp and 700 bp fragments were detected. However, all *P. deltoides* ssp. *monilifera* samples had one fragment approximely 710 bp in length. All studied *P.* × *canadensis* trees showed patterns of products which were similar with *P. deltoides* ssp. *deltoides* (Figure 1b).

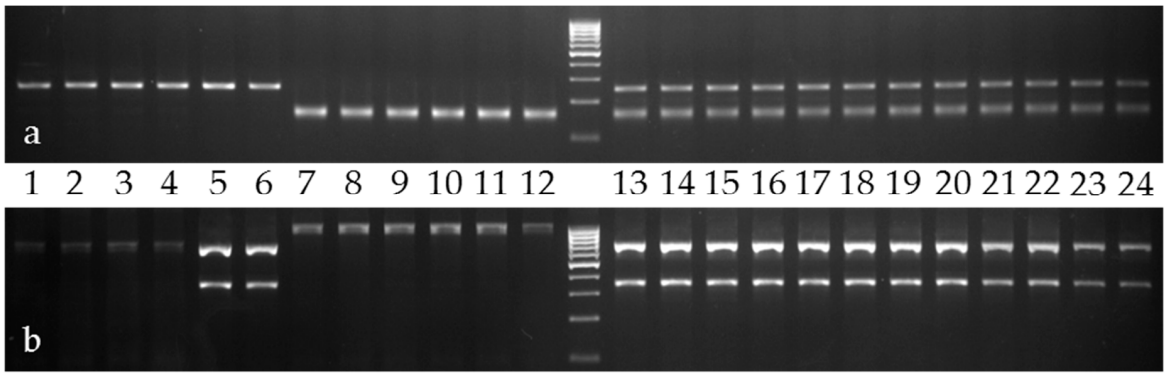


Figure 1. The results of *win3* (**a**) and *trn*L/*trn*F (**b**) marker experiments with *P. deltoides* ssp. *monilifera* (1–4), *P. deltoides* ssp. *deltoides* (5,6), *P. nigra* (7–11), *P.nigra* var. *italica* (12) and *P.* × *canadensis* (13–24) samples. The marker of molecular weight has a 100 bp step. The numbers of samples correspond to Table S1.

3.2. Morphological Analysis

The authors made an attempt to collect and analyze a representative set of samples, taking into consideration all the listed above and newly discovered facts. At the early stages of leaf ontogenesis, the marginal glands may not be fully developed, while the trichomes are already fully developed; at the later stages of ontogenesis, leaves may have glands, but no longer have trichomes [38,39]. In this connection, the correct research requires leaves of different categories: leaves on early ontogenesis stage, leaves on late ontogenesis stage, leaves of abbreviated shoots, leaves of long shoots. Leaves of these categories differ in shape and size, as well as in micromorphological characters (shape and size of their marginal teeth, pubescence density, presence/absence of bazilaminar glands).

In this study, two general trichome types in *Populus* have been found: glandular and non-glandular. Glandular trichomes include marginal glands (colleter) and bazilaminar nectariferous glands. They differ in cell number, length, shape. Non-glandular trichomes can be acicular and ribbon. Acicular trichomes occur mostly on the abaxial midrib or sometimes on the entire abaxial surface of the leaf. We distinguished two types of acicular trichomes in the *Populus*: long and short. Long and short trichomes consisted of a single stalk cell. The surface ornamentation of the trichome body was smooth. Ribbon trichomes can be also of two types: long and short. They are flattened and have a shape as a flowing ribbon. However, they do not persist into the adult stage in all species. Simple trichomes represented in mature leaves are usually short, often curved and closely appressed to the leaf surface. Though the base of the trichome is usually swollen, there is generally little or no cell differentiation of the surrounding epidermal cells. It is possible that the simple trichomes longer than 400 μm always have one cell of the base.

3.2.1. The Features of *P. nigra* Samples

Marginal Glands

In *P. nigra* samples, salicoid teeth consist of glandular roundish spherical or hemispherical and extended apical part, subapical contraction and basis (Figure S1a). The glandular part is presented by palisade-like epidermal cells which secrete resin (Figure S1b).

