RESEARCH ARTICLE

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Genetic, morphological, and spectral characterization of relictual Niobrara River hybrid aspens (*Populus ×smithii*)[1]

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PREMISE OF THE STUDY: Aspen groves along the Niobrara River in Nebraska have long been a biogeographic curiosity due to morphological differences from nearby remnant *Populus tremuloides* populations. Pleistocene hybridization between *P. tremuloides* and *P. grandidentata* has been proposed, but the nearest *P. grandidentata* populations are currently several hundred kilometers east. We tested the hybrid-origin hypothesis using genetic data and characterized putative hybrids phenotypically.

METHODS: We compared nuclear microsatellite loci and chloroplast sequences of Niobrara River aspens to their putative parental species. Parental species and putative hybrids were also grown in a common garden for phenotypic comparison. On the common garden plants, we measured leaf morphological traits and leaf-level spectral reflectance profiles, from which chemical traits were derived.

KEY RESULTS: The genetic composition of the three unique Niobrara aspen genotypes is consistent with the hybridization hypothesis and with maternal chloroplast inheritance from *P. grandidentata*. Leaf margin dentition and abaxial pubescence differentiated taxa, with the hybrids showing intermediate values. Spectral profiles allowed statistical separation of taxa in short-wave infrared wavelengths, with hybrids showing intermediate values, indicating that traits associated with internal structure of leaves and water absorption may vary among taxa. However, reflectance values in the visible region did not differentiate taxa, indicating that traits related to pigments are not differentiated.

CONCLUSIONS: Both genetic and phenotypic results support the hypothesis of a hybrid origin for these genetically unique aspens. However, low genetic diversity and ongoing ecological and climatic threats to the hybrid taxon present a challenge for conservation of these relictual boreal communities.

KEY WORDS clonal growth; common garden; hybridization; *Populus*; Nebraska; Niobrara River; relictual communities; Salicaceae; Smith's aspen; spectra

Interspecific hybridization is widespread across the flowering plants (Mallet, 2005), often leading to the formation of genetically diverse hybrid zones where interfertile parental populations meet (Barton and Hewitt, 1985). It is increasingly recognized that interspecific hybridization can play an important role in adaptation to changing environments, particularly in long-lived plants (Holliday, 2006). Especially at the range boundaries of one or more species, or in other cases in which intraspecific sexual reproduction may be limited, hybridization can present critical opportunities for adaptive evolution and conservation of threatened stands (Allendorf et al., 2001; Seehausen, 2004; Bridle and Vines, 2007). Lotsy (1925) originally hypothesized the nature and importance of this phenomenon using the term syngameon, referring to lineages capable of adaptive introgression.

The phenotypic outcomes and genetic architecture of hybridization can vary widely, even in separate cases involving the same parental species (Rieseberg, 1991; Steen et al., 2000). Among phenotypic traits, leaf morphology is readily accessible for identifying and classifying species and has long been used for this purpose, but it can be influenced by environmental variation (Klingenberg, 2002; Cristofolini and Crema, 2005; González-Rodríguez and Oyama, 2005; Ives et al., 2007; Viscosi et al., 2009; Hulshof and Swenson,

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2010). Common garden comparisons eliminate uncertainty related to the effect of the environment and allow for comparisons between species based on genetically controlled variation (Rowland, 2001; Givnish and Montgomery, 2014; Zohner and Renner, 2014). Advances in molecular techniques have also made it possible to determine the genetic architecture of hybridization. Characterization of biparentally inherited nuclear genomes and uniparentally inherited chloroplast and mitochondrial genomes can indicate the pattern of hybridization where morphological evidence is unclear or cryptic (Isoda et al., 2000). Depending on the directionality of hybridization, hybrid genomes—and especially cytoplasmically inherited components of the genome like the chloroplast—can be predominantly derived from one parental species (Hamzeh et al., 2007; Hamilton and Aitken, 2013) or relatively equally from both (Hersch-Green et al., 2014; Floate et al., 2016), with commensurate impacts on morphology (Wu et al., 1997; Floate, 2004; Tovar-Sánchez and Oyama, 2004).

Therefore, while phenotypic information can be informative in identifying hybrids, individual traits are rarely sufficient, and the effort to measure dozens of traits individually is intractable for field observations. Spectral characteristics of plants measured across a wide range of wavelengths could potentially provide an integrated means to describe their phenotype, given that anatomical properties of plant cells and tissues have wavelength-specific absorbance, reflectance, and transmittance features (Li et al., 2014). Leaf spectra are also strongly associated with genetic and phylogenetic information, even able to differentiate different populations of the same species (Madritch et al., 2014; Cavender-Bares et al., 2016). Leaf spectra are determined by leaf tissue composition: plant pigments absorb most of the incident light in the visible part of the electromagnetic spectrum (VIS, ca. 400–700 nm), while changes in vibrational and rotational frequencies of larger molecules, such as carbohydrates, proteins, and water cause characteristic absorption features in the near-infrared (NIR, ca. 700–1400 nm) and shortwave infrared (SWIR, ca. 1400–2400 nm) regions of the spectrum. In addition, internal and external structural components of leaves, such as air–cell interfaces, trichomes, and the cuticle, cause absorption and light scattering at these longer wavelengths (Ollinger, 2011). Classification methods based on leaf spectra may thus aid in distinguishing parental species and their hybrids, and also in detecting potential chemical, structural, and/or physiological differences among them. However, seasonal and ontological variation presently make that sort of classification difficult (Cavender-Bares et al., 2017).

Aspens (*Populus* spp.) are dioecious, cosmopolitan, economically important (Balatinecz and Kretschmann, 2001) and frequently dominant species (Campbell and Bartos, 2001; Schweitzer et al., 2004), known to hybridize promiscuously with congeners (Eckenwalder, 1996). Though widely distributed, North American aspens, including bigtooth (*P. grandidentata* Michx.) and quaking (*P. tremuloides* Michx.) aspen, are adapted to cool, and often mesic conditions. Quaking and bigtooth aspen have distinct morphologies (Barnes, 1969), and hybrids between these two species, usually denoted as Smith's aspen (*Populus ×smithii* Boivin), are present in areas where their ranges presently overlap (Pauley, 1956; Barnes, 1961; Barnes and Pregitzer, 1985).

The aspen stands found along the Niobrara River Valley (NRV) in north-central Nebraska have long been hypothesized to be hybrid Smith's aspens based solely on leaf morphological traits (Churchill et al., 1988; Kaul et al., 1988; Eckenwalder, 1996). If this is the case, these hybrids are isolated from the nearest stand of quaking aspen by 60 km and from the western range edge of bigtooth aspen by 650 km. Yet before the eastward range contraction of bigtooth aspen at the end of the Pleistocene, both species were abundant in the Midwestern United States (Wright et al., 1985). As such, the NRV aspens would be not only an unusual Pleistocene relict, but also of special conservation interest since hybrids occurring at the "rear edge" of a retracting parental range (Petit et al., 2005) and/or in proximity to or associated with relatively isolated, peripheral stands of either parent (Leppig and White, 2006) can harbor unique adaptive genetic diversity (Kramer et al., 2008). Despite considerable treatment of aspen phylogenetics (Hamzeh and Dayanandan, 2004; Cervera et al., 2005; Wang et al., 2015) and hybridization (Hamzeh et al., 2007; Hersch-Green et al., 2014; Floate et al., 2016) as well as efforts to produce Smith's aspen artificially as a potential timber cultivar in areas where the parental ranges currently overlap (Reighard and Hanover, 1984; Reighard and Hanover, 1990), to our knowledge, systematic molecular and morphological analyses have yet to demonstrate that NRV aspens are hybrids formed during historical range overlap between *P. grandidentata* and *P. tremuloides* in this region.

