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Structural adaptations of willows (*Salix*; Salicaceae) endemic to the Athabasca sand dunes

by

Ranessa L. Cooper ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Plant Biology

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Date: 23 July 2001

"Two roads diverged in a wood, and I – I took the one less traveled by and that has made all the difference." --Robert Frost

"Every tomorrow has two handles. We can take hold of it by the handle of anxiety, or by the handle of faith." --Author Unknown

"Bloom where you are planted." –Author Unknown

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Structural adaptations of willows (*Salix*; Salicaceae) endemic to the Athabasca sand dunes* by *Ranessa L. Cooper* in partial fulfillment of the requirements for the degree of *Doctor of Philosophy* in *Plant Biology*.

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Date: 21 July 2001

For my family (especially my brother Matthew), for those who have inspired me, and for those who share my passion in botany

ABSTRACT

The Athabasca sand dunes, located on the south shore of Lake Athabasca in northern Alberta and Saskatchewan, formed (after deglaciation) about 10,000 years ago and were vegetated soon thereafter. Only 40 plant species occur on open sands, including ten endemics; four of these endemics are willows (*Salix*; Salicaceae). These endemic willows (*Salix brachycarpa* Nutt. var. *psammophila* Raup, *S. planifolia* Pursh ssp. *tyrrellii* (Raup) Argus, *S. silicicola* Raup, and *S. turnorii* Raup) are morphologically similar to their putative progenitors. However, endemic *Salix* taxa have certain structural features that are considered to be adaptive to the open sand habitat. Light and scanning electron microscopy were used to study leaf epidermal features and vessel element (VE) characters in the four endemic *Salix* taxa. These data were compared with those of the respective putative progenitors. *Salix planifolia* ssp. *tyrrellii* and *S. turnorii* have amphistomatic leaves, and *S. brachycarpa* var. *psammophila* and *S. silicicola* have the greatest trichome densities. Endemic willows have thicker cuticles than their widespread progenitors, as well as abaxial epicuticular wax deposits. *Salix brachycarpa* var. *brachycarpa* and its derived endemic, var. *psammophila*, had the highest VE densities of all endemic-progenitor pairs in this study. Vessel element lumen diameter of the endemic *S. planifolia* ssp. *tyrrelli* was significantly less than that of its putative progenitor, and *S. turnorii* had significantly greater values for both VE lumen diameter and length than its associated progenitor. Vessel element clustering did not differ significantly between endemic and progenitor taxa with the exception of *S. silicicola* and its progenitor. Amphistomatic leaves, dense trichome coverings, and thick, waxy cuticles exhibited by the endemic willows suggest adaptation to high light intensity, while VE observations correlate with the sandy environment. This is the first detailed study addressing structural adaptations of endemic willows to the Athabasca sand dunes. Structural similarities within each endemic-progenitor *Salix* pair provide additional support for the close, evolutionary relationships. Structural data, combined with the use of molecular techniques, will aid in elucidating the putative taxonomic status of each endemic-progenitor *Salix* pair.

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CHAPTER 1: INTRODUCTION

The Athabasca sand dunes lie on Lake Athabasca's south shore in northern Alberta and Saskatchewan (Figure 1-1). These dunes are the largest northern dunes in the world. The dunes in Saskatchewan were designated as part of the Athabasca Sand Dunes Provincial Wilderness Park in 1992. The park covers a stretch of about 100 km along the south shore of Lake Athabasca (Saskatchewan Provincial Parks, 1997).

The Athabasca sand dunes have been studied for sand dune formation (Hermesh, 1972), dune terrain and potential land usage (Smith, 1978), and descriptions of both land and vegetation (Raup and Argus, 1982). The Athabasca sands were formed after deglaciation approximately 9,000 years ago, and they have desert-like topography, gravel pavements, and large open sands (Smith, 1978). Although over 200 vascular plant taxa occupy this boreal, sand dune region (Hermesh, 1972), only 40 of these occur on open sands (Hermesh, 1972; Smith, 1978; Raup and Argus, 1982). Of the 40 plant taxa in the open sand vegetation, ten are endemic, including five herbaceous dicots (*Achillea millefolium* L. ssp. *megacephala* (Raup) Argus, *Armeria maritima* ssp. *interior* (Raup) Pors., *Silene acaulis* f. *athabascensis* Argus, *Stellaria arenicola* Raup, *Tanacetum huronense* var. *floccosum* Raup), one grass (*Deschampsia mackenzieana* Raup), and four willows (*Salix brachycarpa* Nutt. var. *psammophila* Raup, *S. planifolia* Pursh ssp. *tyrrellii* (Raup) Argus, *S. silicicola* Raup, and *S. turnorii* Raup).

Endemism is defined as a form of rarity that characterizes a group of organisms confined to a specific habitat or geographic region (Kruckeberg and Rabinowitz, 1985). Mountains and islands are common locations for endemic plants, while it is rare to find endemic distributions in boreal and arctic regions due to such a short time period (since the last deglaciation) for speciation to occur (Kruckeberg and Rabinowitz, 1985). The Athabasca sand dunes serve as home to ten endemic plant taxa. These endemic plants were described by Raup (1936), and they are confined primarily to open, active sand dunes. Following deglaciation, arctic, boreal, and northern Great Plains species vegetated this region (Raup and Argus, 1982). The Athabasca sand dune endemics are derivatives of these progenitor species, resulting from their isolation in active dunes, and endemics have certain features that distinguish them from their associated putative progenitors (Raup, 1936; Raup and Argus, 1982).

The high number of endemics and recent origin of the sand dunes suggest that rapid evolution has taken place (Hermesh, 1972). Recent studies of genetic diversity support the idea that speciation has occurred in some endemic taxa (Purdy et al., 1994; Purdy and Bayer, 1995a, b, 1996). In particular, the endemics that were investigated from the dunes include two of the herbaceous dicots (*S. arenicola*, Purdy et al., 1994; *A. millefolium* ssp. *megacephala*, Purdy and Bayer, 1996), a single grass species (*D. mackenzieana*, Purdy and Bayer, 1995b), and one willow (*S. silicicola*, Purdy and Bayer, 1995a), and their levels of genetic diversity were compared to that of their respective boreal or arctic progenitors. Only one other investigation has examined one willow endemic and its progenitor to elucidate taxonomic relationships (Argus and Steele, 1979).

In many studies of the Lake Athabasca sand dune endemics, suggestions have been made about plant evolution in and adaptation to this rare habitat. Certain structural features of these endemics were suggested to be adaptive to the open sand environment (Hermesh, 1972; Argus and Steele, 1979; Purdy et al., 1994; Purdy and Bayer, 1995a, b, 1996). To my knowledge, no studies have examined structural adaptations of the endemics in terms of their survival in a boreal, sand dune habitat.

Endemic taxa are confined primarily to inner dunes; these active sand areas experience high winds, resulting in sand accretion, and have high light intensity (Raup and Argus, 1982). There are stabilized depressions of sand that exist at or near the water table, known as "dune slacks," where water and moist sand are present for a period of time (G. Argus, personal communication). However, active blowing sand may cover a dune slack, while another dune slack forms in a new depression. Dune slacks are critical areas for seedling establishment of the four endemic willows (Raup and Argus, 1982). If a willow seedling survives the drying of a dune slack, it eventually becomes a woody shrub on open sands.

Willows generally occupy a range of habitats, many of which are physically disturbed areas (Argus, 1973). Willow habitats are diverse, differing in moisture and nutrients (Argus, 1973); habitat range is controlled more by moisture requirements for seed germination (Argus, 1986). Dorn (1976) noted that willows are pioneering species of primarily moist environments. Endemic willows on the active sands on the south shore of Lake Athabasca germinate and become established in dune slacks (Raup and Argus, 1982). Due to the disturbed nature created by active blowing sand and openness of the area, the four endemic willows have most likely developed structural adaptations for survival in this demanding habitat.

Structural features of plants can be correlated with habitat characteristics, and several types of morphology are associated with open, desert-like environments. Endemic willows presumably have adaptive characters, including amphistomatic leaves (i.e., stomata on both leaf surfaces), many trichomes which cover the plant body, and thicker cuticles when compared to their associated putative progenitors (Hermesh, 1972; Argus and Steele, 1979; Purdy et al., 1994; Purdy and Bayer, 1995a, b, 1996).

Stomata are sites of gaseous exchange for photosynthesis, and amphistomatic leaves have been described as being advantageous in the open sand habitat (Argus and Steele, 1979), as well as in high light conditions (Mott et al., 1982). High trichome densities are also adaptive to open environments, and a plant's cuticle serves as an important structure as it is the boundary layer between the plant body and its environment. Cuticular roles include water conservation, protection from wind and physical abrasion, as well as radiation (Martin and Juniper, 1970). Cuticle structure and chemical composition may be even more important than thickness (Martin and Juniper, 1970). Sometimes plants may produce waxes exterior to the existing cuticle known as epicuticular waxes. If the epicuticular waxes are randomly oriented, they can scatter light to a greater degree (Metcalfe and Chalk, 1979).

The endemic willows from the Lake Athabasca sand dunes are known only from this open sand environment, while their associated putative progenitors are widespread in their geographic distribution. Plants growing in different habitats exhibit distinct structural differences that may be referred to as "evolutionary adaptations" to that habitat's conditions (Esau, 1977). Endemic willows appear to have leaves with thicker cuticles or greater trichome densities, as well as amphistomatic leaves that are adaptations to the exposed nature of the inner dunes. These rare willows have features of their water-conducting cells, or vessel elements, (that differ from their putative progenitors) that may be more efficient for water transport in the dune environment.

This study is the first investigation of structural adaptations of the four endemic willows (*Salix brachycarpa* var. *psammophila*, *S. planifolia* ssp. *tyrrellii*, *S. silicicola*, and *S. turnorii*) to the Athabasca sand dunes of northern Saskatchewan, Canada, as compared to features of their respective associated boreal or arctic progenitors (Table 1-1). The Lake Athabasca habitat provides the unique opportunity to investigate structural adaptation and variation in recently derived plant taxa, and evaluation of these adaptive characters will lead to a greater understanding of the role they play to survival in an open sand dune environment.

The primary objectives of my thesis research were: (1) to identify structural features of endemic willows (from the Athabasca sand dunes in Saskatchewan) that may be adaptations to a boreal, open sand dune environment, (2) to compare the structure and morphology of progenitor *Salix* species to their endemic derivatives, (3) to study *S. planifolia* ssp. *tyrrellii* from the Athabasca sand dunes in Alberta, and (4) to carry out a molecular analysis to clarify taxonomic and evolutionary relationships of endemic-progenitor *Salix* pairs.

The Athabasca sand dune habitat provides the unique opportunity to investigate structural adaptation in recently derived plant taxa. This study is the first extensive detailed structural investigation of the four endemic *Salix* taxa. Adaptation can be examined by comparing anatomical structure of endemic willows to their associated boreal or arctic progenitors. Written permission has been granted to selectively collect endemic willow specimens from the park for this particular study. Furthermore, this research will provide a more complete understanding of the dynamics that exist between the Athabasca endemic plant taxa and their boreal, sand dune environment.

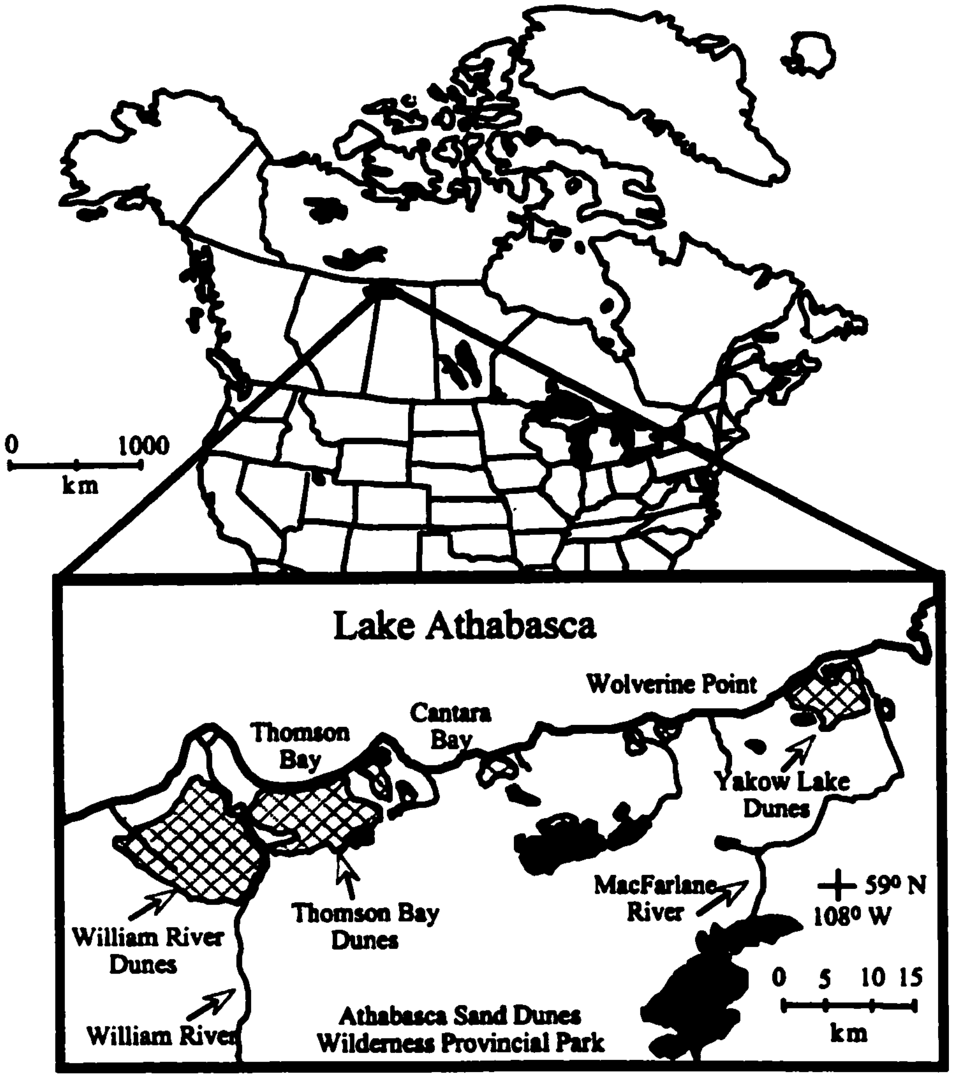

FIGURE 1-1. Map of the Athabasca sand dunes in northern Saskatchewan. (Map courtesy of Brett Purdy.)

TABLE 1-1. List of endemic *Salix* taxa from the Athabasca sand dunes and their respective putative progenitors.

Endemic	**Putative progenitor**
S. brachycarpa Nutt. var. *psammophila* Raup	*S. brachycarpa* Nutt. var. *brachycarpa*
S. planifolia Pursh ssp. *tyrrellii* (Raup) Argus	*S. planifolia* Pursh ssp. *planifolia*
S. silicicola Raup	*S. alaxensis* (Anderss.) Coville
S. turnorii Raup	*S. lutea* Nutt.