The glandular part carries trichomes of different forms and lengths (Figure S2a–l). Possibly, these trichomes increase surface for transpiration and transpiration induced increasing of apoplastic solutes in palisade-like cells for resin secretion. Later, the palisade-like cells are lignified and reduced. Subapical contraction is presented by the elongate cells.

Later, epiglandular trichomes and the glandular part of trichomes reduces and, therefore, were not noticed by the researchers earlier (Figure S3b).

The basis of teeth is covered with epidermal cells and one-two stomata as a relict feature (Figure S5a), which is similar to Idesia polycarpa one adaxial hydathode [40]. Belin-Depoux (1989) considered that nectary and hydathode are relictual and "the foliar glandularization is considered as more recent than hydatherous elements from phylogenetical point of view" [40]. Possibly, a specific unspecialized type of hydathode may act as nectary in specific conditions [41]. *P. nigra* has one vascular bundle per tooth (Figure S3c). The spiral and poriferous elements of a xylem end in tips (Figure S3d). Venation is eukamptodromous. The verge is between salicoid teeth of the leaf (Figure S3e). The verge is represented by several rows of cells without chloroplasts which perform mechanical function.

Basilaminar Nectaries-Glands

It was generally accepted that glands or nectarines are absent at leaf base or on the petiole of *P. nigra*. Most leaves have no glands on the leaf basis (Figure S4a,b). Some samples have basal glands or glands near the petioles (Figure S4c,d). The surface of basilaminar glands is smooth. This species has heterophyllous leaves: most leaves without basilaminar glands and a minor number of leaves carry basilaminar glands.

Non-Glandular Trichomes

We studied leaves of *P. nigra* of several ontogenetic stages. The adaxial surface of a young leaf with a diameter about 1 cm has smooth short simple unicellular acicular trichomes of 50–100 μm long (Figure S5a–c) and 5 μm thick and medium acicular trichomes 100–200 μm long (Figure S5d,e). The trichomes longer than 250 μm are ribbon (Figure S5f). The abaxial surface of veins, leaf basis and petiole have acicular and ribbon trichomes more often.

3.2.2. The Features of *P. deltoides* ssp. *deltoides* Samples

Marginal Glands

The glandular part of salicoid teeth has different forms and is presented by palisade-like epidermal cells which secrete resin (Figure S6). The glandular part carries epiglandular short acicular trichomes of ciliate indumentum. These trichomes fall off later. Subapical contraction is presented by the elongate cells. The basal part of salicoid teeth and verge of leaf are covered by acicular trichomes. One bundle ends near the base of the tooth and includes spiral elements of a xylem.

Basilaminar Nectaries-Glands

Two–three large stalked nectaries glands are located at the leaf base or at the petiole for resin and nectar secretion (Figure S7).

Trichomes

The marginal part of leaf is ciliate, it has smooth short acicular trichomes 50–100 μm long and 5 μm thick (Figure S6a–c). The abaxial and adaxial surfaces, veins, basis of leaf are glabrous (Figure S7d). The petioles have short acicular trichomes 50–100 μm long and very rare long ribbon trichomes 500–1000 μm long (Figure S7e,f).

3.2.3. The Features of *P. deltoides* ssp. *moniliferae* Samples

Marginal Glands

The glandular part of salicoid teeth has different forms and is presented by palisade-like epidermal cells which secrete resin (Figure S8a–d). The glandular part carries epiglandular short acicular trichomes of ciliate indumentum. These trichomes fall off later. Subapical contraction is presented by the elongated cells. Basal part of salicoid teeth and margins of leaf are covered by acicular trichomes. One bundle ends near the base of the tooth and includes spiral elements of a xylem.

Basilaminar Nectaries-Glands

Two basilaminar nectaries-glands are located at the leaf base or at the petiole for resin and nectar secretion. The surface of glands is covered by smooth short acicular trichomes 100 μm long and 5 μm thick (Figure S8d).