Currently, NRV aspen stands appear to suffer from various threats, including decline related to herbivore browsing, disease, and cedar encroachment (Robertson, 2015). Their decline mirrors that of NRV paper birch (*Betula papyrifera*) populations, which have similar physiological tolerances to aspens, and their decline has been attributed to climate change (Stroh and Miller, 2009; Stroh, 2011). Numerous western quaking aspen stands have already collapsed due to a complex combination of physiological stress and infestation by pathogens and insect pests in a phenomenon termed sudden aspen decline (SAD, Rehfeldt et al., 2009; Anderegg et al., 2012; Anderegg et al., 2013; Worrall et al., 2013, 2015), and both quaking and bigtooth aspen are predicted to experience range retraction with further climate change (Iverson and Prasad, 2002; Iverson et al., 2008). The entire range of the NRV aspen population is within the boundaries of the National Park Service's Niobrara National Scenic River unit, and land managers require a more complete understanding of the identity and diversity of the stands to protect and restore them. The preservation of these unique populations—including genetic rescue via enrichment of local genetic diversity (Tallmon et al., 2004) and live, ex situ assisted migration and conservation of genets (Gray et al., 2011)—requires a thorough understanding of threats to the long-term viability of these populations.

In this study, we used molecular genetic analysis, traditional leaf morphological traits, and spectral phenotyping to compare the putative Smith's aspen hybrids of the Niobrara River Valley to genets of the two putative parent species gathered from across the Upper Midwest and also grown in common environments. Our objective was to classify the NRV aspen population by describing the genetic diversity, hybrid status, and ancestry of the Niobrara stand and to compare the leaf morphology and reflectance of the putative parent species and the Niobrara stand. Ultimately, examination of genetic information is critical for determining the hybrid status. Yet, integrative phenotypic information can provide supporting evidence for hybridization and provide insight into the phenotypic consequences of introgression.

We tested four potential alternative hypotheses regarding the identity of these putative hybrids, specifically, whether the Niobrara stand of aspens is (1) a subpopulation of regionally sparse quaking

aspen (*P. tremuloides*), (2) a subpopulation of bigtooth aspen (*P. grandidentata*) that has become geographically isolated from the species' main range, (3) a hybrid of both quaking and bigtooth aspen, or (4) not closely related to either quaking or bigtooth aspen. We used molecular genetic analysis of nuclear microsatellites and chloroplast sequences, integrative phenotypic information using hyperspectral data at the leaf level, and a suite of morphological and physiological traits to test these hypotheses.

MATERIALS AND METHODS

Study system—The Niobrara River Valley in the Sandhill region of northern Nebraska (Fig. 1A), presents a unique opportunity for the study of the consequences of historical hybridization between bigtooth (*Populus grandidentata*) and quaking aspen (*P. tremuloides*) in a former zone of range overlap. As a result of glacial history and biogeography, and in contrast to the surrounding Great Plains, the NRV contains numerous springbranch canyons, shaded, mesic ravines formed by springs and tributaries that cut through the Valley's southern wall (Churchill et al., 1988; Kaul et al., 1988; Kantak, 1995; Rolfsmeier and Steinauer, 2010). On north-facing slopes of the NRV, these conditions have created Pleistocene refugia (Keppel et al., 2012), biotic communities that include species formerly common in the region, but now more typical of boreal, western coniferous, and eastern deciduous forests (Kaul et al., 1988). Nearby, such isolated, relictual communities contain the state's only remaining natural quaking aspen stands, as the species was far more common regionally under cooler, wetter climatic conditions. Presently, these isolated pockets of aspen, sometimes consisting of only a few dozen stems, connect the species' continuous continental range in Canada and the northern and northeastern United States to populations in the South Dakota Black Hills and the Rocky Mountains.

Sampling locations for molecular typing—To characterize both the putative hybrid of interest, Smith's aspen, as well as adjacent populations of its potential parent species in the Upper Midwest, we collected both rhizome cuttings and leaf tissue across five states (Fig. 1B, 1C; Appendix S1, see Supplemental Data with this article). We collected most intensively at and adjacent to Smith Falls State Park (Valentine,

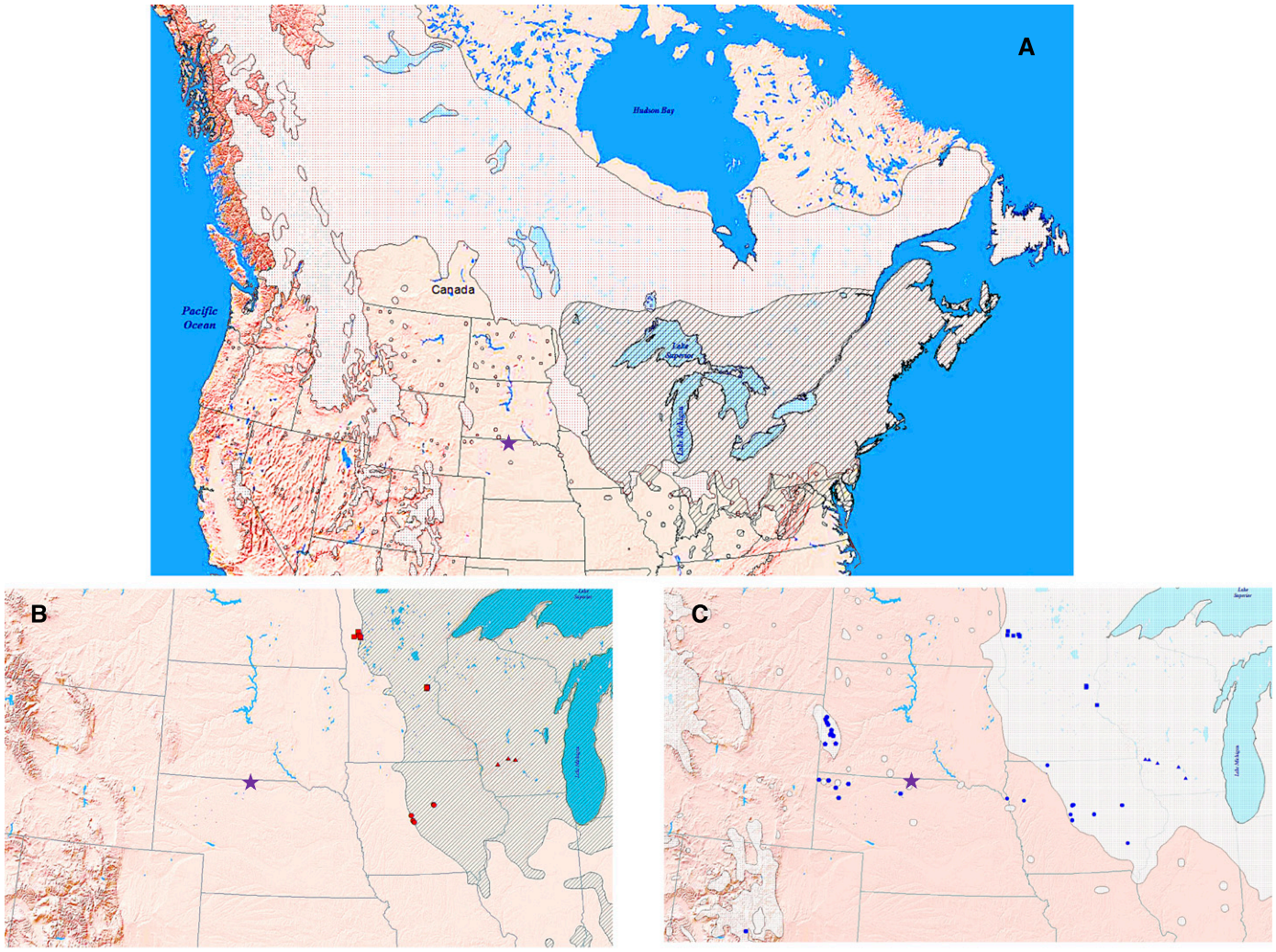

FIGURE 1 (A) Current range maps of *Populus tremuloides* (stippled) and *P. grandidentata* (hatched). Niobrara River Valley (NRV) putative hybrid population of *P.* ×*smithii* indicated by the star. (B) Collection locations for *P. grandidentata* from its western range edge used in genetic, morphological, and spectral analyses. (C) Collection locations for *P. tremuloides* from its range near the putative NRV hybrid population.