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CHAPTER 2: COMPARATIVE EVALUATION OF LEAF EPIDERMAL FEATURES OF *SALIX* SPECIES ENDEMIC TO THE ATHABASCA SAND DUNES[1]

INTRODUCTION

Four *Salix* species are endemic to the Athabasca sand dunes of northern Saskatchewan, Canada. The dunes, located on Lake Athabasca's south shore, were formed after deglaciation 9,000 – 10,000 years ago (Raup and Argus, 1982). Many habitats are represented on the dunes, including dune slacks (areas for establishment of endemic willow seedlings), gravel pavements, and open, active sands. The endemic willow shrubs, as well as six other endemic taxa, are confined primarily to dune interiors (Hermesh, 1972; Smith, 1978; Raup and Argus, 1982). Raup (1936) first described this rare group of boreal endemics, while surveying vegetation of the dunes and surrounding areas.

The recent origin of the Athabasca sand dunes and a high number of endemic plants, suggest that rapid evolution has taken place (Hermesh, 1972). Hermesh (1972), Argus and Steele (1979), Purdy et al. (1994), and Purdy and Bayer (1995a, b, 1996) commented on plant evolution in this unique habitat and suggested that structural characters might be adaptive to the open sands. Such features include increased trichome densities on the plant body or leaves that are amphistomatic (i.e., stomata on both leaf surfaces). To my knowledge, no structural investigations have quantified and qualified these epidermis observations for the endemic *Salix* taxa.

Argus and Steele (1979) examined the taxonomic relationship of the endemic *Salix planifolia* Pursh ssp. *tyrrellii* (Raup) Argus and its putative progenitor *S. planifolia* Pursh ssp. *planifolia*; their study included leaf anatomy and confirmed that the endemic *S. planifolia* ssp. *tyrrellii* is amphistomatic. However, it is not clear whether or not the other three endemic willows have amphistomatic leaves. The open sand habitat is characterized by high light intensity, and amphistomaty is known to be adaptive to intense light regimes (e.g., Parkhurst, 1978).

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[1] **Data from this chapter have been published in an abstract: Cooper, R. L. and D. D. Cass. 2000. American Journal of Botany. 87 (6, supplement): 24-5.**

The primary objectives of this study were (1) to document the presence of amphistomatic leaves for each endemic-progenitor *Salix* pair and (2) to examine leaf epidermal characters for each *Salix* species. The amphistomatous condition of the four endemic willows (*S. brachycarpa* Nutt. var. *psammophila* Raup, *S. planifolia* ssp. *tyrrellii*, *S. silicicola* Raup, and *S. turnorii* Raup) will be assessed using light microscopy (LM) and scanning electron microscopy (SEM). To better understand the adaptive significance of the amphistomatic leaves, its occurrence is noted and then compared to the respective widespread *Salix* progenitors (*S. brachycarpa* Nutt. var. *brachycarpa*, *S. planifolia* ssp. *planifolia*, *S. alaxensis* (Anders.) Coville, and *S. lutea* Nutt.).

Leaf epidermal features of endemic *Salix* species and their associated progenitors were examined. The plant epidermis is exposed to and can respond to surrounding environmental conditions. Stomatal frequency, stomatal index, and trichome density were quantified and evaluated within each endemic-progenitor pair. This detailed structural investigation within each endemic-progenitor *Salix* pair will add new morphological data, as well as identify diagnostic features of the endemic willows.

This study will provide information about the Athabasca sand dune endemics in two ways. First, the findings will complement previously described taxonomic affinities within each endemic-progenitor *Salix* pair. Second, the results will permit an assessment of the adaptive significance of amphistomatic leaves (and related diagnostic leaf epidermal features) of endemic *Salix* species in this boreal, sand dune habitat.

MATERIALS AND METHODS

Stem cuttings were collected from endemic *Salix* taxa at Yakow Lake dune, as well as Thomson Bay dune shore, of the Athabasca sand dunes of northern Saskatchewan, Canada during July 1998 and June 1999. Cuttings of the widespread putative progenitor *Salix* species were obtained from various riparian and wetland localities in Alberta. *Salix planifolia* ssp. *planifolia* was also sampled from Thomson Bay dune shore, where it occurs sympatrically with the endemic derivative *S. planifolia* ssp. *tyrrellii*. Leaves (~1.5 cm X .75 cm) were dissected from cuttings and stored in 70% ethanol or dried in a plant press. Leaves were collected from all taxa.

To determine if leaves were amphistomatic, scanning electron microscopy (SEM) was employed. Leaves from each endemic-progenitor *Salix* pair were air-dried and mounted onto aluminum stubs with colloidal graphite tape. Leaves were sputter-coated with gold-palladium and viewed using a JEOL JSM-6301FXV SEM. At least 5 leaves from each *Salix* species were observed for the occurrence of adaxial (AD) and abaxial (AB) stomata. If the AD epidermis had stomata distributed on the entire leaf surface (along with AB stomata), it was considered to be amphistomatic.

Stomatal frequency (number of stomata per mm^2) was also generated from SEM micrographs. Five or more leaves were examined using SEM, and stomata were counted from 5 fields of view per leaf surface. All of the stomatal counts were averaged per leaf, and the averages were calculated on a per area basis for each species.

Analysis of variance (ANOVA) was used to compare AD and AB stomatal frequencies (per leaf) separately among the nine species sampled. ANOVA was followed by post-hoc pair-wise comparisons among all species using the Bonferroni correction. Significant differences, if present, were presented within each endemic-progenitor *Salix* pair only.

Although care was taken to use leaves of similar developmental age, differing collection times and/or differing phenologies for each *Salix* species may have affected results for stomatal frequency. Thus, stomatal indices were calculated for each endemic-progenitor *Salix* pair to compensate for differences in leaf age. Stomatal indices were calculated as the number of stomata per mm^2 divided by the total number of epidermal cells per mm^2; resulting values were then multiplied by 100. Epidermal cell counts were obtained isolating cuticles from 5 leaves of each taxon in chromium trioxide (Alvin and Boulter, 1974) and counting cell imprints on the remaining cuticle. Air-dried cuticles were mounted on aluminum stubs for observation with SEM. Stomata and epidermal cell imprints were counted from SEM micrographs, such that stomatal indices for AD and AB leaf surfaces could be calculated. Previously determined significant differences in AD or AB stomatal frequency of an endemic-progenitor pair were then re-assessed in light of any differences in stomatal index.

Due to the destructive nature of the cuticle isolation technique, cuticles were torn and could not be associated with a particular leaf. Thus, the average of individual fields of view were used to calculate stomatal indices (of AD and AB leaf surfaces) for each *Salix* species. ANOVA was used to compare AD and AB stomatal indices separately among the nine species sampled, followed by post-hoc pair-wise comparisons among all species using the Bonferroni correction. If there were significant differences for stomatal indices, they were presented within endemic-progenitor *Salix* pairs only.

For trichome density (number of trichomes per mm^2), a method similar to the one described above was used. Five or more leaves from each *Salix* species were examined using SEM. From SEM micrographs, fields of view for both AD and AB leaf surfaces were evaluated, trichome counts were obtained, and average trichome densities were calculated per leaf of each species. In some cases, trichomes were removed mechanically, and the remaining trichome bases were counted. Trichome density data (for AD and AB surfaces) per leaf were compared separately using ANOVA, followed by post-hoc pair-wise comparisons among all nine species using the Bonferroni correction. Significant differences, when present, were presented within endemic-progenitor *Salix* pairs only.

For the endemic, *Salix silicicola*, the methods described did not produce interpretable results. The tomentose nature of the AB leaf surface of *S. silicicola* impaired viewing of the underlying epidermis. Mechanical removal of the trichomes resulted in tearing of the epidermal tissue. To generate values for stomatal frequency and trichome density, paradermal sections were used.

Ten leaves of *Salix silicicola* were dehydrated in an ethanol series and embedded in paraffin, but only 8 leaves produced true paradermal sections. Paradermal sections (5 – 7 μm in thickness) of the AB leaf surface were made using a Spencer 820 microtome. The sections were stained using Safranin O and Light Green (Johansen, 1940) and mounted in DPX mounting with cover slips. The AB paradermal sections of *S. silicicola* were viewed using light microscopy (LM). Counts of stomata, trichomes, and ordinary epidermal cells were made from digital images of the paradermal sections. Stomatal frequency and trichome density were averaged per leaf for both AD and AB surfaces, while stomatal indices were obtained per field of view as described above. These data were analyzed using ANOVA as specified above for each leaf epidermal character.

RESULTS

Amphistomatic leaves—Adaxial (AD) leaf surfaces were examined using SEM for each endemic *Salix* species and its putative progenitor, and two of the four endemic willows are amphistomatous (Table 2-1). Many of the willows in this study had some degree of AD stomatal distribution; however, only those which had stomata distributed over the entire AD leaf surface were considered to be amphistomatic. The endemic, *S. planifolia* ssp. *tyrrellii*, and the Thomson Bay progenitor *S. planifolia* ssp. *planifolia*, have amphistomatic leaves, while the widespread progenitor does not (Figs. 2-1a, b, c). *Salix turnorii* is also amphistomatic (Fig. 2-1d), but its putative progenitor is not. The endemic-progenitor pair (*S. silicicola* and *S. alaxensis*) is not amphistomatic, nor is the endemic *S. brachycarpa* var. *psammophila* (Table 2-1). However, the widespread putative progenitor, *S. brachycarpa* var. *brachycarpa*, has amphistomatous leaves (Table 2-1).

Stomatal frequency—Stomatal frequencies (on adaxial (AD) and abaxial (AB) leaf surfaces) for each endemic-progenitor *Salix* pair are presented in Table 2-1. The *S. brachycarpa* endemic-progenitor pair has significant differences in stomatal frequency for both AD and AB leaf surfaces; the endemic *S. brachycarpa* var. *psammophila* has a greater density of AB stomata, while the putative progenitor *S. brachycarpa* var. *brachycarpa* has a significantly greater number of AD stomata (Table 2-1).

Adaxial stomatal frequencies of both *S. planifolia* ssp. *tyrrellii* and Thomson Bay progenitor *S. planifolia* ssp. *planifolia* were significantly greater than the widespread progenitor (Table 2-1). Both progenitors of *S. planifolia* ssp. *tyrrellii* had greater densities of AB stomata than their endemic derivative, but AB stomatal frequency was significantly greater in the widespread progenitor *S. planifolia* ssp. *planifolia* (Table 2-1). Neither *Salix silicicola* nor its putative progenitor had AD stomata. However, *S. silicicola* did have a significantly greater AB stomatal frequency than its progenitor, *S. alaxensis* (Table 2-1). The endemic *S. turnorii* had a greater AD stomatal frequency than its progenitor, while AB stomatal frequency was greater in the progenitor, *S. lutea*; both differences were statistically significant (Table 2-1).

Stomatal index—Stomatal indices for the Athabasca endemic *Salix* taxa and their putative progenitors are shown in Table 2-2. Stomatal indices correspond to the trends in stomatal frequencies (refer to Table 2-1) of each endemic-progenitor *Salix* pair, with some exceptions. The AD and AB stomatal index in the *S. planifolia* group do not correlate with their associated stomatal frequencies (Tables 2-2; 2-1).

Trichome density—Trichome densities (of AD and AB leaf surfaces) for each endemic-progenitor *Salix* pair are presented in Table 2-3. Density of both AD and AB trichomes was significantly greater in the endemic *S. brachycarpa* var. *psammophila* than its putative progenitor (Table 2-3). Trichome densities were low and variable in the *S. planifolia* group (Table 2-3). The endemic *S. silicicola* had significantly greater AD and AB trichome densities than its progenitor, *S. alaxensis* (Table 2-3); the AB trichome density of *S. silicicola* was the highest of all *Salix* taxa in this study (Table 2-3; Fig. 2-1e). There were no differences between the endemic *S. turnorii* and its progenitor for either AD or AB trichome densities (Table 2-3).

DISCUSSION

Few studies have examined amphistomatic versus hypostomatic (i.e., stomata primarily on lower leaf surface) patterns of plants in relation to their natural environments (Mott et al., 1982). However, there have been investigations, both experimental (*Helianthus annuus* L. and *Xanthium strumarium* L., Mott and O'Leary, 1984; *Ambrosia cordifolia* (Gray) Payne, Mott and Michaelson, 1991) and theoretical (Parkhurst, 1978; Mott et al., 1982), that looked at functional and adaptive significance of amphistomatic leaves. My study is the first to document amphistomatic leaves in closely related pairs of *Salix* taxa, a subset of which are endemic to the Athabasca sand dunes. Parkhurst (1978) and Mott and Michaelson (1991) presented evidence that the amphistomatic condition is adaptive to high light intensities. The putative *Salix* progenitors (in this study) were obtained from primarily open riparian habitats, and the endemic derivatives are confined mainly to dune interiors.

The endemics, *S. planifolia* ssp. *tyrrellii* and *S. turnorii*, have stomata on both leaf surfaces, as does the widespread *S. brachycarpa* var. *brachycarpa* and the Thomson Bay progenitor *S. planifolia* ssp. *planifolia* (Table 2-1; Fig. 2-1b). Based on the amphistomatous condition of the endemic *S. planifolia* ssp. *tyrrellii* and the Thomson Bay progenitor, I suggest that amphistomatic leaves are adaptive to the open sand/dune shore environment. This conclusion is supported further by the hypostomatic condition of the widespread progenitor *S. planifolia* ssp. *planifolia* (Table 2-1; Fig. 2-1a) and a similar pattern of stomatal occurrence in the endemic-progenitor pair of *S. turnorii* and the widespread *S. lutea* (Table 2-1; Fig. 2-1d).

It is possible that the amphistomatous character may be a phenotypic adaptation to this boreal, open sand habitat, as other willows in this study had AD stomatal distributions. However, results of a common garden study of the endemic *S. planifolia* ssp. *tyrrellii* and the closely related widespread *S. planifolia* ssp. *planifolia* suggest that there is a genetic basis for amphistomatic leaves (Argus and Steele, 1979). The endemic *S. planifolia* ssp. *tyrrellii* maintained amphistomatic leaves throughout a cultivation period of three to five years (Argus and Steele, 1979).

Distributions of adaxial (AD) stomata are not always uniform. Some populations of the endemic *S. planifolia* ssp. *tyrrellii* exhibit evenly distributed AD stomata while others show a more dense distribution of AD stomata at leaf apices; AD stomata distributions can vary among leaves on an individual plant (J. Gould and R. L. Cooper, personal observation). This variation also exists in some geographically widespread populations of the putative progenitor *S. planifolia* ssp. *planifolia* (Argus and Steele, 1979; personal observation).

I suggest that the lack of uniform distribution of AD stomata in endemic *Salix* species could be related to light intensity differences during leaf development. First, leaf position and orientation on the stem may affect angles of incident light on individual leaves as they are developing. If a given leaf area is subjected to differing light regimes, then a non-uniform occurrence of AD stomata may result. My observations support the ideas of Mott et al. (1982) that the presence of AD stomata in some plants may be dependent on light intensity during leaf initiation, as leaves prior to light experiments did not produce AD stomata.