Trichomes

The marginal part of leaf is ciliate (Figure S9a–c) with smooth short acicular trichomes of 50–100 μm long and 5 μm thick and long acicular and ribbon trichomes of 500–1000 μm long (Figure S9a,b). The abaxial and adaxial surfaces, veins, basis of leaf are covered with rare short acicular (Figure S9c,d) and long ribbon trichomes (Figure S9e). The petioles have short acicular trichomes of 50–100 μm long and very rare long ribbon trichomes of 500-1000 μm long as in *P. deltoides ssp. deltoides* samples (Figure S7e,f).

3.2.4. The Features of *P.* × *canadensis* Samples

Marginal Glands

The glandular part of salicoid teeth has triangular or spherical forms and is presented by palisade-like epidermal cells, which secrete resin (Figure S10a). The glandular part carries epiglandular trichomes of various shapes and lengths (Figure S10b,c). The basis of teeth covered with epidermal cells and one-two stomata as a relict feature. Later the palisade-like cells are lignified and epiglandular trichomes and glandular part of trichomes are reduced (Figure S10d). Therefore, they were not noticed by the researchers earlier. One bundle ends near the base of the tooth and includes spiral elements of the xylem. The verge is between salicoid teeth of leaf. The border is represented by several rows of cells without chloroplasts which perform mechanical function.

Basilaminar Nectaries-Glands

One or sparsely two glands can be located at the leaf base or at the petiole for resin and nectar secretion. The surface of glands is covered with smooth short acicular trichomes 100 μm long and 5 μm thick (Figure S11a).

Trichomes

The edge, adaxial and abaxial surfaces of *P.* × *canadensis* leaves are covered with sparse smooth short acicular trichomes 50–100 μm long and ribbon trichomes 500–1000 μm long and 5 μm thick (Figure S11b,c). The veins and leaf petioles have trichomes more often (Figure S11d–f). The *P.* × *canadensis*

samples have marginal part of leaves with smooth short acicular trichomes, which form ciliate indumentum. The basis, petiole and basal marginal part of late leaf do not have trichomes.

3.2.5. The Observed Indumentum Types in Studied Poplars

We furthermore discerned four main indumentum types of young leaves of the *P. nigra*, *P. deltoides* and *P.* × *canadensis*:

1. Strigose indumentum. The leaf surface is covered with appressed, rigid, bristle-like, straight trichomes, or is often confined to the lamina and the midrib of the leaf (Figure S12a–c).
2. Pilose indumentum. The leaf surface is covered with hairs which are soft, weak, thin and clearly separated. The hairs are usually defined as long and sometimes ascending (Figure S12d–f).
3. Ciliate indumentum. The leaf has fine eyelash-like hairs at the edge (Figure S12c).

4. Discussion

In this work, we used two molecular markers to validate the species of the studied samples. The nuclear marker *win3* worked as it was presented by Heinz and Holderegger et al., (2005) [25,26]. However, the second marker (the plastid marker *trn*L/*trn*F) showed the interesting picture in *P. deltoides* ssp. *monilifera* and *P. deltoides* ssp. *deltoides* samples. Holderegger et al., (2005) described such results as we observed in *P. deltoides* ssp. *deltoides* trees [26]. However, the *P. deltoides* ssp. *monilifera* results were untypical (we detected one fragment approximately 710 bp in length instead of two expected 370 bp and 700 bp fragments). We found out for the first time that the used *trn*L/*trn*F marker can work this way. In our opinion, it would be promising to conduct an in-depth study of *trn*L/*trn*F region among a large number of *P. deltoides* trees of different subspecies in a separate publication. First, it is necessary to establish precisely that the observed fragment is not a nonspecific artifact of PCR. Second, it is important to examine the sequence of the region to be amplified to explain the observed work of the marker. Additionally, the *trn*L/*trn*F marker allowed us to conclude that one of the parents of the studied *P.* × *canadensis* samples was *P. deltoides* ssp. *deltoides*.