Nebraska) to characterize all known stands of the putative Smith's aspen in the NRV. We also sampled intensively from as many isolated stands of quaking aspen as was feasible in northern Nebraska and southern South Dakota, and from quaking aspen stands from the northern to the southern extent of the species' Black Hills distribution in western South Dakota. Likewise, we took representative samples from both quaking and bigtooth aspen stands from north to south along the species' roughly equivalent western range edge in Minnesota and Iowa. Finally, we collected leaves and rhizomes from well within both species' distribution in Minnesota and Wisconsin and from one quaking aspen stand in Colorado.

Common garden design and establishment—Rhizomes were collected to populate a common garden with aspen genotypes representative of the study area. Rhizomes were dug up by hand from May 2013 through June 2015 and clipped to between 10 and 50 cm in length. They were kept moist and transported to the University of Minnesota Plant Growth Facilities (St. Paul, Minnesota), where they were propagated following root cutting protocols (Luna, 2003). New vegetative cuttings from rhizomes were potted and maintained under typical greenhouse conditions with a minimum of 12 h of light per day until outplanting in spring 2015. The common garden site is located at Cedar Creek Ecosystem Science Reserve (East Bethel, Minnesota), well within the ranges of both bigtooth and quaking aspen and adjacent to stands of both species (45°24′17.4″N and 93°11′25.5″W). The common garden was fenced to exclude deer, mowed, sprayed with glyphosate, burned, and harrowed. Cuttings were planted in a randomized design at a spacing of 1 m^2 and watered following establishment. Herbaceous vegetation in the common garden was controlled with periodic mowing. Over 500 propagated cuttings were planted in the common garden, and approximately 380 survived. Of those that survived, 96 unique genotypes were identified by microsatellites (see Results), and subsets of those were measured for morphological and spectral analyses.

Molecular methods—Leaves were collected for molecular genetic analysis from either mature trees (when rhizome cuttings were not feasible) or from plants in the common garden. Because it was not always possible to collect rhizomes and because root cuttings often failed to produce viable plants, we collected leaves from many more plants than were represented in the common garden. Leaves were stored in moist, dark conditions close to freezing while in transport to the University of Minnesota, then frozen at −20°C until lyophilization and DNA extraction could occur.

We extracted DNA from 385 total leaves representing the complete NRV population and nearest putative parental species populations using Qiagen DNeasy Plant Mini Kits in laboratory facilities at the University of Minnesota. Extracted DNA was quantified using an UV/VIS spectrophotometer at the University of Minnesota and diluted with distilled water to a concentration of approximately 10 ng/μL for PCR amplification.

Twelve microsatellites were chosen from published literature based on linkage group coverage and ability to be amplified in multiplex PCR reactions (Table 1) (Smulders et al., 2001; Cole, 2005). A multiplex PCR approach was taken in which each PCR reaction included four primer pairs. Each 10-μL PCR reaction consisted of 1.0 μL of the fluorescently labeled forward primer and 1.0 μL of the reverse (10 μM each), 5.0 μL Qiagen Master Mix, 1 μL (~10 ng) DNA, and sterilized water. The thermal cycles for this reaction were 94°C for 15 min, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. A final extension step of 10 min at 72°C was added after the last cycle (Deacon and Cavender-Bares, 2015). An ABI 377 Automated Sequencer at the University of Minnesota's Genomics Center was used to measure the length of the fluorescently labeled PCR product. We used the program Geneious version 9 (Kearse et al., 2012) to visualize and characterize alleles by the length of the amplified fragments.

Statistical analysis of molecular data—Diversity statistics were calculated for all unique genotypes of each species. We used the clonal function in GENALEX to identify exact matches of multilocus genotypes (Peakall and Smouse, 2006), and repeated clonal genotypes were removed from further analysis. This approach does not attempt to identify somatic mutation or PCR artifacts, and genotyping errors were minimized by hand calling all peaks in the Geneious software and validating those calls by having coauthors assess peaks for subsamples of the data. Subsequent analysis in GENODIVE, where the threshold for identification of clonal genotypes was adjusted using the method of Meirmans and Van Tienderen (2004), increased the genetic distance for clonal assignment from 0 to 4. We calculated the percentage of polymorphic loci, average number of alleles per locus, observed and expected heterozygosities, and a fixation index averaged over all loci for all individuals of each species in GENALEX (Peakall and Smouse, 2006). We examined evidence of admixture in allele frequencies of putative hybrids by testing for significant differences between each parental species and the hybrid using AMOVA (GENALEX) quantified by R_{ST} using a stepwise-mutation model. Significance of R_{ST} was tested through 10,000 permutations of the data.

The number of genetically distinct clusters was estimated using a Bayesian approach in which a Markov chain Monte Carlo method clustered individuals based on the 12 microsatellite allele frequencies to minimize Hardy–Weinberg disequilibrium and linkage disequilibrium (STRUCTURE; Pritchard et al., 2000). This method does not assume a particular mutation model, and posterior probabilities of belonging to each K group are calculated for each individual. We tested $K = 1$ through $K = 4$ for 10 runs with a burn-in length of 500,000 and a MCMC of 1,000,000. The K supported by the data was determined by comparing the magnitude of the change in the log likelihood values (ΔK method) averaged over 10 runs (Evanno et al., 2005). Finally, a Bayesian model-based clustering program, NewHybrids, that computes posterior probabilities that samples are from a parental class or various hybrid classes (F1, F2, etc.) was used (Anderson and Thompson, 2002). The z and s options were used to indicate morphologically identified parental individuals of both species, and results were based on 1,000,000 MCMC sweeps after a burn-in period of 500,000.

Sequence data were collected at a 794-bp, noncoding chloroplast region (*trnD-trnT*) in 94 plants (Table 2). The region was amplified using universal primers (Shaw et al., 2007). Reaction volumes of 25 μL included 1 μL of template DNA, 5 μM of dNTP, 2.5 μL of 10× Standard Taq Reaction Buffer, 1 μL of $MgCl_2$, 0.2 μM of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), 5.0 μL Q solution (Qiagen, Valencia, California, USA), and 0.125 μL *Taq* DNA Polymerase (New England BioLabs, Ipswich, Massachusetts, USA). The thermal cycler program consisted of 1 cycle of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min. PCR products were verified on 1% agarose gels before cleaning with the Qiaquick PCR cleaning kit (Qiagen). DNA sequencing reactions were performed with the ABI Prism Big Dye

TABLE 1. Microsatellite primer details and multiplex PCR groupings. Dye labels were used on the 5′ end of forward primers in all cases. Linkage group (LG), repeat motif, primer sequences, melting point (MP), and approximate length of repeat unit are shown. Multiplexed primers were grouped by length and dye color.