Results obtained for the *S. brachycarpa* endemic-progenitor pair indicated that the putative progenitor *S. brachycarpa* var. *brachycarpa* was amphistomatic, while the endemic was not (Table 2-1). Few stomata were present on the AD leaf surface of the endemic, but AD stomatal distribution was consistently confined to the leaf apex (R. L. Cooper, personal observation). Leaves of the endemic *S. brachycarpa* var. *psammophila* are upright on the stem, with leaf tips curled away from the plant body. The presence of stomata on AD leaf apices may be a phenotypic response to the intense light condition of the inner dune habitat. Specimens of the progenitor *S. brachycarpa* var. *brachycarpa* were collected from exposed riparian areas; fewer trichomes covering the AD surface (Table 2-3) may expose the epidermis to more light, resulting in AD stomatal development. Trichomes are known to reflect incident light (e.g., Karabourniotis and Borman, 1999). A denser trichome covering may reduce penetration of light rays, but an exposed AD leaf apex of the endemic *S. brachycarpa* ssp. *psammophila* might explain why stomata are only distributed at the AD leaf tip. Thus, these combined features may allow for a greater intensity of light to penetrate the trichome layers at the AD leaf apex, resulting in a restricted stomatal distribution.

The amphistomatic progenitor *S. planifolia* spp. *planifolia* from Thomson Bay dune shore had a significantly greater AD stomatal frequency than the widespread *S. planifolia* ssp. *planifolia*; the endemic derivative was clearly amphistomatic (Table 2-1). My results are not in agreement with those of Argus and Steele (1979) who reported that the endemic, *S. planifolia* ssp. *tyrrellii*, had an equal stomatal frequency on both leaf surfaces. As mentioned previously, the discrepancy between these findings may be due to uneven distributions of AD stomata. Personal observations have revealed that some specimens of this endemic have more AD stomata at the leaf apex. In addition, I averaged stomatal counts from fields of view for each leaf surface, while Argus and Steele (1979) examined epidermal peels from the leaf center to assess stomatal occurrence. The combination of different methodologies in the examination of stomatal occurrence, along with a documented variation in AD stomatal distributions of *S. planifolia* ssp. *tyrrellii*, may explain the inconsistency between my results and those of Argus and Steele (1979).

This is the first report of stomatal frequency, stomatal index, and trichome density for the Athabasca sand dunes endemic *Salix* taxa and their associated putative progenitors. Only *S. planifolia* ssp. *tyrrellii* and *S. turnorii* have amphistomatic leaves (Table 2-1). Much of the discussion has been focussed on stomatal occurrence, but a brief statement on trichome density is necessary. Both amphistomatic endemic *Salix* species are highly cuticularized, but have few to no trichomes (Fig. 2-1c, d; Table 2-3). In contrast, the two endemics, *S. brachycarpa* var. *psammophila* and *S. silicicola*, have dense trichome coverings (Table 2-3; Fig. 2-1e); their leaves are not amphistomatic (Table 2-1). Trichomes scatter and reflect light. If light intensity is an important factor in the occurrence of AD stomata, then trichome coverings of the pubescent *Salix* taxa may well impede incident light. This idea is supported by the studies of Liakoura et al. (1997) and Karabourniotis and Borman (1999).

Liakoura et al. (1997) provided experimental evidence that trichomes might function collectively as a "UV-B radiation screen." Trichome coverings in two Mediterranean taxa filtered UV light and attenuated blue light from the visible spectrum (Karabourniotis and Borman, 1999). Since the open sand habitat is characterized by high light intensity, the dense covering of leaf hairs of the endemic *Salix* species could function in the same way.

The occurrence of amphistomatic leaves and dense trichome coverings are likely correlated with the exposed nature of the inner dunes. Differences in morphology can be addressed as they relate to the taxonomy of the endemic-progenitor *Salix* pairs. Recently, Argus (1999) recognized four subgenera of *Salix*. The *S. brachycarpa* endemic-progenitor pair was placed in *Salix* subgenus *Chamaetia* (Dumort.) Nasarov, while the other pairs were placed in *Salix* subg. *Vetrix* (Dumort.) Dumort; more specifically, each endemic-progenitor *Salix* pair is classified in separate taxonomic sections of the genus (Argus, 1999). Morphological differences documented among endemic-progenitor *Salix* pairs reflect their taxonomic placements within the genus.

TABLE 2-1. Stomatal frequencies for Athabasca endemic *Salix* species and their putative progenitors.

Taxon	**Stomatal frequency (#/mm^2)**			
	Adaxial		**Abaxial**	
	n	mean ± SD	*n*	mean ± SD
S. brachycarpa var. *psammophila*	12	38 ± 37	12	505 ± 99**
S. brachycarpa var. *brachycarpa*	5	130 ± 22***	5	286 ± 38
S. planifolia ssp. *tyrrellii*	24	126 ± 36^{a}	22	228 ± 68^{a}
S. planifolia ssp. *planifolia* (T-Bay)	12	144 ± 66^{a}	12	299 ± 92^{a}
S. planifolia ssp. *planifolia*	7	36 ± 44^{b}	7	610 ± 142^{b}
S. silicicola	12	0 ± 1	8	581 ± 135***
S. alaxensis	5	11 ± 12	5	201 ± 24
S. turnorii	11	127 ± 43**	13	195 ± 54
S. lutea	5	44 ± 27	5	942 ± 235***

T-Bay = Thomson Bay dune shore; Results of ANOVA: *** = $P < 0.001$; ** = $P < 0.01$ for comparison within endemic-progenitor pairs. $P < 0.001$ for comparison within *Salix planifolia* endemic-progenitor group.[1]

[1] Different letters indicate significant differences (post-hoc test after ANOVA).

TABLE 2-2. Stomatal indices for Athabasca endemic *Salix* species and their putative progenitors.

Taxon	**Stomatal index**			
	Adaxial		**Abaxial**	
	n	mean ± SD	*n*	mean ± SD
S. brachycarpa var. *psammophila*	28	0.63 ± 1.51	34	8.77 ± 3.77***
S. brachycarpa var. *brachycarpa*	34	1.85 ± 1.62*	33	4.58 ± 1.54
S. planifolia ssp. *tyrrellii*	27	4.67 ± 1.42^{a}	28	7.20 ± 2.45^{a}
S. planifolia ssp. *planifolia* (T-Bay)	29	2.09 ± 2.09^{b}	31	8.63 ± 1.82^{a}
S. planifolia ssp. *planifolia*	32	0.60 ± 1.50^{b}	26	11.18 ± 1.94^{b}
S. silicicola	24	0 ± 0	25	9.89 ± 4.61***
S. alaxensis	34	0.24 ± 0.47	34	2.85 ± 1.06
S. turnorii	18	3.19 ± 2.26***	25	5.16 ± 2.51
S. lutea	28	0.14 ± 0.51	28	8.43 ± 4.41**

T-Bay = Thomson Bay dune shore; Results of ANOVA: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$.

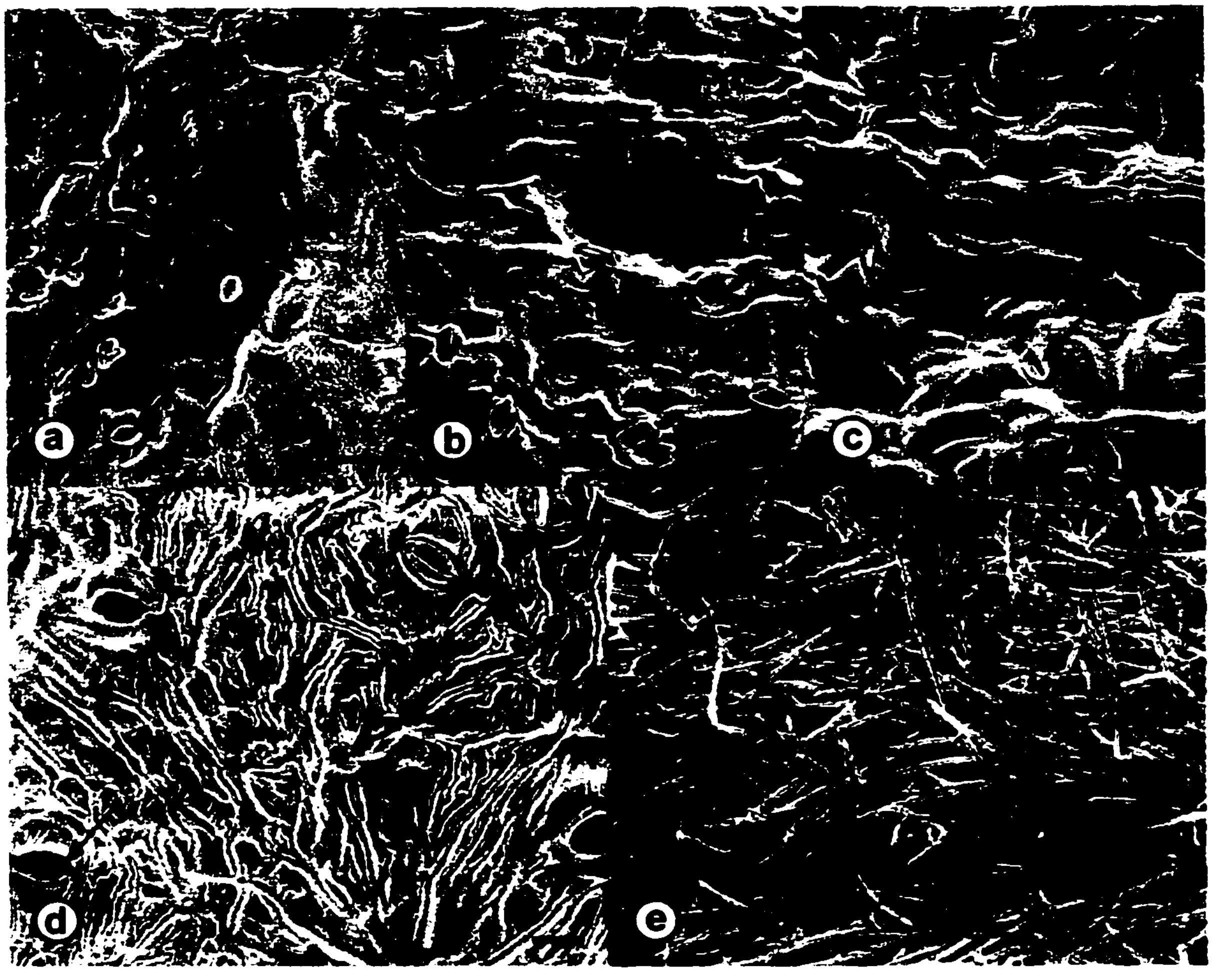
Note: For *S. planifolia* group, different letters indicate significant differences (post-hoc test after ANOVA).

TABLE 2-3. Trichome densities for Athabasca endemic *Salix* species and their putative progenitors.

Taxon	**Trichome density (#/mm^2)**			
	Adaxial		**Abaxial**	
	n	mean ± SD	*n*	mean ± SD
S. brachycarpa var. *psammophila*	12	270 ± 102***	12	245 ± 95**
S. brachycarpa var. *brachycarpa*	5	58 ± 11	5	108 ± 38
S. planifolia ssp. *tyrrellii*	24	0 ± 1	24	2 ± 2
S. planifolia ssp. *planifolia* (T-Bay)	12	1 ± 1	12	1 ± 2
S. planifolia ssp. *planifolia*	7	15 ± 14	7	52 ± 25
S. silicicola	12	366 ± 93***	8	903 ± 192***
S. alaxensis	5	57 ± 19	5	112 ± 10
S. turnorii	11	31 ± 22	14	9 ± 10
S. lutea	5	0 ± 1	5	0 ± 1

T-Bay = Thomson Bay dune shore; Results of ANOVA: *** = $P < 0.001$; ** = $P < 0.01$.

FIGURE 2-1. Scanning electron micrographs of *Salix* species showing epidermal features. **a.** AD leaf surface of progenitor *S. planifolia* ssp. *planifolia*. Bar = 20 µm. **b.** AD leaf surface of Thomson Bay progenitor *S. planifolia* ssp. *planifolia* (from Thomson Bay dune shore). Bar = 20 µm. **c.** AD leaf surface of endemic *S. planifolia* ssp. *tyrrellii*. Bar = 20 µm. **d.** AD leaf surface of endemic *S. turnorii*. Bar = 20 µm. **e.** AB leaf surface of endemic *S. silicicola* showing dense trichome covering. Bar = 100 µm. Arrows indicate AD stomata.


a
b
c
d
e

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CHAPTER 3: CUTICLE THICKNESS AND EPICUTICULAR WAX MORPHOLOGY OF *SALIX* SPECIES (SALICACEAE) ENDEMIC TO THE ATHABASCA SAND DUNES[1]

INTRODUCTION

The plant cuticle is an important component of the leaf epidermis, as it forms a continuous, protective layer over the leaf epidermal cells (Martin and Juniper, 1970). Wax exudates, or epicuticular waxes, are usually embedded in the cuticle and deposited on the cuticle exterior. Epicuticular waxes and the underlying cuticle are beneficial to a plant by providing a barrier against wind (Martin and Juniper, 1970), protection against mechanical damage and physical abrasion (Eglinton and Hamilton, 1967; Martin and Juniper, 1970), as well as excessive insolation (Martin and Juniper, 1970). Another cuticular function is to minimize water loss. In exposed areas and drier habitats, plants tend to have thicker cuticles and crystalline waxes (Martin and Juniper, 1970).

The Athabasca sand dunes in northern Saskatchewan (formed about 10,000 years ago) have gravel pavements, areas of boreal forest, and large expanses of open sand (Smith, 1978). The open sand habitat of the dunes has high light intensity (including sand reflectivity) and is home to 10 endemic plant taxa. Four are *Salix* species (Salicaceae), first described by Raup (1936). Argus and Steele (1979) observed that endemic *Salix* species appear to have thicker cuticles than their associated widespread putative progenitors. The abaxial (AB) leaf surfaces of two *Salix* endemics, *S. planifolia* Pursh ssp. *tyrrellii* (Raup) Argus and *S. turnorii* Raup, are 'bluish' in appearance indicative of a dense, wax bloom (R. L. Cooper, personal observation).

Thick, waxy cuticles may be an adaptation of endemic *Salix* species to this open boreal sand dune environment. My goal was to quantify cuticle thickness of endemic *Salix* taxa (*S. brachycarpa* Nutt. var. *psammophila* Raup, *S. planifolia* ssp. *tyrrellii*, *S. silicicola* Raup, and *S. turnorii*) as compared to that of their respective putative progenitors (*S. brachycarpa* Nutt. var. *brachycarpa*, *S. planifolia* Pursh ssp. *planifolia*, *S. alaxensis* (Anders.) Coville, and *S. lutea* Nutt.) using histology and light microscopy (LM) techniques. Scanning electron microscopy (SEM) was used to determine

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[1] **Data from this chapter have been published in an abstract: Cooper, R. L. and D. D. Cass. 2000. American Journal of Botany. 87 (6, supplement): 24-5.**

epicuticular wax occurrence and its morphology (if present) for each endemic-progenitor *Salix* pair.