We observed non-glandular and glandular trichomes on all two *Populus* species from *Aigeiros* section (*P. deltoides*, *P. nigra*, and their hybrid *P.* × *canadensis*). The non-glandular trichomes did not show a wide morphological variability; they all represent uniseriate, uni- and/or oligocellular trichomes—short and long acicular and short and long ribbon. Unicellular short acicular trichomes are the most simple ones to be found in *P. deltoides*, *P. nigra*, *P.* × *canadensis* and, possibly, the oldest in this genus. Long acicuar trichomes are common type of *P. deltoides*, *P. nigra*, *P.* × *canadensis*, but are rare on the leaves' surfaces. *P. nigra* and *P.* × *canadensis* species present a characteristic arrangement of rare non-glandular long ribbon trichomes on abaxial surface. *P. deltoides* and *P.* × *canadensis* species present a characteristic arrangement of rare non-glandular short ribbon trichomes on marginal part of leaves.

P. deltoides and *P.* × *canadensis* have ciliate marginal indumentum. *P. nigra* do not have ciliate marginal indumentum. *P. deltoides* ssp. *deltoides* have stalked basilaminar glandular trichomes. *P. deltoides* ssp. *moniliferae* have sessile basilaminar glandular trichomes. The complex characters (basilaminar glands and ciliate indumentum) identify *P. deltoides* species. The ciliate indumentum without basilaminar glands identifies *P.* × *canadensis*. The blades without ciliate indumentum and basilaminar glands characterize *P. nigra*.

In the case of glandular trichomes (collectors), the ancestral features of trichomes can be reconstructed by the structure of the former Flacourtiaceae. This, apparently, is a transformation from sessile marginal glands of Flacourtiaceae and Salicaceae into complex stalked basilaminar nectaries-glands of *Populus deltoides*. Possibly, stalked nectaries-glands are derived from sessile nectaries-glands and arise independently in some Salicaceae taxa.

The epiglandular trichomes are common glandular trichomes for *P. nigra* and *P.* × *canadensis* and possibly represent a variety of hydathodes [42,43]. The epiglandular trichomes of *Populus deltoides* are short and acicular. They form a part of the continuous ciliate indumentum. This is likely due to an

adaptive trait often exhibited in resin secreting plants to increase the active water secretion of glandular trichomes. Our data correlate with the opinion of Ponzi and Pizzolongo (1992) for *Rhinanthus minor* and *Odontites vema* [44,45], who wrote that glandular trichomes, participating in the active secretion of water as trichome hydathodes, direct solutions from the stem to the young parts of the shoot.