Primer	LG	Motif	Forward primer	Reverse primer	Bases	MP	Group	Dye	Length (bp)
WPMS_16 *	7	GTC	CTCGTACTATTTCCGATGATGACC	AGATTATTAGGTGGGCCAAGGACT	24	54.8	A	Bl	145
WPMS_15 *	5	CCT	CAACAAACCATCAATGAAGAAGAC	AGAGGGTGTTGGGGGTGACTA	24	52.8	A	Gr	193
ORPM_344 §	10	[TC]8	GGAGATTGTCGGAGAATGGA	TGGACGTTACGATAGGAGTGG	20	54	A	Gr	229
WPMS_14 *	5	CGT	CAGCCGCAGCCACTGAGAAATC	GCCTGCTGAGAAGACTGCCTTGAC	22	60.9	A	Bl	245
PMGC_0575 §	1	GA	TAAATTCATGTAGATTGACG	CTTACTATTTCATGGTTGTC	20	45.1	B	Gr	145
ORPM_206 §	19	[GCT]7	CCGTGGCCATTGACTCTTTA	GAACCCATTTGGTGCAAGAT	20	55	B	Bl	196
ORPM_127 §	4	[TG]8	TCAATGAGGGGTGCCATAAT	CTTTCCACTTTTGGCCCTTT	20	53.9	B	Gr	200
ORPM_149 §	Unk.	[AT]4[CT]4	GTCTCTGCCACATGATCCAA	CCCGAAATGGATCAAACAAG	20	54.8	B	Bl	216
PMGC_667 †	2	GA	CATTCGTTCAGTAGTTAAGGC	GGTTAAGCTACCTCTGCTAC	21	52.9	C	Gr	220
WPMS20 *	13	[TTCTGG]*n*	GTGCGCACATCTATGACTATCG	ATCTTGTAATTCTCCGGGCATCT	22	55.6	C	Gr	204–235
GCPM970-1 †	Unk.	[TGC]*n*	CTCATCCATCGTAACCATTT	CGAGTATGTTAGGAGGTTGG	20	50	C	Bl	119–134
PMGC2571 †	10	[GA]*n*	TCTCGCAGATTCATGTAACCC	GACTGTATGTTGACCATGCCC	21	54.6	C	Bl	84–120

Notes: Primer sequence sources: * Smulders et al., 2001; § Cole 2009; † http://ornl.gov/sci/ipgc/ssr_resource.htm. Bl = blue; Gr = green; Unk. = unknown.

Terminator Cycle Sequencing Ready Reaction kit and subjected to capillary electrophoresis (Applied Biosystems, Foster City, California, USA) at the University Minnesota Genomics Center. Both forward and reverse directions were sequenced. Sequence chromatograms were edited, and forward and reverse sequences were assembled to produce a single sequence for each sample and aligned using Geneious v.9 (Kearse et al., 2012) with the inclusion of gaps to accommodate insertions/deletions across species. Sequences can be found in Appendix S1. A network diagram illustrating sequence similarity was constructed using statistical parsimony in TCS (POPART; Leigh and Bryant, 2015).

Phenotype screening—We used three to five leaves from 77 common garden plants for morphological analyses during the summer of 2016 (Table 2). All plants had been growing in the common garden environment for 1 year after spending 1–2 yr in the greenhouse. Petiole length (cm), blade length (cm), blade width (cm), blade area (cm^2), blade perimeter (cm), and blade circularity were all measured using the program ImageJ (Rasband, 1997) using the LeafJ plugin (Maloof et al., 2013). These traits were manually measured using ImageJ for specimens that could not be effectively measured by LeafJ. Blade width at widest point (cm) and distance from petiole insertion point to the widest point (cm) were also manually measured. Tooth number was manually counted from the backside of the leaf, starting to the left of the petiole insertion point and ending at the distal end of the blade. Pubescence was scored as a discrete categorical index with scores ranging between one and six (1 = no hair, 2 = hairs around edges of leaf, 3 = very sparse, but evenly distributed hairs, 4 = more abundant, evenly distributed hairs, 5 = dense hair, 6 = densely matted so much so that individual hairs could not be discerned). Photosynthetic capacity (A_{max}; $\mu mol \cdot m^{-2} \cdot s^{-1}$) was measured using a LI-COR (Lincoln, Nebraska, USA) 6400 portable photosynthesis system under full sun between 09:00 and 13:00. From directly measured traits, we derived the following trait values: specific leaf area (SLA; $cm^2 \cdot g^{-1}$), teeth per unit perimeter (TPP; cm^{-1}), blade length to width (L:W; $cm \cdot cm^{-1}$), blade length to area (L:A; $cm \cdot cm^{-2}$).

TABLE 2. Sample sizes for each molecular and morphological test in this study. Number of individual plants (ramets) and of the number of distinct genotypes (genets) measured are given.

	P. grandidentata		*P. ×smithii*		*P. tremuloides*	
Test	All	Unique	All	Unique	All	Unique
Molecular analysis						
trnD-trnT	20	20	7	3	67	63
Morphological analysis						
Pubescence	7	6	4	3	65	50
Petiole length	7	6	4	3	65	50
Blade length	7	6	4	3	65	50
Blade width	6	5	4	3	62	48
Blade area	7	6	4	3	65	50
Blade perimeters	7	6	4	3	65	50
Blade circularity	7	6	4	3	65	50
Distance from petiole insertion to widest point	6	5	4	3	62	48
Tooth number	6	5	4	3	62	48
SLA	7	6	4	3	64	50
Teeth per perimeter	6	5	4	3	61	48
Length to width ratio	6	5	4	3	62	48
Length to area ratio	7	6	4	3	65	50
Leaf carbon	5	5	4	3	62	47
Leaf nitrogen	5	5	4	3	60	45
C:N ratio	5	5	4	3	60	45
Leaf cellulose	4	4	4	3	62	47
Photosynthetic capacity	7	6	4	3	60	47
Spectral analysis	5	5	4	3	63	48

Spectral data—We collected spectral data from two to five fully mature, recently expanded leaves per individual from the same 77 common garden plants used for morphological analyses on 16 and 17 June 2016 (Table 2). All measurements were taken from the leaf adaxial surface using a portable spectroradiometer (SVC HR-1024i, Spectra Vista Corp., Poughkeepsie, New York, USA) covering the wavelength region from 350–2500 nm in 1024 contiguous spectral bands with a leaf-clip assembly (SVC LC-RP PRO) including an internal, calibrated light source. We corrected artifacts in the spectral data at the sensor overlap regions, interpolated

to 1 nm spectral resolution, and used vector normalization to correct for brightness effects (Feilhauer et al., 2010). Noisy regions at the beginning and end of the spectrum (center wavelengths smaller than 400 nm and greater than 2400 nm) were excluded from analysis. We used the spectrolab package for all processing steps (Meireles et al., 2017).

Traits from spectra—We predicted a range of biochemicals from spectra using partial least squares regression (PLSR; (Wold et al., 1983) models developed based on a separate data set consisting of chemical assays and spectra of 65 (for pigments), 127 (for carbon and nitrogen), and 114 (for fiber components) individual tree and prairie plant leaves from over 20 species collected at the Cedar Creek Ecosystem Science Reserve (East Bethel, Minnesota) where *P. tremuloides* and *P. grandidentata* currently occur (A. K. Schweiger, personal observation). Pigment concentrations of chlorophyll *a* ($\mu mol \cdot m^{-2}$), chlorophyll *b* ($\mu mol \cdot m^{-2}$), beta-carotene ($\mu mol \cdot m^{-2}$), lutein ($\mu mol \cdot m^{-2}$), neoxanthin ($\mu mol \cdot m^{-2}$), violaxanthin ($\mu mol \cdot m^{-2}$), antheraxanthin ($\mu mol \cdot m^{-2}$), and zeaxanthin ($\mu mol \cdot m^{-2}$) were determined using high-performance liquid chromatography. Carbon, nitrogen, and fiber component (neutral detergent fiber [NDF, %], acid detergent fiber [ADF, %], acid detergent lignin [ADL, %], solubles [%], hemicellulose [%], cellulose [%], lignin [%]) concentrations were determined using standard laboratory methods (TruSpec CN Analyzer, Leco Corp., St Joseph, Michigan, USA; Fiber Analyzer 200, Ankom Technology, Macedon, New York, USA). Spectra from the same leaves were collected as described above. Partial least squares regression (PLSR) models were established using the plsRglm package (Bertrand et al., 2014) in R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). For each chemical component, we performed 10-fold cross-validation 100 times and used the mean PLSR coefficients for subsequent predictions (Appendix S2).