MATERIALS AND METHODS

Stem cuttings from each endemic *Salix* species were collected from Yakow Lake dune in northern Saskatchewan (June 1998 and July 1999); the endemic willow *S. planifolia* ssp. *tyrrellii* and its putative progenitor, *S. planifolia* ssp. *planifolia* were also sampled at Thomson Bay dune shore (Fig. 1-1). During mid- and late summer of 1998, the remaining associated progenitors were sampled from riparian and peatland Alberta localities. Leaves (~1.0 – 1.5 cm X 0.5 cm) were dissected from the willow stem cuttings.

Twenty or more leaves from each *Salix* species were prepared for LM. Leaves were fixed in the field with FAA (formalin acetic acid) and brought back to laboratory facilities at the University of Alberta. Leaves were then rinsed in running tap water for at least 1 hr, followed by dehydration in an ethanol series. Leaves were embedded in paraffin and mounted into blocks for sectioning with a Spencer 820 microtome. Sections (7 μm – 10 μm) were made from the mid-section of each leaf.

Sections were mounted on glass slides and stained with a Safranin O/Light Green protocol modified from Johansen (1940). Cuticle thickness was determined at the mid-rib of each leaf for both adaxial (AD) and abaxial (AB) leaf surfaces by taking measurements with a light microscope. Cuticles were measured from only those leaves that remained intact after sectioning. Values for cuticle thickness were averaged per AD and AB leaf surface for each leaf of all *Salix* species.

Adaxial and AB data sets were analyzed separately. Analysis of variance (ANOVA) was used to compare cuticle thickness (per leaf) among the nine species sampled. The use of ANOVA was followed by post-hoc pair-wise comparisons among all species using the Bonferroni correction. Significant differences (if present) were presented within endemic-progenitor *Salix* pairs only.

Leaves (~ 1.5 cm X .75 cm) from a previous epidermis study were examined for the occurrence of epicuticular wax for all endemic-progenitor *Salix* pairs using SEM and cryo-SEM. For SEM, leaves that had been dried in a plant press were used for study, while fresh leaves were used for examination with cryo-SEM. After SEM observation, morphology of the epicuticular wax was described for each *Salix* species in this study.

RESULTS

Cuticle thickness—Adaxial (AD) and AB cuticle thicknesses were determined for each Athabasca sand dune *Salix* endemic and its associated putative progenitor (Table 3-1). The endemic, *S. brachycarpa* var. *psammophila*, had thinner AB cuticle thickness than the widespread progenitor, *S. brachycarpa* var. *brachycarpa*, but AD cuticle thickness was similar in both (Table 3-1). For both AD and AB cuticle thicknesses in the *S. brachycarpa* endemic-progenitor pair, the differences were not significant (Table 3-1).

Salix planifolia ssp. *tyrrellii* had the thickest AD and AB cuticles, as compared to both the Thomson Bay and widespread progenitor, and the AD cuticle of the endemic was significantly thicker than that of its widespread putative progenitor (Table 3-1). *Salix planifolia* ssp. *planifolia* from Thomson Bay dune shore had the thinnest AB cuticle in the group, while the widespread *S. planifolia* ssp. *planifolia* had the thinnest AD cuticle thickness (Table 3-1).

The endemic, *S. silicicola*, had thinner AD and AB cuticles than the putative, widespread progenitor, *S. alaxensis* (Table 3-1). *Salix turnorii* had a thinner AD cuticle and a thicker AB cuticle than its associated widespread progenitor, *S. lutea* (Table 3-1). For these endemic-progenitor *Salix* pairs, AD and AB cuticle thicknesses were not significantly different (Table 3-1).

Epicuticular wax morphology—Using SEM, the presence of epicuticular wax on AD and AB leaf surfaces was determined for each species of the four endemic-progenitor *Salix* pairs, and morphology of the epicuticular wax was described (Table 3-2). In general, epicuticular wax is similar with respect to either AD or AB leaf surfaces within each endemic-progenitor pair. Overall, AD epicuticular wax morphology was alike in two of the endemic-progenitor *Salix* pairs (Table 3-2; Fig. 3-1a). Abaxial wax morphology was similar among each endemic-progenitor *Salix* group, with the exception of *S. silicicola* and its putative progenitor (Table 3-2; Figs. 3-1b, c, d, e, f).

Epicuticular wax is present on both leaf surfaces in the *S. brachycarpa* endemic-progenitor pair, as well as in the *S. planifolia* endemic-progenitor group. In the *S.*

brachycarpa endemic-progenitor pair, wax is sparse on AD leaf surfaces and exists as narrow slivers, while AB leaf surfaces are covered with a dense layer of columnar wax plates (Table 3-2; Fig. 3-1b).

The AD leaf surface of the endemic *S. planifolia* ssp. *tyrrellii* has sparse, narrow plates (Fig. 3-1a); the AB leaf surface of this endemic is covered in a dense crust of plates (Fig. 3-1c) that have a raised, column-like structure (Fig. 3-1d). Wax morphologies of the progenitor *S. planifolia* ssp. *planifolia* are similar to the endemic derivative *S. planifolia* ssp. *tyrrellii* (Table 3-2; Fig. 3-1c, d).

Salix silicicola and its putative widespread progenitor, *S. alaxensis*, had no detectable epicuticular wax deposits on AD leaf surfaces. A smooth wax covered AB leaf surfaces in this endemic-progenitor *Salix* pair (Table 3-2; Fig. 3-1e).

The AD leaf surfaces of *S. turnorii* did not have any wax deposits, nor did those of the associated putative progenitor, *S. lutea*. However, AB leaf surfaces of this endemic-progenitor pair have a dense covering of columnar wax plates (Fig. 3-1f). This type of AB epicuticular wax morphology is also similar to that of the *S. brachycarpa* endemic-progenitor pair and *S. planifolia* group (Table 3-2; Figs. 3-1b, c, d).

DISCUSSION

Cuticle thickness is correlated with a plant's environment (e.g., Martin and Juniper, 1970), and an epicuticular wax coating has been regarded as an important land plant adaptation that reduces water loss (Dodd and Afzal-Rafii, 2000). Willows are known to be highly shade-intolerant (G. Argus, personal communication), but the open sand habitat presents a demanding set of environmental conditions, including high insolation and high winds (Macdonald et al., 1987). In this study, cuticle thickness and epicuticular wax morphology were examined in the four endemic *Salix* species from the Athabasca sand dunes of northern Saskatchewan, Canada. To assess adaptive and functional significance of these characters, structural data were compared to those of the associated widespread boreal or arctic progenitor of each endemic derivative.

As mentioned previously, two willow endemics have a glossy, waxy appearance, as do their associated progenitors (personal observation). The *Salix planifolia* endemic-progenitor group, as well as *S. turnorii* and its putative progenitor, both shared similar AB epicuticular wax morphologies (Table 3-2; Figs. 3-1c, d, f). However, the *S. turnorii*

– *S. lutea* pair did not have AD epicuticular wax deposits; the absence of AD epicuticular wax, presence of AB epicuticular wax, and similar morphology of AB epicuticular wax support the presumably close evolutionary relationship between these two taxa. The endemic *S. turnorii* had a thinner AD cuticle than its putative widespread progenitor (Table 3-1).

The endemic willows, *S. brachycarpa* var. *psammophila* and *S. silicicola*, had thinner AD and AB cuticles than their respective putative progenitors (Table 3-1). A recent investigation of the epidermis of each *Salix* endemic-progenitor pair confirmed significantly greater trichome densities in these two endemics as compared to their widespread progenitors (Cooper, Thesis Ch. 2, 2001). Dense trichome coverings reflect and scatter light (Karabourniotis and Borman, 1999). I suggest that *S. brachycarpa* var. *psammophila* and *S. silicicola* have adapted to intense light conditions of the Athabasca open sands by increasing trichome numbers rather than cuticle thickness. These two tomentose endemic-progenitor *Salix* pairs belong to distantly-related sections of the genus (Dorn, 1976; Argus, 1999). The presence of a plate-like AB epicuticular wax in the *S. brachycarpa* endemic-progenitor pair and a smooth AB wax in *S. silicicola* and its putative progenitor may reflect differences in the genetic identity and wax chemistry of each pair.

Baker (1982) proposed that epicuticular wax morphology was determined mainly by the chemical composition of the wax exudates. Rashotte and Feldmann (1998) demonstrated correlations between chemistry and epicuticular wax structures in *Arabidopsis thaliana* using gas chromatography-mass spectroscopy (GC-MS) analysis and SEM, respectively. Epicuticular wax deposits on AB leaf surfaces of the Athabasca sand dune endemic willows impeded examination of the epidermis for determination of stomatal frequencies and indices in a previous study (Cooper, Thesis Ch. 2, 2001). The wax was not dissolved in chloroform or toluene, even after leaves were submersed for over 2 min with sonication and attempts at mechanical removal. Rashotte et al. (1997) were able to dissolve and extract the epicuticular wax of *A. thaliana* in hexane after 30 seconds of being submersed. The solvent hexane was used in an attempt to characterize the chemistry of epicuticular waxes of each endemic-progenitor *Salix* pair; examination with SEM showed that AB epicuticular wax was still persistent on AB leaf surfaces (Cooper and Cass, unpublished data).

Preliminary GC-MS analyses of epicuticular wax components (of each *Salix* species in this study) have been done. However, only those chemical components that were dissolvable in hexane were analyzed; these components were primarily alkanes, some acids, ketones, and aldehydes (Cooper and Cass, unpublished data). Wax chemistry results were similar within endemic-progenitor *Salix* pairs, and each pair shared some of the same chemical components (Cooper and Cass, unpublished data). It is important to emphasize the preliminary nature of these results. As epicuticular wax does not completely dissolve in primary solvents, these results should be regarded as preliminary.

For the Athabasca sand dune endemic *Salix* taxa, I conclude that the persistent AB epicuticular crust serves as a protective barrier, not only against insolation, but also against sand reflectivity and abrasion from blowing sand. Furthermore, chemical characterization of the epicuticular waxes is necessary to address functional significance of the persistent AB crusts, as well as to better understand adaptation of these recently-derived willows to this boreal, sand dune habitat.

TABLE 3-1. Cuticle thickness for the Athabasca endemic *Salix* species and their putative progenitors.

Taxon	**Cuticle thickness (μm)**			
	Adaxial		**Abaxial**	
	n	mean ± SD	*n*	mean ± SD
S. brachycarpa var. *psammophila*	19	2.37 ± 0.57	19	2.72 ± 0.73
S. brachycarpa var. *brachycarpa*	22	2.42 ± 0.46	22	3.25 ± 1.01
S. planifolia ssp. *tyrrellii*	33	2.47 ± 0.50^{a}	33	3.11 ± 1.17
S. planifolia ssp. *planifolia* (T-Bay)	17	2.31 ± 0.51^{ac}	17	2.54 ± 0.50
S. planifolia ssp. *planifolia*	27	1.87 ± 0.44^{bc}	27	2.77 ± 1.19
S. silicicola	24	1.98 ± 0.28	24	2.29 ± 0.51
S. alaxensis	7	2.15 ± 0.40	7	2.49 ± 0.39
S. turnorii	18	2.20 ± 0.67	18	2.95 ± 0.72
S. lutea	9	2.40 ± 0.33	9	2.68 ± 0.18

T-Bay = Thomson Bay dune shore; Results of ANOVA: $P < 0.001$ for comparison within *Salix planifolia* endemic-progenitor group for AD cuticle thickness.[1]

[1] For adaxial cuticle thickness values in the *S. planifolia* group, different letters indicate significant differences (post-hoc test after ANOVA).

TABLE 3-2. Epicuticular wax morphology for the Athabasca endemic *Salix* species and their putative progenitors.

Endemic-progenitor pair	**Epicuticular wax morphology** **AD**	 **AB**
S. brachycarpa var. *psammophila*	sparse, narrow plates	dense, columnar plates
S. brachycarpa var. *brachycarpa*		
S. planifolia ssp. *tyrrellii*	sparse, narrow plates	dense, columnar plates
S. planifolia ssp. *planifolia* (T-Bay)		
S. planifolia ssp. *planifolia*		
S. silicicola	absent	smooth
S. alaxensis		
S. turnorii	absent	dense, columnar plates
S. lutea		

T-Bay = Thomson Bay dune shore

FIGURE 3-1. Scanning electron micrographs of endemic *Salix* species showing epicuticular wax morphology. **a.** *Salix planifolia* ssp. *tyrrellii* showing AD leaf surface with sparse, sliver-like wax plates (arrows). Bar = 3 µm. **b.** *Salix brachycarpa* var. *psammophila* showing AB leaf surface with a dense covering of columnar wax plates. Bar = 20 µm. **c.** *Salix planifolia* ssp. *tyrrellii* showing AB leaf surface showing dense crust of epicuticular wax. Bar = 30 µm. **d.** *Salix planifolia* ssp. *tyrrellii* AB columnar wax plates. Bar = 3 µm. **e.** *Salix silicicola* showing AB leaf surface of smooth epicuticular wax. Bar = 30 µm. **f.** *Salix turnorii* showing AB leaf surface of columnar wax plates. Bar = 10 µm.


a
b
c
d
e
f

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CHAPTER 4: COMPARATIVE EVALUATION OF VESSEL ELEMENTS IN *SALIX* SPECIES (SALICACEAE) ENDEMIC TO THE ATHABASCA SAND DUNES OF NORTHERN SASKATCHEWAN, CANADA[1]

INTRODUCTION

The Athabasca sand dunes are located on the south shore of Lake Athabasca in northern Alberta and Saskatchewan, Canada. These dunes are the largest northern dunes in the world and were formed after deglaciation approximately 10,000 years ago (Raup and Argus, 1982). Lake Athabasca's south shore has been a study site for sand dune formation (Hermesh, 1972), dune terrain and potential land usage (Smith, 1978), and detailed descriptions are available for both land and vegetation (Raup and Argus, 1982). Although over 200 plant taxa occupy this boreal sand dune region (Hermesh, 1972), only 40 of these occur on the open sands (Hermesh, 1972; Smith, 1978; Raup and Argus, 1982). Ten endemic taxa were first described by Raup (1936), of which four are *Salix* species.

Several studies have examined the origin and evolution of genetic variation within these endemics (Purdy et al., 1994; Purdy and Bayer, 1995a, b, 1996). Only one study has critically evaluated the taxonomic relationship of *Salix planifolia* Pursh ssp. *tyrrellii* (Raup) Argus and its progenitor, *S. planifolia* Pursh ssp. *planifolia*, from the Athabasca sand dunes (Argus and Steele, 1979). Purdy and Bayer (1995a) examined allozyme variation in the endemic *S. silicicola* Raup and its widespread arctic progenitor, *S. alaxensis* (Anderss.) Coville. Morphological similarities shared within endemic-progenitor pairs indicate close taxonomic associations (e.g. Argus and Steele, 1979).

There have been no detailed structural investigations of Lake Athabasca's endemic willows. Structural information may provide insight into functional strategies of these endemic sand dune species. The habitat of the Athabasca sand dunes can be described as desert-like, with endemic willows occurring on large expanses of open, actively blowing sand. Dune slacks are critical areas for establishment of endemic willows (Raup and

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[1] A version of this chapter has been published: Cooper, R. L. and D. D. Cass. 2001. American Journal of Botany. 88: 583-587.