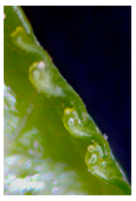
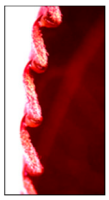
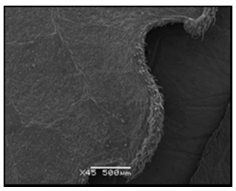
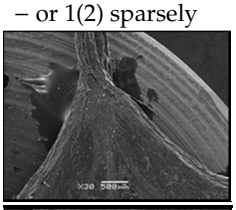
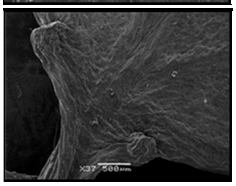
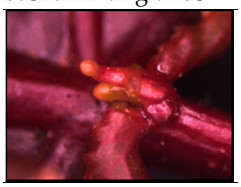
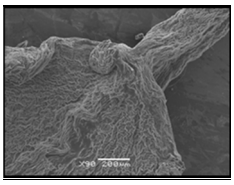
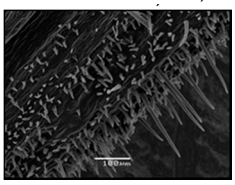
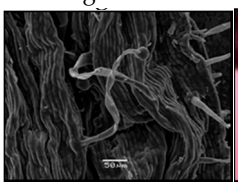
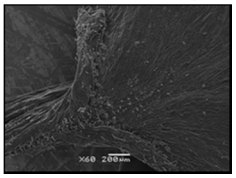
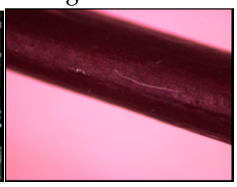
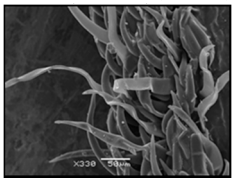
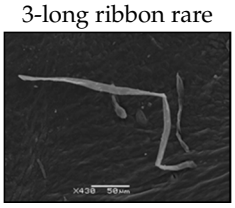
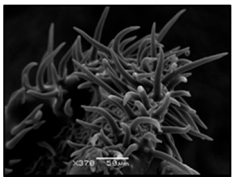
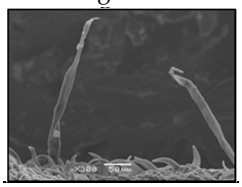
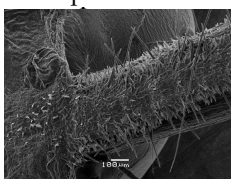
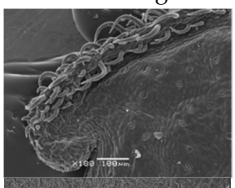
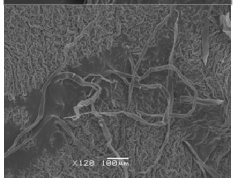
The earlier studies did not find all trichome types reported here. We have described, for the first time, that poplar's young leaves have straight, non-glandular trichomes (acicular and ribbon) in addition to marginal and basilaminar glandular trichomes. The early and late leaves are formed in different seasons, but they develop in the same vegetative season. The dense trichome becomes thinner with leaf size increasing. During the foliage transition from a juvenile to an adult form, the trichomes of adaxial and abaxial surfaces fall off, and the basilaminar and marginal glandular trichomes are destroyed. The presence of dimorphic indumentum (juvenile leaves with non-glandular trichomes and nude adult leaves) is often mistakenly used as a diagnostic and taxonomic character. The short needle-like trichomes of the marginal hairs of the cilia of *P. deltoides* and *P.* × *canadensis* persist for a long time and bring *P. deltoides* closer to balsamic poplars. Therefore, a clear distinction between the two species groups can be suggested here. Taking into account the characteristic features of indumentum, the adopted sectional subdivision of the genus *Populus* looks artificial.

Nevertheless, the pubescence and trichome types are characters of a diagnostic significance. Short and long ribbon trichomes are the feature of *P. nigra*; those short ribbon trichomes are typical for *P. deltoides* along with cilia at the margin. *P. deltoides* ssp. *deltoides* and ssp. *moniliferae* confidently differ in basilaminar gland types (stalked in ssp. *deltoides* and sessile in ssp. *moniliferae*) and their quantity. In the *P. deltoides* and *P. nigra* hybrid (*P.* × *canadensis*) two types of trichomes occur: short and long needle-like, while marginal cilia are facultative. Basilaminar glands cannot be used for identification of the hybrid, as their occurrence overlaps in the involved species.

The presence of several types of *P. nigra* trichomes is probably the evidence of an ancient hybridogenic origin of this species, one of its parents may be a poplar of balsamic group.

The plant trichomes can be used for the taxonomy of species of *Aigeiros* section (for example, see Table 1). There is no clear presense/absence of the trichome type pattern that could allow a unambiquous taxon delimitation, because all types are present in all the three species (*P. deltoides*, *P. nigra*; *P.* × *canadensis*). The exception is the lack of long ribbon trichomes in *P. deltoides*. These trichomes are the derivates of acicular trichomes as a basic type. Each trichome type can include several groups with different size. This can be the distinguishing characteristic between taxa. In contrast, ciliate foliar indumentums, especially the persistence of trichomes on the marginal side of the leaves, differentiates the American and Eurasian groups in the *Aigeiros* section. *P.* × *canadensis* consistently differs from both species in that it has all the characteristics of both types of trichomes, and is probably a hybrid adapted to wider climatic conditions. We believe that a large number of American *P. deltoides* and *P.* × *canadensis* samples, covering the entire distribution area, should be studied to capture natural variation. Such studies will provide more knowledge about evolutionary issues and the origin of various types of trichomes and their functional properties in the *Populus* genus.