Statistical analyses of phenotypic data—All leaf trait analyses were conducted in R version 3.3.1. Replicated measurements of morphological traits were averaged on a per-tree basis, and individual trait values among bigtooth, quaking, and Smith's aspen were compared using a one-way ANOVA with species (as identified at collection and confirmed with simple sequence repeats [SSRs]) as the predictor variable. Significant ANOVA results were followed by Tukey's honestly significant difference (HSD) post hoc testing using the agricolae package (de Mendiburu and de Mendiburu, 2016). We also used principal components analysis (PCA) to explore trait variation among species holistically. We conducted PCA for a subset of all measured traits, excluding traits that were highly correlated with another trait that was already included in the analysis. We log-transformed, centered, and scaled to unit variance all trait values; trees that did not have complete records for all traits of interest were excluded from analysis. We then used species designation (described above) to predict informative principal components (PCs) from the analysis in a one-way ANOVA to assess the relationship between species identity and leaf phenotype.

Statistical analysis of spectral data—To compare spectrally derived trait values, water content index, and water-stress index among bigtooth, quaking, and Smith's aspen, we used one-way ANOVAs, followed by Tukey's HSD post hoc test and PCAs. For classifications, we used partial least squares discriminant analysis (PLSDA) as implemented in the caret package (Kuhn, 2016) in R, using the leaves × morphological traits, leaves × chemical traits, and leaves × spectra as predictor variables, and the two species and their putative hybrid as the three response classes. Fifty percent of the data were used for model calibration (training), using 10 repeats of 10-fold cross validations. The optimal number of components was chosen based on the maximum value of the kappa statistic, which compares the observed classifications to a random classification. We report model statistics based on the 50% of data kept for independent validation (testing). We also calculated a water content index (WI = r900 / r970; Peñuelas and Filella, 1998), and a water-stress index [WCRI = rSWIRmin / (rNIRmax − rSWIRmin); Sun et al., 2008], for each leaf and tested whether indices related to leaf water status differed among the three species groups.

RESULTS

Molecular genetic results—Microsatellite amplification of 379 separately collected leaves (each from a unique ramet) indicated that these 379 ramets represented 96 distinct genotypes (genets; hereafter, "individuals"). When the threshold for clonal identification was relaxed by increasing the genetic distance for clonal assignment from zero to four, 93 genets were identified. Only one of the collapsed genotypes from the strict matching protocol was considered likely to be accurate due to geographic proximity with the rest of its genet. For subsets of individuals from these genets, we measured morphological traits ($N = 77$) and sequenced the chloroplast *trnD-trnT* region ($N = 94$). Results presented below pertain to these subsets of sampled individuals (Table 2, Appendix S1).

Of the 285 *P. tremuloides* collected, 71 had unique genotypes; 22 of the 50 *P. grandidentata* collected were unique, and only 3 of the 44 putative Smith's aspen (*P. ×smithii*) collected had unique microsatellite genotypes. One of these Smith's aspen genotypes collapsed into the neighboring genet when a relaxed clonal assignment was used. All 12 loci were polymorphic for *P. tremuloides*, while 92% and 83% were for *P. grandidentata* and Niobrara aspens, respectively. *Populus grandidentata* was monomorphic at ORPM127, and the putative *P. ×smithii* was monomorphic at P667 and WPMS 15. Average number of alleles per locus was lowest for the Niobrara aspens (2.167, *P. grandidentata* = 5.417, *P. tremuloides* = 9.500), but *P. grandidentata* had the lowest expected heterozygosity (0.377, putative *P. ×smithii* = 0.440, *P. tremuloides* = 0.650, Table 3). Only one allele appeared in the putative *P. ×smithii* that was not sampled in either parental population (226 at ORPM344). The parental species have significantly different allele frequencies from each other at all polymorphic loci (Table 4). The putative hybrid's allele frequencies are significantly different from each parent at five loci each (Table 4).

The STRUCTURE results showed $K = 2$ to be the most likely number of genetic clusters according to the ΔK method, and *P. tremuloides* and *P. grandidentata* individuals made up the two clusters, respectively (Appendix S3). Putative *P. ×smithii* individuals shared assignment to both parental genetic clusters (Fig. 2A). Posterior probabilities of the three putative *P. ×smithii* individuals in the NewHybrids analysis indicated membership in the F1 hybrid genotype category (posterior probabilities = 0.99, 0.99, and 0.83 respectively, Fig. 2B). The analysis also suggested F1 hybrid status of one individual previously identified as *P. grandidentata* from Minnesota (MN) and one F2 hybrid previously identified as *P. tremuloides* from Iowa (IA) (posterior probabilities = 0.88 and 0.58

TABLE 3. Genetic diversity for each species at 12 nuclear microsatellite loci.

Taxon	Statistic	*N*	Na	Ne	*I*	H_o	H_e	uHe	*F*
P. grandidentata	Mean	22	5.417	2.392	0.851	0.280	0.377	0.385	0.173
	SE	NA	0.925	0.670	0.198	0.065	0.082	0.084	0.081
P. ×smithii	Mean	3	2.167	2.012	0.670	0.722	0.440	0.528	–0.670
	SE	NA	0.241	0.201	0.109	0.107	0.064	0.077	0.100
P. tremuloides	Mean	71	9.500	4.158	1.475	0.607	0.650	0.655	0.067
	SE	NA	1.790	0.880	0.195	0.059	0.062	0.063	0.049

Notes: Na = no. of different alleles; Ne = no. of effective alleles = 1 / ($\Sigma\pi^2$); *I* = Shannon's information index = $-1\cdot\Sigma(\pi\text{Ln }\pi)$; H_o = observed heterozygosity = No. of hets / $N\Sigma$; H_e = expected heterozygosity = $1 - \Sigma\pi^2$; uHe = unbiased expected heterozygosity = $[2N / (2N - 1)]\cdot H_e$; *F* = Fixation index = $(H_e - H_o) / H_e = 1 - (H_o / H_e)$; where π is the frequency of the *i*th allele for the population ,and $\Sigma\pi^2$ is the sum of the squared population allele frequencies.

respectively, Fig. 2B). Chloroplast sequences showed high similarity between NRV *P. ×smithii* and *P. grandidentata* sequences (Fig. 3), as did the IA individual identified as a hybrid. The MN individual identified as a hybrid clustered with *P. tremuloides*.

Phenotypic results—Some morphological traits clearly distinguished among bigtooth, quaking, and Smith's aspen, while others did not (Fig. 4A; Appendix S4). Generally, quaking aspen leaves were smooth, smaller, and had many teeth, while bigtooth aspen leaves were large and pubescent with few teeth. Putative Smith's aspen hybrids from the NRV were more similar to one parent for some traits and were intermediate in others. Putative hybrids were intermediate to parents in pubescence (and nonsignificantly in teeth per unit perimeter), similar to bigtooth aspen in two traits (SLA, blade length to width), and similar to quaking aspen in six traits (blade length, width, area, and perimeter; distance from the petiole to the widest point of the blade; and tooth number). There were no differences among species in petiole length, blade circularity, or maximum photosynthetic rate. Overall, pubescence emerged as the best diagnostic trait for differentiating parents from the putative Smith's aspen hybrid and each other (Appendix S4).

A PCA of uncorrelated morphological traits explained 57% of variation in two PCs (Fig. 4A). The second principal component (PC2), which was associated with pubescence and tooth number per unit of leaf perimeter, was especially strongly associated with species group and explained 22.1% of the total variation. An individual's loading on PC2 was a significant univariate predictor of its species identity (Fig. 4B; One-way ANOVA: $F_{2, 54} = 51.4$, $P < 0.001$), and post hoc testing indicated that each parental species and the NRV Smith's aspen were all associated with significantly different values of PC2.