Argus, 1982). If a willow seedling survives sand accretion, it eventually becomes a woody shrub on the open sands. Endemic willows are rooted in the water table, which may be several meters below the sand surface (G. Argus, personal communication). Overall, the open sand habitat can be described as xerophytic, although these endemic shrubs have access to water from the water table and precipitation.

Endemic willows (*S. brachycarpa* Nutt. var. *psammophila* Raup, *S. planifolia* ssp. *tyrrellii*, *S. silicicola*, and *S. turnorii* Raup) are confined to open sand areas on the dune interiors. However, *S. planifolia* ssp. *tyrrellii* is also found along the shoreline of Lake Athabasca, occurring sympatrically with its putative progenitor, *S. planifolia* Pursh ssp. *planifolia*. Thomson Bay is a site on Lake Athabasca's shoreline where these two willows co-occur, and collections were taken from this site, while other endemics were collected from the Yakow Lake dune. The progenitor taxa are thought to be *S. brachycarpa* Nutt. var. *brachycarpa*, *S. planifolia* ssp. *planifolia*, *S. alaxensis*, and *S. lutea* Nutt., respectively.

The primary objectives of this study were: (1) to evaluate quantitatively and qualitatively vessel element (VE) characters in endemic willows, (2) to compare VE structure in endemic willows with those of their associated boreal or arctic progenitors, (3) to relate VE structural information in endemic willows to functional significance in this northern, open sand habitat, and (4) to address taxonomic implications of structural data in each endemic-progenitor pair.

This study is the first extensive investigation of internal structure of the Lake Athabasca sand dune endemic willows as compared with their closely-related boreal or arctic putative sister species. Evaluation of adaptive VE characters of endemic willows will lead to a greater understanding of the role VEs play in survival in a northern, open sand habitat. In particular, we chose to examine VE characters correlated with water conduction (Carlquist, 1988). The Lake Athabasca sand dune habitat provides a unique opportunity to investigate structural adaptation in recently derived plant taxa.

MATERIALS AND METHODS

Stem cuttings from endemic willows, as well as *Salix planifolia* ssp. *planifolia* (a progenitor from Thomson Bay dune shore), were collected from the Lake Athabasca sand dunes in Saskatchewan, Canada (July 1998 and June 1999). During mid- to late summer 1998, stem cuttings for widespread progenitor taxa were collected from riparian and peatland sites in central and southern Alberta.

Stems from each species were softened in boiling water. Cross-sectional and radial-longitudinal sections were made using fresh razor blades. Sections were air-dried, coated with gold-palladium, and observed using a JEOL JSM-6301FXV scanning electron microscope.

Stems were also prepared for light microscopy (LM). After boiling, stems were cut in cross-section and stored in 70% ethanol. Sections (15 to 24 µm in thickness) were prepared using a sliding microtome, mounted on glass slides, and stained with Safranin O/Light Green using a modified protocol from Johansen (1940).

General observations of VE characters were made. Fields of view (at 300X magnification) were examined from at least 10 stems of each species. One to three fields of view (per stem) from electron micrographs were used to obtain quantitative data for VE density (#VE/mm^2) and VE clustering (#VE/group). For both VE density and VE clustering, fields of view were averaged per stem of each taxon, and these values were then averaged per *Salix* species.

For measuring VE lumen diameter and VE length, LM was employed. Vessel element lumen diameters were examined from stem cross-sections and were measured from early wood vessels in the most recent growth increment in at least 5 stems of each willow species. Measurements were taken at the widest region of the lumen, and all individual lumen measurements were averaged per taxon.

Jeffrey's method (Johansen, 1940) for wood macerations was used to obtain VE length measurements. Five stems from each species were macerated, and at least 25 vessel elements were measured per stem. Individual VE length measurements were averaged per willow species.

For each VE character (density, lumen diameter, clustering, and length), analysis of variance (ANOVA) was used to compare each data set separately for all nine species sampled. The use of ANOVA was followed by post-hoc pair-wise comparison among all species using the Bonferroni correction for each VE character data set. Significant differences, if present, were presented within endemic-progenitor *Salix* pairs only.

RESULTS

General observations—All willows examined in this study are diffuse-porous. Vessel elements (VEs) have simple perforations on both end walls, and some VEs have narrow tails on their end walls while others are blunt. Vessel elements from all species exhibit primarily alternate intervascular pitting patterns. Some transitional and scalariform lateral wall pitting was observed. Vessel element density, VE lumen diameter, VE length, and VE clustering were evaluated within each endemic-progenitor pair (Table 4-1).

Vessel element density—Vessel element densities vary both within endemic-progenitors pairs and among the pairs. *Salix brachycarpa* var. *brachycarpa* and the derived endemic *S. brachycarpa* var. *psammophila* had the highest densities of all willows examined (Fig. 4-1a, b). *Salix silicicola* and *S. turnorii* had smaller VE densities compared with their respective widespread progenitors. *Salix planifolia* ssp. *tyrrellii* also had VE density less than that of its progenitor *S. planifolia* ssp. *planifolia*. However, *S. planifolia* ssp. *planifolia* from Thomson Bay had more VEs/mm^2 than the widespread ssp. *planifolia*. Vessel element densities were not significantly different within any of the endemic-progenitor *Salix* pairs (Table 4-1).

Vessel element lumen diameter—Vessel element lumen diameter varied among endemic-progenitor pairs, and in some cases, significant differences were noted between an endemic and its putative progenitor. *Salix brachycarpa* var. *psammophila* and its progenitor had the smallest VE lumen diameters (Table 4-1).

Lumen diameters for VEs of the widespread *S. planifolia* ssp. *planifolia* were significantly greater than the endemic *S. planifolia* ssp. *tyrrellii* and *S. planifolia* ssp.

planifolia from Thomson Bay dune shore (Table 4-1; Figs. 4-1c, d, e). *Salix alaxensis* had a greater VE lumen diameter than that of its derived endemic, *S. silicicola*, but this difference was not significant (Table 4-1). Lumen diameter, however, for VEs of *S. lutea* was significantly less than that of the closely related endemic, *S. turnorii* (Table 4-1; Figs. 4-1f, g).

Vessel element clustering—Vessel element clustering, or the number of VEs per group, varied among all willow taxa in this study. The *S. brachycarpa* endemic-progenitor pair had similar clustering values, although the endemic *S. brachycarpa* var. *psammophila* had more VEs per group than its associated putative progenitor.

The number of VEs per group was greatest in the endemic, *S. planifolia* ssp. *tyrrellii*, as compared to both the widespread and Thomson Bay progenitor (Table 4-1). A significant statistical difference was observed between *S. alaxensis* and its derived endemic, *S. silicicola*, as the progenitor had greater VE clustering value than the endemic (Table 4-1; Fig. 4-1h, i). *Salix lutea* had a similar VE clustering value than the endemic *S. turnorii*.

Vessel element length—Vessel element length varied within endemic-progenitor *Salix* pairs, and some of these differences were significant. *Salix brachycarpa* var. *brachycarpa* had an average VE length value that was significantly less than that of the endemic derivative (Table 4-1; Figs. 4-1j, k). In the *S. planifolia* endemic-progenitor group, both the endemic and the widespread progenitor had similar values for VE length, and these values were greater than the average length of *S. planifolia* ssp. *planifolia* from Thomson Bay dune shore (Table 4-1). Values of VE length of *S. silicicola* and its progenitor were similar, but the average value was greater in *S. silicicola*, and average VE length for the progenitor *S. lutea* was significantly less than that of its derived endemic, *S. turnorii* (Table 4-1; Figs. 4-1l, m).

DISCUSSION

The morphology of a plant is determined in part by its environment; however, little is known about environmental effects on a plant's internal structure (Arnold and Mauseth, 1999). Endemic willows occur primarily in open sand areas of dune interiors. The desert-like property of this habitat is also characterized by high light intensity and actively blowing sand. Although one might presume this to be a xerophytic environment, the endemic willows are rooted in the water table. Thus, access to available water allows for a more mesic situation for these endemics. Vessel elements characters were assessed to see if they were more correlated with a xerophytic or mesic environment. This is the first study to address internal anatomy of the Lake Athabasca sand dune endemic willows.

Quantitative data for VE density, lumen diameter, clustering, and length in all four endemic willows were compared to those of the associated boreal or arctic putative progenitors. Densities varied within endemic-progenitor pairs, as well as among them. Statistical analyses revealed some significant differences within endemic-progenitor pairs for VE lumen diameter, VE clustering, and VE length (Table 4-1). Significant differences were interpreted as being linked with either habitat or growth habit. Although statistical manipulations did not show more variation within endemic-progenitor pairs, differences were noted and are discussed below. The minor degrees of variation in VE characters examined reflect the taxonomic affinity of the progenitor willows and their associated derived endemics.

Correlations with habitat—In mesic habitats, tension is lower in conductive systems, so VEs tend to be longer and wider; in xeric environments, VEs are generally shorter and narrower (Carlquist, 1975). In our study, results do not reveal distinct trends when comparing VEs from endemic willows with those of their respective boreal or arctic putative progenitors. Overall, endemics had longer VEs than their progenitors, with the exception of the widespread *S. planifolia* ssp. *planifolia*; this progenitor had a similar VE length value as compared to the endemic *S. planifolia* ssp. *tyrrellii*. Based solely on VE length data, the below-ground environment of the open sands may be considered mesic as willows are rooted in the water table.

Endemics appear to have greater VE clustering than the progenitors, with the exception of *Salix silicicola*. This endemic had significantly fewer VEs per group than its widespread progenitor, *S. alaxensis*. As many of the progenitors were collected from peatlands and riparian sites, the significant difference in VE length between *S. silicicola* and *S. alaxensis* may be due to growth habit rather than habitat. *Salix alaxensis* has a rather prostrate growth form (at its collection site by a stream), while the habit of *S. silicicola* is upright.

Studies have shown that VE diameter can decrease due to water stress (*Vitis*; Lovisolo and Schubert, 1998), as well as low watering and low nutrient regimes (*Cereus*; Arnold and Mauseth, 1999). The sandy substrate does not hold water; the only standing water on the open sands is that of the exposed water table. Thus, one might expect the endemic willows to have narrower vessels than their putative progenitors due to this sandy substrate, which is also low in nutrients (S. E. Macdonald, personal communication, University of Alberta). This hypothesis was supported by data from two of the four Lake Athabasca endemic willows. Both *Salix planifolia* ssp. *tyrrellii* and *S. silicicola* had narrower VE lumen diameters than their widespread sister species. In contrast, the endemic *S. brachycarpa* var. *psammophila* had a greater average VE lumen diameter than its putative progenitor, but no statistical significance was detected. *Salix turnorii* had a significantly greater VE lumen diameter than its progenitor, *S. lutea*. For the endemic *S. turnorii*, a wider lumen may be a structural adaptation for more efficient water transport in this open sand environment.

Three endemic willows had lower VE densities than their associated progenitors; however, none of these differences was statistically significant. The only endemic having a greater VE density than its progenitor was *Salix brachycarpa* var. *psammophila*, but their density values were quite similar.

Salix planifolia ssp. *planifolia* from Thomson Bay had a greater VE density than both the endemic and the widespread *S. planifolia* ssp. *planifolia*, and it also had the smallest VE length of the three subspecies. During collection of the *S. planifolia* ssp. *planifolia* from Thomson Bay, stem cuttings were taken from shrubs that had fewer stomata on adaxial leaf surfaces. The endemic *S. planifolia* ssp. *tyrrellii* is amphistomatic (Argus and Steele, 1979; Cooper and Cass, unpublished data), but the Thomson Bay progenitor had few to no stomata on its adaxial leaf surface. At this site, it may be possible that we collected stem cuttings from hybrids as well as *S. planifolia* ssp. *planifolia*. The two subspecies are quite similar morphologically, and the stomatal character has been useful in elucidating the identity of each (Argus and Steele, 1979; personal observation). Furthermore, Orians et al. (1999) suggested that increased water availability may enhance relative performance of *Salix* hybrids. We feel that we collected pure *S. planifolia* ssp. *planifolia* from this shore-line site; however, the structural data from hybrid stem cuttings might explain the variation in the data sets for VE density and VE length. If the latter is true, additional morphological characters need to be evaluated to properly identify the subspecies of *S. planifolia* at the shore of Thomson Bay.

Taxonomic implications—The similarities of VE character data within endemic-progenitor pairs illustrate the close taxonomic relationships between the Lake Athabasca sand dune endemics and their widespread boreal or arctic putative progenitors. However, there were some notable differences among endemic-progenitor pairs. *Salix brachycarpa* var. *psammophila* and its progenitor had the highest VE densities of all willows in this study. In our opinion, this is most likely indicative of the taxonomic position of the *S. brachycarpa* endemic-progenitor pair. In a recent classification of New World *Salix*, Argus (1999) divided *Salix* into four subgenera. *Salix brachycarpa* var. *psammophila* and var. *brachycarpa* were placed in *Salix* subgenus *Chamaetia* (Dumort.) Nasarov, while the other three endemic-progenitor pairs were placed in *Salix* subgenus *Vetrix* (Dumort.) Dumort (Argus, 1999). We believe that little to no detectable variation in VE characters reflects the close taxonomic relationships within endemic-progenitor pairs. Furthermore, internal structural similarities support the putative evolutionary relationship between derived, endemic willows from the Athabasca sand dunes and their widespread progenitors.