Table 1. The major trichomes diagnostic features of the *Aigeiros* section poplars.

Features	*Popilus nigra*	*P. deltoides* ssp. *deltoides*	*P. deltoides* ssp. *moniliferae*	*P.* × *canadensis*
Marginal glands	+ 	+ 	+ 	+ 
Basilaminar nectaries-glands	– or 1(2) sparsely  	+ 2-3-4-stalked, hemistalked basilaminar glands 	+ 2 sessile basilaminar glands; surface is ciliate, late smooth 	– or one sessile sparsely 
Non-glandular trichome type	1-short acicular, 2-medium acicular,  3-long ribbon 	1-short acicular (ciliate), 2-short ribbon,  3-long ribbon rare 	1-short acicular (ciliate), 2-short ribbon (ciliate),  3-long ribbon rare 	1-short acicular (ciliate), 2-medium acicular,  3-long ribbon 
Indumentum	pilose 	ciliate and strigose  	ciliate and strigose	1-pilose, 2-ciliate and strigose

5. Conclusions

In this work, we formulated a whole range of morphological and anatomical features of the leaf, which can distinguish the studied species: shape and number of basilaminar glands, non-glandular

trichome types and their distribution on the leaf. *P. deltoides* ssp. *deltoides* leaves have big stalked and hemistalked basilaminar glands (*P. deltoides* ssp. *moniliferae* also have big basilaminar glands, but they are sessile) and *P. nigra* sparsely have small sessile basilaminar glands (often, basilaminar glands are absent). The presence of ciliate type of indumentum in *P. deltoides* is a clear character that discretize this species and *P. nigra* which have not ciliate trichomes. In *P.* × *canadensis* leaves, these features are selectively combined. Basilaminar glands in this hybrid are as in *P. nigra* and it have ciliate trichomes as *P. deltoides*.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/12/1267/s1, Table S1: Poplar trees samples, origin, collectors and Herbarium voucher location and number, Table S2: The used molecular markers and their characteristics, Figure S1: Marginal glands of *P. nigra*, Figure S2: Differences in forms and lengths of glandular parts of *P. nigra* marginal glands, Figure S3: The external and internal structure of salicoid teeth of *P. nigra*, Figure S4: Leaves of *P. nigra* without basilaminar glands and with basilaminar glands, Figure S5: Trichomes on *P. nigra* leaves, Figure S6: Edge, basal part and petiole of *P. deltoides* ssp. *deltoides* leaves, Figure S7: Basilaminar glands, abaxial surface and petiole of *P. deltoides* ssp. *deltoides* leaves, Figure S8: The micromorphological features of *P. deltoides* ssp. *moniliferae* leaves, Figure S9: Trichomes of *P. deltoides* ssp. *moniliferae* leaves, Figure S10: The features of edge of *P.* × *canadensis* leaves, Figure S11: Glands and trichomes of *P.* × *canadensis* leaves, Figure S12: Types of leaf indumentum.

Author Contributions: T.A.F. and O.S.A. created main ideas of work, conceived and designed the experiments and formulated the discussion; T.A.F. collected plant materials, conducted microscopy investigations and wrote the paper; O.S.A. collected plant materials, performed the PCR experiments, analyzed the data, corrected and supplemented the text of the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Russian Foundation for Basic Research, contract No. 19-04-00744\20 of March 31, 2020.

Acknowledgments: The authors are grateful to A.N. Sorokin (Department of tropical and subtropical plants, Main Botanical Garden named by N.V. Tsitsin, RAS), D.A. Ipatova (student of Biology department, Moscow State University), staff of Herbarium of Lomonosov Moscow State University (MW) and Herbarium of Main Botanical Garden named by N.V. Tsitsin, RAS (MHA), staff of Laboratory of Electron Microscopy of MSU, T.V. Alexandrova for critically reading the English version of the manuscript, and two anonymous reviewers for helpful comments that improved this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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