Spectrally derived traits—We found few significant differences in leaf chemistry among the three species groups based on Tukey tests ($P < 0.05$) (Appendix S4). Leaf chlorophyll *a* and beta-carotene content were higher for *P. tremuloides* than the putative hybrid, *P. grandidentata* was not different. *Populus tremuloides* also had lower NDF and hemicellulose content than in *P. grandidentata*, with the putative hybrid ranging in between and not significantly different. In addition, *P. tremuloides* had higher water content based on the WI than in *P. grandidentata* and *P. ×smithii*, which did not differ. The water stress index (WCRI) was marginally higher for the putative hybrid than for *P. grandidentata* and *P. tremuloides*, which were again not different from each other.

Using the four least correlated pigments (Pearson's correlation coefficient $R < 0.8$), antheraxanthin, violaxanthin, beta-carotene, and total chlorophyll (chlorophyll *a* + chlorophyll *b*), in a PCA revealed no clear pattern separating three species groups (Appendix S5). Similarly, there was no clear pattern in a PCA based on the four least correlated "structural" leaf characteristics (C, N, NDF, and lignin content). *Populus grandidentata* and *P. tremuloides* differed significantly along the third PC axis (PC3), with the putative hybrid ranging in between and not significantly different ($F_{2, 242} = 10.02$, $P < 0.001$), but this axis only explained 11.4% of the total variance. Unsurprisingly, *P. tremuloides*, *P. grandidentata*, and the putative hybrid were neither distinguishable in a PLSDA based on the measured pigments, nor in a PLSDA based on C, N, and the five fiber components that were not perfectly correlated (NDF, ADF, hemicellulose, cellulose, and lignin) (kappa = 0.0, in both cases).

The PLSDA based on leaf spectra successfully distinguished the two parental species and the putative *P. × smithii,* with few misclassifications (kappa = 0.90, Table 5, Appendix S6). Interestingly, the three species groups did not differ consistently across the spectrum. When splitting the leaf spectra into regions, the SWIR separated the three species groups best (kappa = 0.76), followed by the NIR (kappa = 0.57), while considerable misclassifications occurred when using the VIS only (kappa = 0.32, Table 5, Appendix S6).

TABLE 4. Pairwise analysis of locus-by-locus differences in allele frequencies using R_{ST} values between *Populus grandidentata* (G), *P. tremuloides* (T), and *P. ×smithii* (S).

Locus	G-S	G-T	S-T
PMGC0575	0.169	**0.266**	0.000
ORPM206	0.000	**0.269**	0.025
ORPM127	**0.796**	**0.282**	0.068
WPMS14	**0.153**	**0.565**	**0.222**
WPMS16	**0.617**	**0.851**	**0.490**
ORPM149	0.387	**0.749**	**0.380**
WPMS15	0.000	0.000	0.000
ORPM344	**0.746**	**0.948**	**0.468**
PMGC2571	0.074	**0.420**	**0.249**
G970	**0.453**	**0.313**	0.000
P667	0.003	**0.162**	0.000
W20	0.132	**0.044**	0.000
TOTAL	0.286	0.531	0.154

Note: Significant values in bold based on 10,000 permutations of the data $P < 0.05$.

DISCUSSION

The results of this study provide clear evidence supporting the hypothesis that the Niobrara River Valley aspens are the hybrid, *P. ×smithii*, between *P. tremuloides* and *P. grandidentata*, a hypothesis generated from morphological assessment in earlier treatments of the regional flora (Churchill et al., 1988; Kaul et al., 1988; Eckenwalder, 1996). Three separate analyses based on molecular, morphological, and spectral data all support the same conclusion. However, molecular data show that NRV aspens are intermediate relative to parents in nuclear, biparentally inherited microsatellite markers, while the maternally inherited chloroplast likely originates from *P. grandidentata*. Leaf morphological traits that distinguished parental species were intermediate in

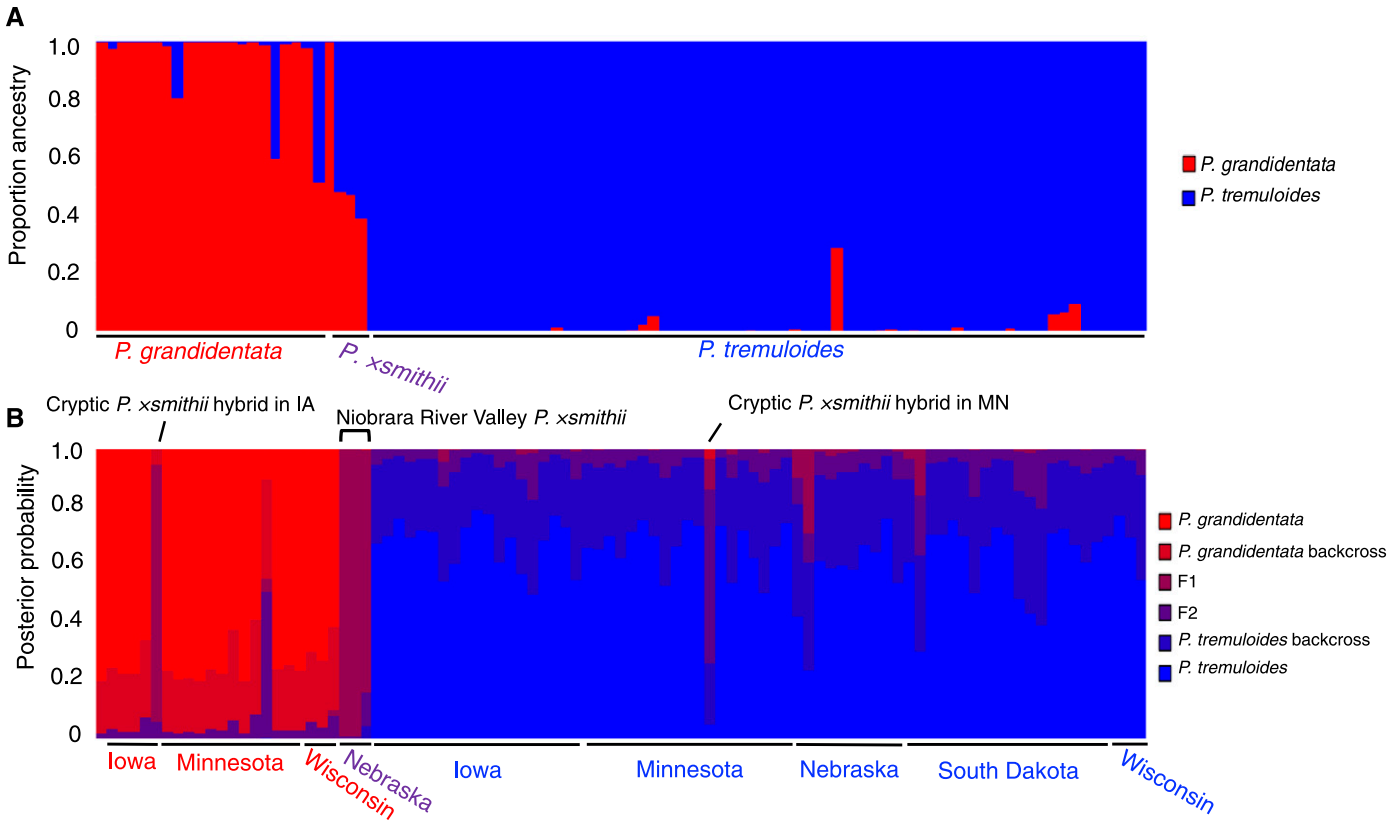

FIGURE 2 (A) Proportion of ancestry for 96 individuals of unique genotype surveyed over 12 microsatellite loci determined by STRUCTURE analysis for the most likely *K* (*K* = 2) based on the Δ*K* method. The two ancestral populations correspond to the two parental species, *Populus grandidentata* (red) and *P. tremuloides* (blue). The ancestry of the Niobrara River Valley (NRV) aspens is split between these two ancestral populations, indicating their hybrid status. (B) NewHybrids classification of each individual genotype to either a parental generation (P1 and P2), first generation hybrid (F1), second generation hybrid (F2), or a backcross category (BCP1 and BCP2). Default genotype frequency class categories were used and the *y*-axis shows the posterior probability of each individual's membership in each of the different genotype frequency categories. Collection locations are shown along the *x*-axis and samples are ordered the same in (A) as in (B).

the hybrid, while highly labile traits such as gas exchange and pigments, were not. Holistic consideration of all three analyses suggests that the NRV aspens are a uniquely identifiable hybrid population. This result is somewhat surprising given that the ranges of these two species no longer overlap in the Niobrara River Valley, and the nearest *P. grandidentata* population is hundreds of kilometers to the east.