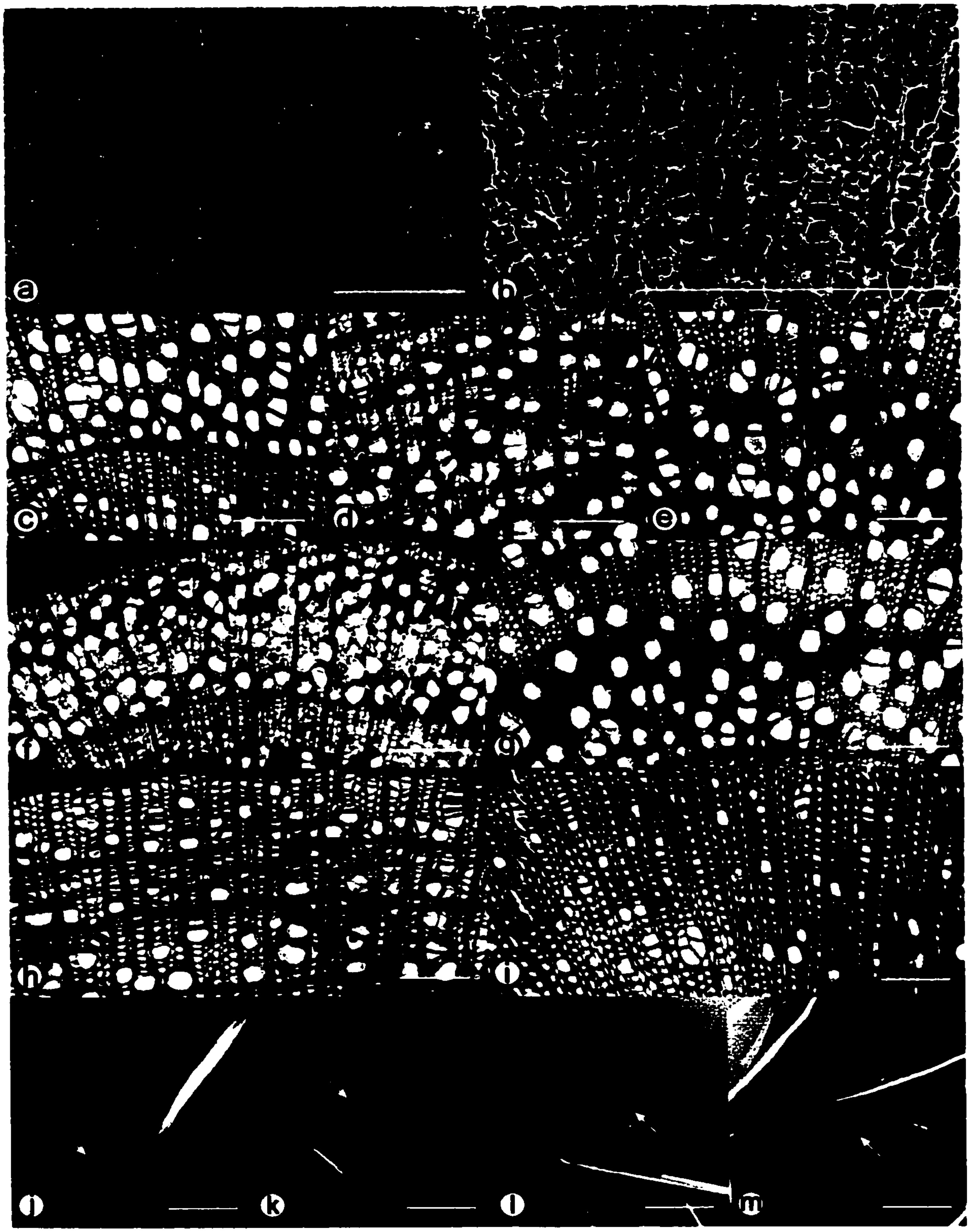
TABLE 4-1. Vessel element (VE) characters for the Athabasca sand dune endemic *Salix* species and their putative progenitors.

Taxon	# VE/mm^2		VE lumen diameter (μm)		# VE/group		VE length (μm)	
	n	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD
S. brachycarpa var. *psammophila*	15	1119 ± 390	55	19.56 ± 4.56	15	1.53 ± 0.17	126	278.16 ± 86.77***
S. brachycarpa var. *brachycarpa*	24	1011 ± 187	137	18.89 ± 4.49	24	1.47 ± 0.17	125	215.65 ± 48.91
S. planifolia ssp. *tyrrellii*	18	605 ± 84	82	22.05 ± 4.99^{a}	18	1.47 ± 0.14	125	278.97 ± 53.64
S. planifolia ssp. *planifolia* (T-Bay)	10	791 ± 288	60	22.77 ± 5.04^{a}	10	1.40 ± 0.14	125	273.15 ± 68.51
S. planifolia ssp. *planifolia*	25	691 ± 136	126	26.03 ± 5.20b	25	1.35 ± 0.14	125	278.97 ± 42.98
S. silicicola	14	553 ± 134	77	22.86 ± 5.34	14	1.36 ± 0.14	125	241.36 ± 68.10
S. alaxensis	10	770 ± 274	63	23.41 ± 7.00	10	1.69 ± 0.32***	125	237.12 ± 49.42
S. turnorii	11	684 ± 175	110	23.80 ± 6.05***	11	1.35 ± 0.14	125	277.14 ± 54.89***
S. lutea	10	837 ± 120	65	20.24 ± 4.41	10	1.32 ± 0.13	126	226.57 ± 55.28

T-Bay = Thomson Bay dune shore; Results of ANOVA: *** = $P < 0.001$ for comparison within endemic-progenitor pairs.

Note: For VE lumen diameter in the *S. planifolia* group, different letters indicate significant differences (post-hoc test after ANOVA).

FIGURE 4-1. Scanning electron and light micrographs of vessel element (VE) characters in *Salix* species. **a.** SEM of stem cross-section of *S. brachycarpa* var. *brachycarpa* depicting VE density. **b.** SEM of stem cross-section of *S. brachycarpa* var. *psammophila* depicting VE density. c-g. Light micrographs of stem cross-sections comparing VE lumen diameter of early wood vessels in the most recent growth increment. **c.** *S. planifolia* ssp. *planifolia*. **d.** *S. planifolia* ssp. *planifolia* from Thomson Bay dune shore. **e.** *S. planifolia* ssp. *tyrrellii*. **f.** *S. lutea*. **g.** *S. turnorii*. h-i. Light micrographs of stem cross-sections comparing VE clustering. **h.** *S. alaxensis*. **i.** *S. silicicola*. j-m. Light micrographs of wood macerations using DIC to compare VE length. **j.** *S. brachycarpa* var. *brachycarpa*. **k.** *S. brachycarpa* var. *psammophila*. **l.** *S. lutea*. **m.** *S. turnorii*. Bars = 100 μm.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CHAPTER 5: A STRUCTURAL STUDY OF *SALIX PLANIFOLIA* SSP. *TYRRELLII* FROM THE ATHABASCA SAND DUNES OF NORTHERN ALBERTA[1]

INTRODUCTION

The Athabasca sand dunes are located on the south shore of Lake Athabasca in northern Alberta and Saskatchewan (Figure 1-1). These dunes are the largest northern dunes and are noted for their desert-like topography, gravel pavements and large open sand areas (Smith, 1978). The dunes were formed after deglaciation approximately 9,000 years ago and vegetated soon thereafter (Raup and Argus, 1982). Of the 40 plant taxa on the sand dunes, ten are endemic, including four willows (Salicaceae).

The high number of endemic plant taxa and recent origin of the sand dunes suggest rapid evolution (Hermesh, 1972). Recent studies have provided support for speciation in some Athabasca sand dune endemics by examining their levels of genetic diversity (*Stellaria arenicola*, Purdy et al., 1994; *Salix silicicola*, Purdy and Bayer, 1995a; *Deschampsia mackenzieana*, Purdy and Bayer, 1995b; *Achillea millefolium* ssp. *megacephala*, Purdy and Bayer, 1996). These investigations evaluated endemics only from the Saskatchewan dunes.

This particular study is part of a larger program in which I have examined structural adaptations of willows (*Salix*; Salicaceae) endemic to the Athabasca sand dunes. Characters that have been examined include stomatal frequency, trichome density, cuticle thickness, epicuticular wax morphology, and vessel elements. Data have been compared among the four endemic *Salix* taxa (*S. brachycarpa* var. *psammophila*, *S. planifolia* ssp. *tyrrellii*, *S. silicicola*, and *S. turnorii*) and their respective widespread putative progenitors (*S. brachycarpa* var. *brachycarpa*, *S. planifolia* ssp. *planifolia*, *S. alaxensis*, and *S. lutea*) to address and better understand adaptation to the open sand habitat.

There have been no systematic botanical surveys of the Athabasca dunes in Alberta; however, an earlier study evaluated the potential recreational usage for the dunes and surrounding areas (Landals, 1978) who noted that the plant life on the Alberta dunes had yet to be studied. The two Athabasca dunes in northeastern Alberta are the Maybelle

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[1] Data from this chapter were provided in a supplemental report to the Natural Resources Service of the Alberta government. 2001.

River and Richardson Dunes. In 1998, both dune areas were designated as wildland parks. The Athabasca Dune Ecological Reserve lies within the boundaries of the Maybelle River Wildland Park, but the Richardson Dune is not protected.

The endemic willow, *S. planifolia* ssp. *tyrrellii*, has been reported for Alberta, but no documentation of specimens had been compiled by the Alberta Natural Heritage Information Centre prior to June 2000 (J. Gould, personal communication). I had the unique opportunity to examine the Athabasca sand dune vegetation in Alberta for the first time as a volunteer with the Natural Resources Service of the Alberta government. Formal identification and an evaluation of diagnostic structural features (i.e., adaxial stomatal frequency) of *S. planifolia* from the Alberta dunes confirmed that the populations of the endemic *S. planifolia* ssp. *tyrrellii* were present on both dunes.

This is the first study evaluating structural variation of *S. planifolia* ssp. *tyrrellii* from the Maybelle River and Richardson Dunes in northeastern Alberta. In addition, structural data were compared for this endemic taxon between specimens from the Athabasca sand dunes in Alberta and Saskatchwen. Structural characters examined in this study include stomatal frequency of both leaf (adaxial (AD) and abaxial (AB)) surfaces, as well as the VE features of density, lumen diameter, clustering, and length. The VE characters evaluated here are those most associated with water conduction (Carlquist, 1988).

This investigation will provide further understanding of adaptation of *S. planifolia* ssp. *tyrrellii* to this boreal, sand dune environment. In Saskatchewan, many of the Athabasca sand dune endemics are considered rare by the Saskatchewan Conservation Data Centre. Furthermore, the documentation of *S. planifolia* ssp. *tyrrellii* from the Alberta dunes can be filed to formally verify its status so that recommendations can be made for conservation and protection of this rare Alberta species.

MATERIALS AND METHODS

Stem cuttings of *Salix planifolia* ssp. *tyrrellii* were collected in June 2000 from the Maybelle River and Richardson dunes in northeastern Alberta (Figure 5-1). Five cuttings were collected from individual plants from each of two sites on the Maybelle River dune, and 10 cuttings were collected from individual plants from one site on the Richardson dune.

Stomatal frequency—Scanning electron microscopy (SEM) was used to observe leaf surfaces to determine the number of stomata per mm^2 (or stomatal frequency). Leaves were prepared for SEM using the methods outlined in this thesis (Cooper, Thesis Ch. 2, 2001). Leaves (~ 1.5 cm X 0.75 cm) were dissected from stem cuttings. Ten leaves from each of the three sites were used, and AD and AB stomatal frequencies were generated for each leaf. Stomata were counted from SEM micrographs for 5 fields of view per leaf surface, and these data were averaged per leaf. Stomatal frequency data from the two Maybelle River Dune sites were combined. Data sets of stomatal frequency from Maybelle River Dune and Richardson Dune were compared using *t*-tests. Adaxial and AB leaf surfaces were analyzed separately.

Vessel element (VE) characters—Both SEM and light microscopy (LM) were used to examine VE characters. Five stem cuttings were taken from two sites on Maybelle River Dune, and 10 cuttings were taken from one site on the Richardson Dune. Stems were prepared for SEM and LM as described by Cooper and Cass (2001). Density (#VE/mm^2) and VE clustering (#VE/group) were obtained from electron micrographs, and 3 fields of view per micrograph were analyzed; averages were calculated per stem.

Light microscopy was used to measure VE lumen diameter and VE length. Lumen diameters were determined from early wood vessels in the most recent growth increment of 5 stems of *S. planifolia* ssp. *tyrrellii* from each Alberta dune site; measurements were taken at the widest region of the lumen. Lumen diameters were averaged for each site.

Measurements of VE length were obtained using Jeffrey's method (Johansen, 1940) for wood macerations. Sections of each stem were macerated, and at least 25 vessel elements were measured per stem. Length values were averaged for each site.

Data sets from the two Maybelle River Dune sites were combined for each VE character. Then, VE character data were compared separately between specimens of *S. planifolia* ssp. *tyrrellii* from each Alberta dune using *t*-tests.

Comparison of* S. planifolia *ssp.* tyrrellii *from Athabasca sand dunes of Alberta and Saskatchewan—Structural data (for AD and AB stomatal frequencies and VE characters) for *S. planifolia* ssp. *tyrrellii* of each Alberta dune were combined. Then, the

Alberta data sets were compared to the *S. planifolia* ssp. *tyrrellii* data sets from Saskatchewan (Cooper, Thesis Ch. 2, 2001; Cooper and Cass, 2001) using *t*-tests.

RESULTS

Table 5-1 shows the stomatal frequency and VE characters for specimens of *Salix planifolia* ssp. *tyrrellii* from the Athabasca sand dunes in northern Alberta. Stomatal frequencies for this willow were similar for both AD and AB leaf surfaces. Specimens from Maybelle River Dune had an AD stomatal frequency value that was slightly greater than the value for the Richardson dune, while the AB frequency was slightly lower.

Overall, values of each VE character for specimens of *S. planifolia* ssp. *tyrrellii* from each Alberta dune were similar (Table 5-1). There was little variation between VE density and VE clustering for specimens from each Alberta dune (Table 5-1), but specimens from Maybelle River Dune had a greater VE density and less clustering. Lumen diameter was significantly greater for specimens from the Maybelle River Dune; VE length was significantly greater in the Richardson Dune specimens (Table 5-1).

Stomatal frequencies and VE character values are presented for *S. planifolia* ssp. *tyrrellii* of the Athabasca sand dunes in Alberta and Saskatchewan (Table 5-2). Alberta dunes specimens had significantly greater AD and AB stomatal frequencies than specimens from Saskatchewan (Table 5-2). The Saskatchewan specimens had a significantly greater VE density and VE length, while VE clustering values were similar between specimens from each provincial site (Table 5-2). In specimens from the Alberta dunes, VE lumen diameter was significantly greater than specimens from Saskatchewan (Table 5-2).

DISCUSSION

The structural data presented in this study for *Salix planifolia* ssp. *tyrrellii* have been provided to compliment a report on rare vascular plants in northeastern Alberta. In addition, this particular endemic has now been studied from four of the Athabasca sand dunes. The structural information for *S. planifolia* ssp. *tyrrellii* from dune fields in both Alberta and Saskatchewan has given insight into the natural variation for stomatal frequency and VE characters (Tables 5-1; 5-2). It is possible that data differences support levels of genetic isolation of each of these populations of *S. planifolia* ssp. *tyrrellii*.

Specimens of *S. planifolia* ssp. *tyrrellii* from both Athabasca dunes in Alberta have amphistomatic leaves (Table 5-1). Variation among AD and AB stomatal frequencies in specimens from each Alberta dune may be due to slight differences in leaf development. The greater AD and AB stomatal frequencies in specimens from Alberta, as compared to *S. planifolia* ssp. *tyrrellii* from Saskatchewan (Table 5-2), suggest habitat or environmental differences between dunes of each province.

Morphologically, *S. planifolia* ssp. *tyrrellii* is quite similar to its widespread putative progenitor, *S. planifolia* ssp. *planifolia* (Argus and Steele, 1979). However, the amphistomatic leaves of *S. planifolia* ssp. *tyrrellii* have been shown to be a useful diagnostic feature in distinguishing it from its putative progenitor (Argus and Steele, 1979; personal observation). Amphistomatic leaves have been described as being adaptive to high light regimes (e.g., Mott and Michaelson, 1991), and the Athabasca open sand habitat is known for high insolation (Macdonald, et al., 1987). Amphistomatic leaves are likely an important adaptation of *S. planifolia* ssp. *tyrrellii* to high light intensity of this northern, open sand environment.