Microsatellite allele frequencies for *P. ×smithii* are significantly differentiated from one parental species or the other at different loci, as would be expected in a hybrid. If the NRV aspens were a subpopulation of *P. tremuloides*, these loci would only be differentiated from *P. grandidentata*, and vice versa (Wallace et al., 2011). The STRUCTURE and NewHybrids analyses clearly designate two separate parental species and indicate that the NRV aspens are composed of a combination of parental alleles. The result that the NRV aspens may even be primarily F1 hybrids can only be explained by persistence of the vegetative clonal suckers of hybrids that formed thousands of years ago when parental ranges overlapped in this region during a cooler and wetter postglacial climate. Though it is not possible to reliably estimate the age of aspen genets, palynological records indicate *P. grandidentata* in the Niobrara River Valley 8900–3600 yr ago (Wright et al., 1985), implying historical hybridization occurred. Barnes (1967) suggested that such ancient hybridizations under different climatic conditions can potentially persist for thousands of years, and others have found evidence for speciation in the *Populus* genus driven by pleistocene glacial cycles (Levsen et al., 2012).

As evidenced by our unintentional collection of hybrids in Iowa and Minnesota, natural hybrids of bigtooth aspen and quaking aspen do occur in areas where their ranges overlap today, but less frequently than might be expected because of differences in time of flowering. When hybridization occurs, it is most likely to be between male quaking aspen and female bigtooth aspen (Graham et al., 1963). This generalization is supported by our analysis of a short segment of the maternally inherited chloroplast. *Populus grandidentata* and NRV *P. ×smithii* are nearly identical in this region compared to only 3 of the 67 *P. tremuloides*. On the other hand, analysis of the biparentally inherited nuclear microsatellites does not result in *P. ×smithii* clustering with one parent. A more comprehensive survey of both the nuclear and chloroplast genomes using next-generation sequencing technologies would more clearly elucidate the exact genetic architecture of the hybrids (Eaton et al., 2015), but that is beyond the scope of this study.

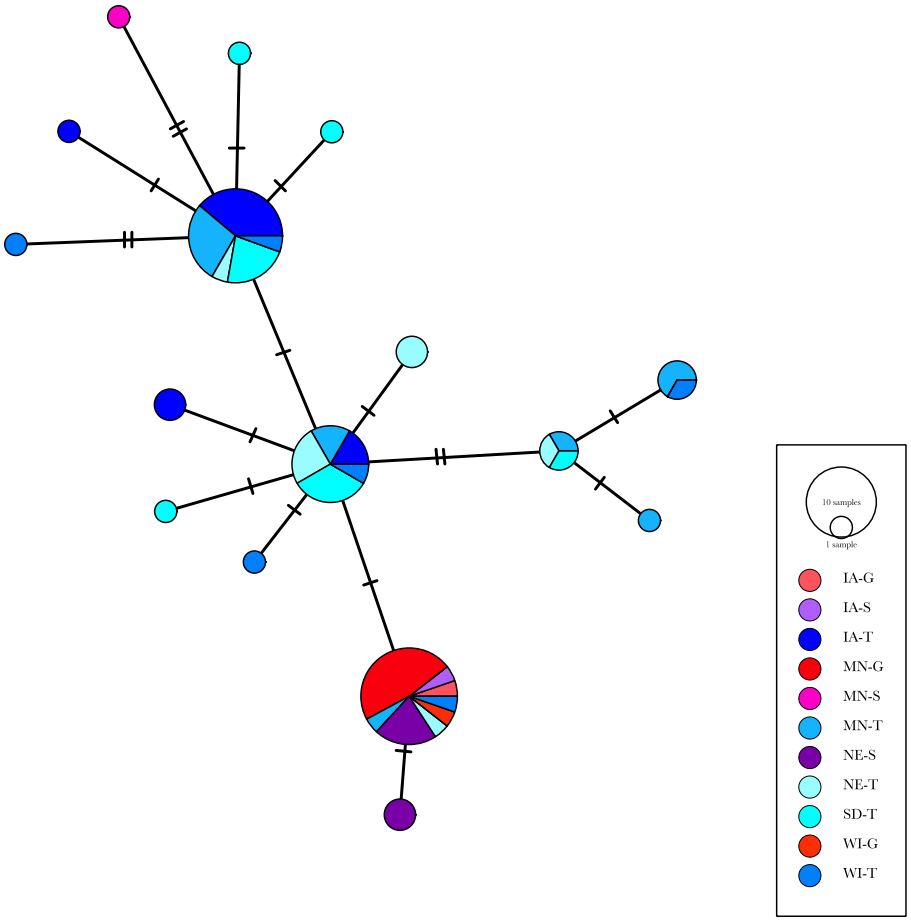

FIGURE 3 TCS network diagram using sequences from the *trnD-trnT* chloroplast region. Blue tones indicate *Populus tremuloides* individuals from different states. Red tones indicate *P. grandidentata* individuals from different states. Purple tones indicate the putative hybrid Niobrara River Valley (NRV) aspens. Size of the circle represents the number of identical sequences, and hatch marks across lines indicate sequence differences. The unintentionally collected hybrid from Iowa groups with the NRV hybrids, while the unintentionally collected hybrid from Minnesota groups with *P. tremuloides*.

Artificial crosses of *P. tremuloides* and *P. grandidentata* in Michigan demonstrated little hybrid vigor of *P. ×smithii* and therefore were of little interest for timber purposes (Reighard and Hanover, 1990). As a consequence, this hybrid species has not been well studied despite observations of its intermediate leaf morphology. Our morphological analysis of fully formed leaves collected from propagated root cuttings and grown for over 1 year in a common garden, support previous descriptions of hybrid leaf shape and structure. Similar to descriptions from Barnes (1961) and Pauley (1956) of naturally occurring hybrids in Michigan and Minnesota, the pubescence and tooth number for the hybrid are intermediate between the two parental states. The two parental species are differentiated by these traits in the final steps of identification using dichotomous keys (Gleason and Cronquist, 1991). The morphological variation we observed was from common garden leaves, allowing us to rule out environmental variation as a causal factor. We can thus infer a genetic basis for the variation in these traits and provide confidence in their utility in identification.

Likewise, spectral variation of leaves was determined from plants grown in the common garden. The spectral response of leaves is determined by the combined effects of chemical and structural characteristics (Curran, 1989; Kokaly et al., 2009). Because spectroscopy integrates structural and chemical characteristics, we thus expected *P. tremuloides*, *P. grandidentata*, and *P. ×smithii* to be easier to distinguish based on their leaf spectra than based on their chemical or morphological characteristics. This was confirmed by the PLSDA result. Leaf chemistry (pigments, fiber components, C, N) did not differ sufficiently to tell *P. tremuloides*, *P. grandidentata*, and *P. ×smithii* apart, but spectra did. The differences in spectral reflectance in the SWIR, and to a lesser degree in the NIR, made it possible to distinguish *P. grandidentata*, *P. tremuloides*, and *P. ×smithii* reasonably well using PLSDA. Misclassifications were most likely for *P. ×smithii*, which is in line with our finding that *P. ×smithii* is phenotypically intermediate. Spectral dissimilarities not only allowed the two species and *P. ×smithii* to become distinguishable in a PLSDA, but also suggested physiological differences in leaf water content and drought tolerance because of the separation of the three groups in SWIR region. In future studies, we will compare differences among these species in water stress tolerance and explore the genomic basis for these differences implied by the spectral results.