Little variation occurred among VE characters for specimens of *S. planifolia* ssp. *tyrrellii* from the Athabasca sand dunes of northern Alberta (Table 5-1). However, between specimens of Alberta and Saskatchewan, it appears that specimens of *S. planifolia* ssp. *tyrrellii* from the dunes in Saskatchewan have significantly greater VE densities and VE lengths, while Alberta specimens have significantly greater lumen diameters (Table 5-2). These results may represent natural variation for this rare willow, differences in environmental and/or ecological conditions among dune fields in each province, or differences may be correlated with genetic isolation of these populations.

Based on previously published data for the widespread *S. planifolia* ssp. *planifolia*, lumen diameters may differ significantly between this widespread putative progenitor (26.03 µm ± 5.20; Cooper and Cass, 2001) and the endemic *S. planifolia* ssp. *tyrrellii* from the Athabasca dunes in Alberta (23.83 µm ± 6.30; Table 5-2). If the open sand habitat, either above- or below-ground, differs ecologically, a smaller lumen diameter may be more adaptive for water conduction in *S. planifolia* ssp. *tyrrellii* in the Alberta sand dune environment. Shorter VE length may be another adaptation to the open sand habitat of the Alberta dunes for this willow, suggesting that water availability may be lower here. Environmental data, combined with ecological studies, from the Athabasca dune fields in both Alberta and Saskatchewan, would further help to understand the range of variation that exists among specimens of *S. planifolia* ssp. *tyrrellii.*

On a conservation note, *S. planifolia* ssp. *tyrrellii* is only known in Alberta from the Maybelle River and Richardson Dunes. During the June 2000 survey, another endemic herb (*Stellaria arenicola*) from the Saskatchewan dunes was documented on both Athabasca dunes in Alberta (J. Gould, personal communication). Thus, there are now reports of two rare plants endemic to the Athabasca sand dunes in northeastern Alberta. The Maybelle River Dune is protected as an ecological reserve, while the Richardson Dune is not. It is hoped that the presence of two rare Alberta plant species on the Richardson Dune may promote legislation to protect and conserve this delicate habitat.

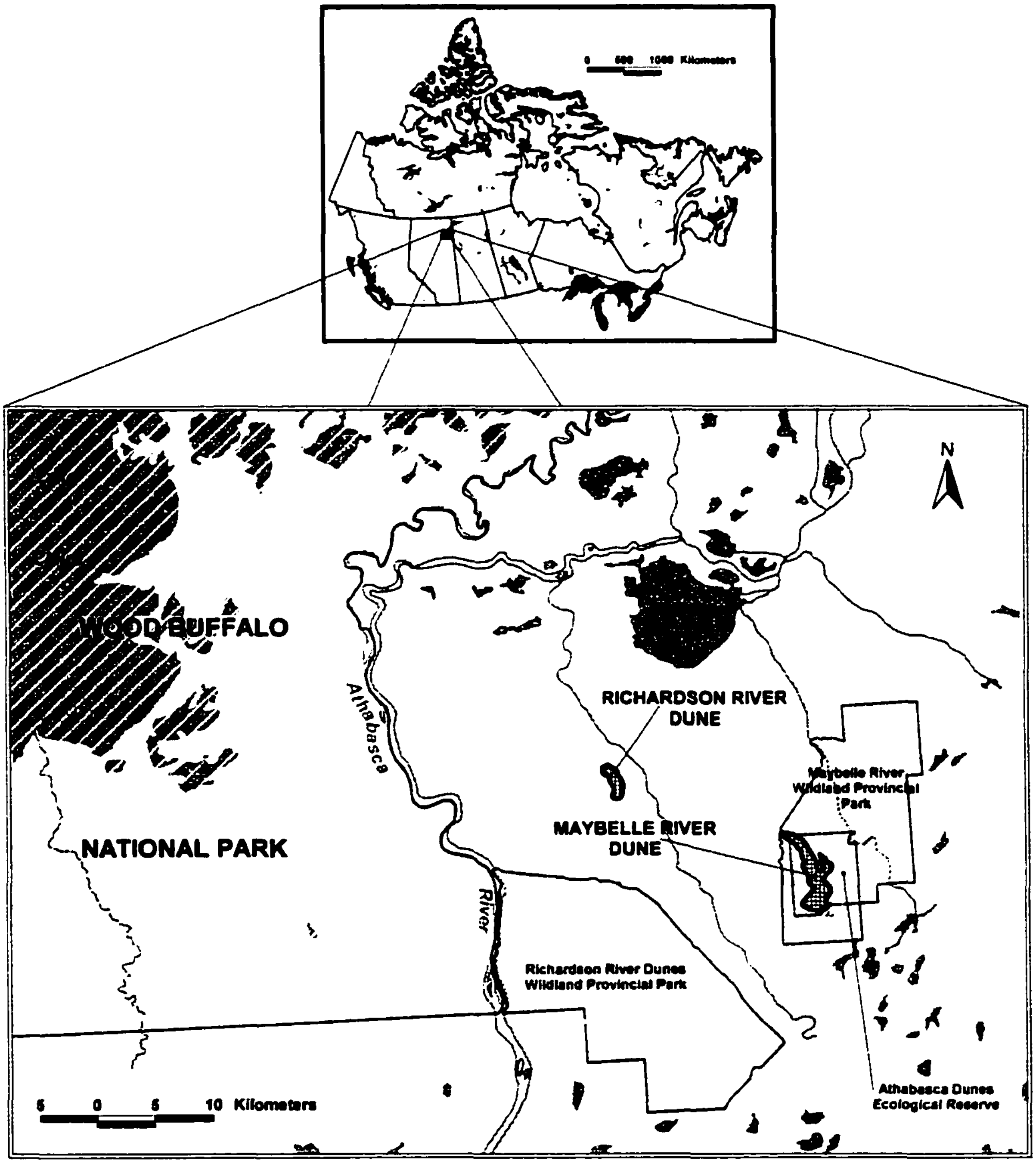

FIGURE 5-1. Map of the Athabasca sand dunes in northeastern Alberta. (Map courtesy of Drajs Vujnovic.)

TABLE 5-1. Stomatal frequency and vessel element (VE) characters for *Salix planifolia* ssp. *tyrrellii* from Athabasca sand dunes in Alberta.

Site	**Stomatal frequency (#/mm²)**				**VE characters**							
	Adaxial		**Abaxial**		**#VE/mm²**		**Lumen diameter (μm)**		**#VE/group**		**Length (μm)**	
	n	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD
Maybelle River Dune	20	159 ± 47	20	331 ± 42	10	576 ± 130	173	24.79 ± 6.79**	10	1.44 ± 0.16	297	226.36 ± 42.47
Richardson Dune	10	146 ± 39	10	343 ± 53	10	502 ± 83	160	22.79 ± 5.55	10	1.50 ± 0.29	286	233.30 ± 46.72*

Results of *t*-tests: ** = $P < 0.01$; * = $P < 0.05$.

TABLE 5-2. Stomatal frequency and vessel element (VE) characters for *Salix planifolia* ssp. *tyrrellii* from Athabasca sand dunes.

Site	Stomatal frequency (#/mm^2)				VE characters							
	Adaxial		Abaxial		#VE/mm^2		Lumen diameter (μm)		#VE/group		Length (μm)	
	n	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD
Alberta	30	154 ± 44*	30	335 ± 45***	20	539 ± 113	333	23.83 ± 6.30**	20	1.47 ± 0.23	583	229.06 ± 44.76
Saskatchewan	24	126 ± 36	22	228 ± 68	18	605 ± 84*	82	22.05 ± 4.99	18	1.47 ± 0.14	125	278.97 ± 53.64***

Results of *t*-tests: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$.

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CHAPTER 6: MOLECULAR ANALYSIS OF THE TAXONOMIC RELATIONSHIP OF ENDEMIC *SALIX* SPECIES AND THEIR ASSOCIATED PUTATIVE PROGENITORS

INTRODUCTION

The willow family, Salicaceae, consists of approximately 400 described species. Salicaceae has been classically divided into two genera, *Salix* (willow) and *Populus* (aspen, cottonwood, and poplar). Dorn (1976) presented a synopsis of American *Salix* by evaluating over 50 characteristics, including both morphological characters and habitat information; he recognized less than 20 sections of *Salix*. Argus (1999) suggested a classification of New World *Salix* taxa, as he examined 90 morphological characters in 103 species and classified 28 sections. Argus (1999) proposed that the New World species be divided into four subgenera, *Salix*, *Longifoliae* (Andersson) Argus, *Chamaetia* (Dumort.) Nasarov, and *Vetrix* (Dumort.) Dumort.

Salix taxa are trees or shrubs; willow trees generally have a subtropical distribution, while shrubs are usually found in boreal and temperate regions (Dorn, 1976). Four willow shrubs (*S. brachycarpa* var. *psammophila*, *S. planifolia* ssp. *tyrrellii*, *S. silicicola*, and *S. turnorii*) are considered to be endemic to the Athabasca sand dunes of northern Saskatchewan, Canada.

Salix brachycarpa belongs to the subgenus *Chamaetia*, while the other three endemic willows have been placed in the subgenus *Vetrix* (Argus, 1999); these taxonomic designations were supported by comparison of several morphological characters. The Athabasca sand dune endemic willows share many similarities with taxa that are their widespread presumed progenitors (Raup, 1936; Argus and Steele, 1979). However, endemic *Salix* taxa primarily occupy dune interiors, and they exhibit structural adaptations to this high light intensity environment. Endemic willows have thicker leaf cuticles than their closely-related progenitors and a dense crust of epicuticular wax on their abaxial leaf surfaces. The two tomentose endemic willows, *S. brachycarpa* var. *psammophila* and *S. silicicola*, have significantly greater trichome densities on both leaf surfaces, as compared to their putative progenitors. In addition, Cooper and Cass (2001) suggested that endemic *Salix* species have vessel element characters that might function for more efficient water transport in this environment. Leaf epidermal characters, including adaxial (AD) stomatal distributions, suggest adaptation to high insolation of the inner dune habitat; two of the four endemic *Salix* taxa have amphistomatic leaves.

The amphistomatic character has proven to be useful in distinguishing *Salix planifolia* ssp. *tyrrellii* from its sympatric and putative progenitor, *S. planifolia* ssp. *planifolia*, from Thomson Bay (R. L. Cooper, personal observation). At this shore-line site on the Athabasca sand dunes, these two taxa co-occur. Although the Thomson Bay progenitor is also amphistomatic, AD stomata are present in a greater frequency in the endemic. Other features of the endemic *S. planifolia* ssp. *tyrrellii* that separate it from its putative progenitor include narrower, thicker leaves and a prolonged juvenile growth habit (Argus and Steele, 1979). These characters may be useful for interpreting the taxonomic status of this putative endemic-progenitor *Salix* pair.

Molecular techniques have been used to examine genetic relationships among numerous plant taxa. One study addressed levels of genetic variation between the Athabasca sand dune endemic, *Salix silicicola*, and its widespread putative progenitor (Purdy and Bayer, 1995). To my knowledge, the other three endemic-progenitor *Salix* pairs have not been studied using molecular techniques. Structural investigations (Cooper, Thesis Ch. 2, 3, and 5, 2001; Cooper and Cass, 2001) of the endemic-progenitor *Salix* pairs support the close evolutionary relationship in each species pair; additional molecular data would help to clarify these putative taxonomic affinities.

I concentrated on the taxonomic relationship of *Salix planifolia* ssp. *tyrrellii* and its putative widespread progenitor because more morphological information is available for this species pair (Argus and Steele, 1979). A rapidly evolving region of the chloroplast genome, *trn*LF (Taberlet et al., 1991), was chosen as the genetic marker for this study. The *trn*LF region consists of the *trn*L gene, its intron, an intergenic spacer between *trn*L and *trn*F, and the *trn*F gene (Taberlet et al., 1991; Figure 6-1).

First, I needed to determine if the *trn*LF region exhibited enough variation to assess interspecific relationships in *Salix*. Three *Salix* taxa (*S. alaxensis*, *S. planifolia* ssp. *tyrrellii*, and *S. turnorii*) were selected from different sections (*Villosae* (Anderson) Rouy, *Phylicifoliae* (Fries) Andersson, and *Cordatae* Barratt ex. Hook, respectively) of the genus, according to Argus (1999); *Populus tremuloides* Michaux was chosen as an outgroup. Preliminary analyses of these four species will provide an idea of the amount of genetic variation that can be determined from the *trn*LF region.

MATERIALS AND METHODS

***Plant material*—**The plant material for this study was collected from sites in Alberta and Saskatchewan. Voucher specimens are deposited in the University of Alberta Vascular Plant Herbarium (ALTA). Species information is presented in Table 6-1.

***DNA extraction and analysis*—**Fresh leaf tissue was silica-dried for each specimen, and DNA was extracted with a similar protocol as described by Doyle and Doyle (1987). The *trn*LF region was amplified with primers "c" and "f" (Taberlet et al., 1991) using the polymerase chain reaction (PCR). The PCR profile consisted of: (1) an initial denaturation step of 5 min at 94°C, (2) 30 cycles of 30 sec at 94°C, 30 sec at 45°C (primer annealment), 1 min at 72°C (primer extension), and (3) a final extension step of 7 min at 72°C. PCR products were purified on QiaQuick PCR columns, followed by double-stranded sequencing of the purified products using "c," "d," "e," and "f." Sequencing reactions were done using 25 cycles of 20 sec at 95°C, 15 sec at 45°C, and 2 min at 60°C. A Perkin-Elmer GeneAmp PCR System 9700 thermocycler was used for PCR amplification and sequencing reactions.

Chromatograms were compiled using Sequencher 4.1 (Gene Codes Corporation, 2000). Alignments were generated manually using Se-Al version 1.0 alpha (Rambaut, 1998) using criteria described in Graham et al. (2000).

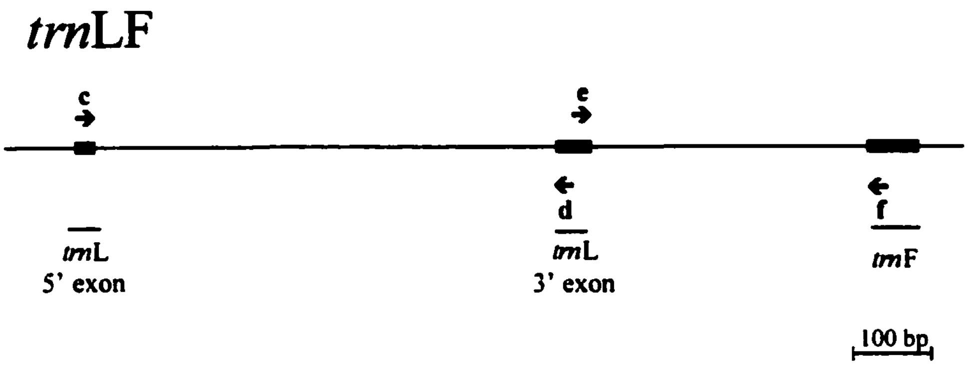
RESULTS AND DISCUSSION

Sequence data matrices are presented in Appendix 1. A total of 12 substitutions and 11 insertion/deletions (or 'indels') were detectable among these salicaceous taxa using the *trn*LF region of the chloroplast genome (Appendix 1). Little variation was shown among *Salix* taxa, as only two indels were present (Appendix 1). However, more variation was evident between *Populus* and *Salix* sequences with 12 substitutions and nine indels (Appendix 1).

This is the first report of *trn*LF sequence data for *Salix* (Appendix 1). Minimal variation was shown for the three *Salix* taxa in this preliminary study, although it is possible that the *Salix* species selected for this study are not variable across *Salix*. However, the *trn*LF region should be examined in additional *Salix* taxa to clarify levels of variation (or lack thereof) within the genus. Additional examinations using the *trn*LF region may confirm the lack of utility of this marker for assessing intrageneric relationships in *Salix*.

If no detectable genetic variation within *Salix* results from further studies with the *trn*LF region, it will be necessary to use another molecular approach. Azuma et al.(2000) used another chloroplast region, the *rbc*L gene, to investigate phylogenetic relationships in *Salix*; they commented that genes with higher rates of sequence evolution should be used to evaluate these relationships. Moreover, the present preliminary molecular investigation using the *trn*LF region revealed potential usefulness of this marker for detecting intergeneric relationships in Salicaceae. Amplified Fragment Length Polymorphism (AFLP) analysis (Vos et al., 1995) may provide enough genetic variation to more closely evaluate taxonomic affinities within *Salix*.

AFLP analyses were useful in other taxonomic and genetic assessments of *Salix* taxa. Morphological data were used to identify two *Salix* species and their hybrid, and AFLP analysis confirmed some of the observations, as well as clarified prior identification of four separate specimens as one *Salix* clone (Beismann et al., 1997 and references therein). Barker et al. (1999) used AFLP analysis combined with random amplified polymorphic DNA (RAPD) markers to assess genetic diversity of several *Salix* clones. Based on their success (Beismann et al., 1997; Barker et al., 1999), I plan to use AFLP analyses to re-address the variation among the salicaceous taxa in this study. If enough variation is detected within *Salix* using AFLP techniques, I will then attempt to elucidate the putative *S. planifolia* endemic-progenitor relationship with this approach. Subsequently, my goal is to provide molecular data to more clearly understand the taxonomic status of each endemic-progenitor *Salix* pair.



Forward	Reverse
C CGAAATCGGTAGACGCTACG	D GGGGATAGAGGGACTTGAAC
E GGTTCAAGTCCCTCTATCCC	F ATTTGAACTGGTGACACGAG

FIGURE 6-1. Map and sequences of primers used to amplify and sequence *trn*L and *trn*F in the large single copy region of the chloroplast genome in salicaceous taxa. (Figure courtesy of Andrea L. Case.)

TABLE 6-1. Plant material used in this study.

Taxon	**Voucher**	**Collection site**
Populus tremuloides	RLCOOPER00012	UA greenhouses
Salix alaxensis	RLCOOPER00007	Cardinal Divide, Alberta
*S. planifolia ssp. tyrrellii**	RLCOOPER00003	Yakow Lake dune; ASD
*S. turnorii**	RLCOOPER00008	Yakow Lake dune; ASD

UA = University of Alberta; * = Athabasca sand dune endemic; ASD = Athabasca sand dunes

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CHAPTER 7: DISCUSSION AND CONCLUSIONS

The Athabasca sand dune habitat provided a unique opportunity to investigate structural adaptation of the four endemic *Salix* species. Several structural characters of endemic willows were examined using LM and SEM. Certain structural features of the endemic willows (*S. brachycarpa* var. *psammophila*, *S. planifolia* ssp. *tyrrellii*, *S. silicicola*, and *S. turnorii*) were different from those of their putative progenitors (*S. brachycarpa* var. *brachycarpa*, *S. planifolia* ssp. *planifolia*, *S. alaxensis*, and *S. turnorii*), respectively.

Leaf epidermis data, including adaxial (AD) stomatal distributions, increased trichome densities, greater cuticle thicknesses, and epicuticular waxes, suggested that endemic *Salix* species have features that are adaptive to a high light intensity environment. For example, the endemic *S. turnorii*, had amphistomatic leaves, as well as a persistent epicuticular wax (Cooper, Thesis Ch. 2 and 3, 2001). Amphistomatic leaves have been shown to be an adaptation to high light regimes (e.g., Parkhurst, 1978; Mott and Michaelson, 1991), and wax deposits can protect against excessive solar insolation (Martin and Juniper, 1970).

Trichomes scatter and reflect light, and dense trichome coverings are likely to be advantageous in exposed environments. *Salix brachycarpa* var. *psammophila* and *S. silicicola* had significantly greater AD and AB trichome densities than their putative progenitors (Cooper, Thesis Ch. 2, 2001). Karabourniotis and Borman (1999) showed that greater trichome densities can effectively reflect incident light, Liakoura, et al. (1997) reported that trichomes can protect against UV-B radiation. Denser trichome coverings could provide similar types of protection for these two endemic *Salix* species.

Evaluation of internal stem anatomy, and in particular, vessel element (VE) characters provided information that can help to understand water transport in this boreal, sand dune habitat. The open sand environment appears to be xeric; however, willows are rooted in the water table. I wanted to determine whether VE characters were more associated with a mesic or xeric habitat by examining VE density, VE lumen diameter, VE clustering, and VE length within each endemic-progenitor *Salix* pair.

No differences were found within species pairs for VE density, while other VE characters showed considerable variation among *Salix* pairs (Cooper and Cass, 2001). It might be expected that endemic willows would have narrower VEs than their progenitors due to their sandy, low-nutrient environment; yet, only two of the four endemics had narrower VEs. The endemic *S. turnorii* had a significantly greater lumen diameter than its progenitor, and thus, for this particular willow, a wider lumen may be adaptive for more efficient water transport. Furthermore, VE similarities within each *Salix* species pair suggest a close evolutionary relationship.

Taxonomic affinities of each endemic-progenitor *Salix* pair are commonly accepted (e.g., Argus and Steele, 1979; Purdy and Bayer, 1995). My studies have shown that structural data support the close relationships within each pair, while distinct differences exhibited by endemic willows are most likely adaptive to the open sand habitat. I am pursuing a molecular study to better understand the close taxonomic association in each *Salix* pair, and I will do AFLP analysis to further investigate these relationships (Cooper, Thesis Ch. 6, 2001). I hope to be able to interpret AFLP results to evaluate associations between endemic *Salix* taxa and their presumed progenitors. Furthermore, I will discuss observations that deserve additional attention, including the occurrence of *S. planifolia* ssp. *tyrrellii* in Alberta, as well as new considerations for two Athabasca sand dune willow endemics. I will conclude with a brief statement of ideas for future study of the Athabasca sand dune *Salix* species.

Salix planifolia ***ssp.*** **tyrrellii** ***in Alberta***—I was able to pursue a structural study of *Salix planifolia* ssp. *tyrrellii* from the Maybelle River and Richardson Dunes in Alberta and compare findings with its populations from the Saskatchewan dunes. As well, one specimen of the endemic *S. turnorii* may have been identified from the Alberta dunes; however, due to the young age of the stem and the absence of reproductive characters, a definitive identification was not possible.

The presence of *S. planifolia* ssp. *tyrrellii* and another endemic herb known only from the Athabasca sand dunes in Saskatchewan allows for attention to be drawn to this Alberta habitat. I hope that conservation efforts will be implemented to preserve the habitat for these rare plants. The recent survey of the Richardson Dune not only revealed the presence of rare endemics, but landscape damage due to destructive use of off-highway vehicles on the dunes (R. L. Cooper, personal observation). I feel that it is important to implement a protected status to the Richardson Dune as soon as policy can become activated.

***New considerations for* Salix planifolia *ssp.* tyrrellii *and* S. silicicola**—Recently, it has been suggested that *Salix planifolia* ssp. *tyrrellii* and *S. silicicola* may have a geographic range outside the Athabasca sand dunes. George Argus (personal communication) has examined herbarium specimens of these two taxa, and he concluded that each of these species occurs in the Pelly Lake region of Nunavut (the former District of Keewatin).

As only herbarium specimens for each of these species have been examined, it is important to consider habitat characteristics, as well as relative abundance of these taxa. Molecular techniques may be effective in addressing this taxonomic examination. Combining structural and molecular data could potentially clarify any uncertainty that may exist regarding these recent observations for *S. planifolia* ssp. *tyrrellii* and *S. silicicola.*

Moreover, multiple specimens of *S. planifolia* ssp. *tyrrellii* from the Pelly Lake region of Nunavut indicate that this species is not restricted to the Athabasca sand dunes, and it should not be subsequently referred to as an endemic (G. Argus, personal communication). Fewer data (i.e., descriptions of a single specimen) are available for *S. silicicola*; thus, consideration for this species as an endemic is discretionary at this time.

Future investigations—This structural study provides comparative data that are useful in implementing ecological, experimental, and molecular studies. Personally, I have gained a broader background in structural botany, as well as developed new interests in plant chemistry, ecology, conservation, and molecular systematics. I plan to pursue a more extensive examination of the functional significance of amphistomatic leaves, and the current molecular study of the endemic *Salix* species and their putative progenitors will be on going. In addition, I have been asked by Steven Brunsfeld (University of Idaho) to participate in a collaborative effort that involves characterization of the chloroplast genome to study introgression in the endemic *S. turnorii*, along with the other sand dune taxa in section *Cordatae*. Many of the observations and ideas presented in this thesis warrant further study to better understand the interactions that exist between the Athabasca sand dune willows and their open sand habitat.

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APPENDIX 1. Aligned Salicaceae sequences for the *trn*LF region.

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[                   10        20        30        40        50        60        70        80        90       100       110     ]
[                    .         .         .         .         .         .         .         .         .         .         .     ]

Salix.turn  -AACTTACTAAGTGATAACTTTCAAATTCAGAGAAACCCCGGAAAAAAAAA--GGGGCAATCCTGAGCCAAATCCTATTTTTCGAAAACAAA------------CAAAGGTTCAT   [100]
Salix.alax  -------------------------------------------------------------------------------------------------------------------   [0]
Salix.plan  ------------------------..........................---.......................................------------...........   [76]
Populus.tr  A..................................................AG.............?.........................CAAAAAAACAAA...........   [115]

[             120       130       140       150       160       170       180       190       200       210       220       230]
[               .         .         .         .         .         .         .         .         .         .         .         .]

Salix.turn  AAAGACAGAATAAGAATACAAAAGGATAGGTGCAGAGACTCAATGGAAGTTGTTCTAACAAATAGAGTTGACTGCGTTCCATTAGTCAAGTACAAGTAAGGGAATCCTTCTGCTA   [215]
Salix.alax  ---------..........................................................................................................   [106]
Salix.plan  ...................?...............................................................................................   [191]
Populus.tr  ......................................................................................------.......A............T..   [224]

[                  240       250       260       270       280       290       300       310       320       330       340     ]
[                    .         .         .         .         .         .         .         .         .         .         .     ]

Salix.turn  AAGTTAAAATTCCAGAAAAAAAGAAAGGTAAAGTAAAGTATAACCCTATATACA--------------TAATACATAGGCATACGTACTGAAATACTATCTCAAATGATTAATGA   [316]
Salix.alax  ......................................................--------------...............................................   [207]
Salix.plan  ......................................................--------------...............................................   [292]
Populus.tr  .....................-.....T......................A...TAATACATAAAACA................A..............................   [338]

[             350       360       370       380       390       400       410       420       430       440       450       460]
[               .         .         .         .         .         .         .         .         .         .         .         .]

Salix.turn  CAACCGACCCAAATCTGTATTTTTTTCTATGTTATATGAAAAATGAAATAATTAATAATTCAAATA------------TCAAA----------TAATAATAAAAAAAAAAACATT   [409]
Salix.alax  ..................................................................------------.....----------.................-....   [299]
Salix.plan  ..................................................................------------.....----------......................   [385]
Populus.tr  ........T.G............A......-.............T.....................ATAATTAATAAT.....TAATAATTAA..................A-..   [451]

[                  470       480       490       500       510       520       530       540       550       560       570     ]
[                    .         .         .         .         .         .         .         .         .         .         .     ]

Salix.turn  GCTTTGATTTGAATCGATTCCAAGTTGACGAAAGGATCGAATATTCATTGATCAGATCATTCACCCCAGAATCTGCTGATTAATTGGACGAGAGTAAAGATAGAGTCCCATTCTA   [524]
Salix.alax  ...................................................................................................................   [414]
Salix.plan  ...................................................................................................................   [500]
Populus.tr  ...............................G......................................G............................................   [566]
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[                 580       590       600       610       620       630       640       650       660       670       680       690]
[                   .         .         .         .         .         .         .         .         .         .         .         .]

Salix.turn      CATGTCAATATCGACAACAAAGAAATTTATAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAAAAAGGGGCCCACCCGACTCCCTAAT    [639]
Salix.alax      ...................................................................................................?...............    [529]
Salix.plan      ...................................................................?.............?......?..........................    [615]
Populus.tr      .................................----------------------------------------------------------------------------------    [599]
......................................................._______________trnL_exon2__________________________

[                      700       710       720       730       740       750       760       770       780       790       800     ]
[                        .         .         .         .         .         .         .         .         .         .         .     ]

Salix.turn      TGTTTATCTTATTCTCTCTTTTCGTTAACGATTCAAAATTCGTTATCTTTCTCATTTATTTTTCTCATTTATTCGAGTTTTTCACAAATGTATCCGGGCTGCATTTTTTCTTTTC    [754]
Salix.alax      ...................................................................................................................    [644]
Salix.plan      ...................................................................................................................    [730]
Populus.tr      -------------------------------------------------------------------------------------------------------------------    [599]

[                 810       820       830       840       850       860       870       880       890       900       910       920]
[                   .         .         .         .         .         .         .         .         .         .         .         .]

Salix.turn      ATTTCTTTTCACAAGTTTTGTGATAGATAGGATAGACATCCAAACGAACTTCTTTGAGTAAAACAAGGAATCCCTTTTAAAATAAAATTGGAATGATTAACAATGATAAGACTTT    [869]
Salix.alax      ...................................................................................................................    [759]
Salix.plan      ...................................................................................................................    [845]
Populus.tr      -------------------------------------------------------------------------------------------------------------------    [599]

[                      930       940       950       960       970       980       990      1000      1010      1020       ]
[                        .         .         .         .         .         .         .         .         .         .       ]

Salix.turn      AGAATAGCTTTAGAATATCTTTTTTTTTTTATTGACTTTTATTGACATAGACTCAAGTCATTTACTAAAATTAGAAAGAAGGCGCGTCGGAAATGGTCGGGATAGCT    [976]
Salix.alax      ..............................-----------------------------------------------------------------------------    [789]
Salix.plan      ...............................................................................................------------    [940]
Populus.tr      -----------------------------------------------------------------------------------------------------------    [599]
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Note: *trn*L exon 2 is marked on this data matrix. ? = unknown base; • = matching character to top sequence; – = gap (with the exceptions of the start and end of individual regions signifying missing data)