The clonal growth form of aspens makes assessments of genetic diversity challenging, and we were surprised to find only three, or possibly even two, unique genotypes within the entire NRV aspen population. This small sample size from our original collection of leaves from over 40 ramets makes drawing statistically robust conclusions difficult. But given our extensive sampling of individuals spanning the spatial extent of all stands of NRV aspens, we are confident that we have exhaustively catalogued the genetic diversity of the population. Relaxing our threshold for identifying clones based on the microsatellite data to accommodate potential somatic mutation or PCR artifacts resulted in only two unique genotypes rather than three. The stands comprising the collapsed genotypes are separated by only ~400 m, so their genotypic differences may be due to somatic mutation or perhaps these two stands are close relatives. The third genotype is approximately 1 km away and remained distinct even under relaxed clonal assignment parameters. These few genotypes and the resultant lack of standing genetic variation suggest that the population is extremely vulnerable to extinction due to changing environmental conditions. Throughout the western United States, *P. tremuloides* populations are already in decline (Bretfeld et al., 2016) and are undergoing climate-induced range shifts (Worrall et al., 2013). Management practices that address these threats may be necessary for the persistence of the NRV aspens.

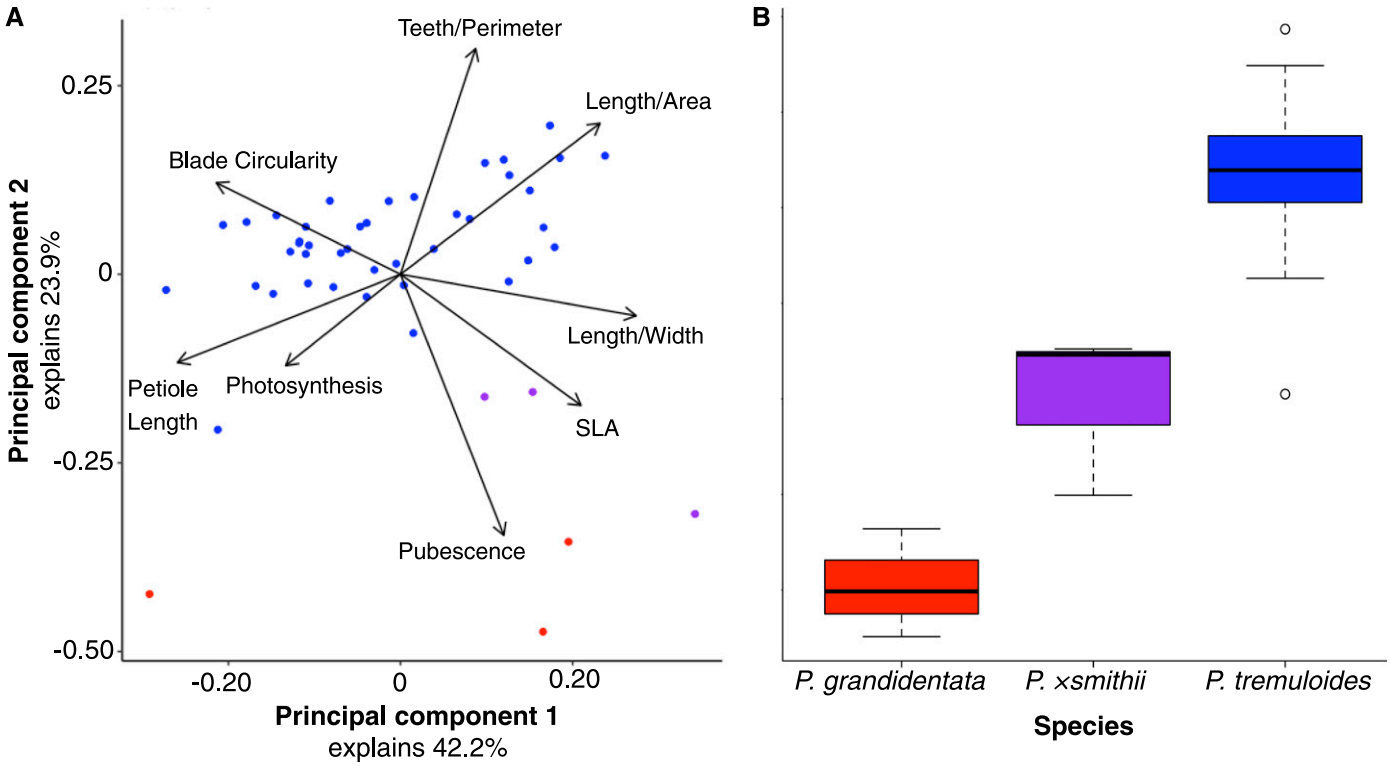

FIGURE 4 (A) PCA from morphological measurements of leaves of the two parental species and hybrid grown in a common garden. *Populus tremuloides* are blue, *P. grandidentata* are red, and the putative *P. ×smithii* is purple. Traits and vectors show what is driving the differentiation along each axis. (B) Box plot of PC2 loadings for each species. There is a significant difference among species along this axis that is driven by pubescence and tooth number.

The NRV *P. ×smithii* have long been recognized as a charismatic component of this unique, disjunct boreal forest relict, and continued ecological management for aspen suckering may allow the population to exist indefinitely. However, detrimental effects of somatic mutation load may occur over time in long-lived aspen genets (Ally, 2008), and continued climate change may eventually become too extreme. The three genets are also situated on three different properties, with one genet entirely confined to either side of Smith Falls State Park, so coordinated monitoring and management activities should be continued (Robertson, 2015). Management of aspen populations increasingly depends on genetic tools to prioritize conservation efforts and to inform other actions (Mock et al., 2013), and future decline in the NRV aspens may require introducing new genetic variation into the population from nearby *P. tremuloides*. It is unclear, however, if any sexual reproduction is occurring in the current stands.

Options for genetic rescue of the NRV aspens in response to future decline may be limited. We never observed hybrid individuals flowering over 3 years and were not able to use new molecular techniques (Pakull et al., 2015) to determine the sex of the three NRV genotypes. As such, we can only speculate that, should some NRV aspens be reproductive, introduction of new genetic variation from nearby *P. tremuloides* populations may represent one management option. Additionally, it is possible that the F1 hybrids at NRV are sterile but highly successful at suckering in their environment—a possibility supported by our observation that they have not flowered and seem, under the right conditions, to be able to reproduce consistently by suckering. This would result in low fitness of hybrids with any additional *P. grandidentata* introgression; we know that this is a challenging environment for aspens, and maybe advanced generation hybrids were wiped out along with *P. grandidentata* as the climate continued to become drier and hotter.

Future studies of these trees should address their likely tolerances to predicted changes in climate to guide decision

TABLE 5. Species-dependent sensitivity (rate of true positives) and specificity (rate of true negatives), and overall accuracy of partial least squares discriminant analysis (PLSDA) classification. All values range from 0 to 1.

Species	Metric	Full spectrum	VIS	NIR	SWIR
P. grandidentata	Sensitivity (true positives)	0.87	0.40	0.53	0.87
	Specificity (true negatives)	0.99	0.96	0.98	0.99
P. ×smithii	Sensitivity	0.75	0.00	0.38	0.38
	Specificity	1.00	1.00	0.98	0.99
P. tremuloides	Sensitivity	1.00	0.97	0.97	0.99
	Specificity	0.91	0.30	0.61	0.74
All species	95% CI accuracy	0.94, 0.99	0.81, 0.92	0.85, 0.95	0.90, 0.98
	Kappa	0.90	0.32	0.57	0.76

Notes: VIS = visible wavelengths (400–700 m), NIR = near-infrared (700–1400 m), SWIR = short-wave infrared (1400–2400 nm), CI = confidence interval.

making and ensure the long-term persistence of this unique hybrid population.

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