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BIOCLIMATIC, ECOLOGICAL, AND PHENOTYPIC INTERMEDIACY AND HIGH GENETIC ADMIXTURE IN A NATURAL HYBRID OF OCTOPLOID STRAWBERRIES[1]

ISABELLA SALAMONE[2], RAJANIKANTH GOVINDARAJULU[2], STACEY FALK[3], MATTHEW PARKS[4], AARON LISTON[4], AND TIA-LYNN ASHMAN[2,5]

[2]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, USA; [3]South Park High School, Pittsburgh, Pennsylvania 15129, USA; and [4]Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, USA

- *Premise of the Study:* Hybrid zones provide "natural laboratories" for understanding the processes of selection, reinforcement, and speciation. We sought to gain insight into the degree of introgression and the extent of ecological–phenotypic intermediacy in the natural hybrid strawberry, *Fragaria* × *ananassa* subsp. *cuneifolia.*
- *Methods:* We used whole-plastome sequencing to identify parental species-specific (*Fragaria chiloensis* and *F. virginiana*) chloroplast single-nucleotide polymorphisms and combined the use of these with nuclear microsatellite markers to genetically characterize the hybrid zone. We assessed the potential role of selection in the observed geographic patterns by bioclimatically characterizing the niche of the hybrid populations and phenotypically characterizing hybrid individuals of known genomic constitution.
- *Key Results:* Significant admixture and little overall maternal bias in chloroplast or nuclear genomes suggest a high degree of interfertility among the parental and hybrid species and point to a long history of backcrossing and genetic mixing in the hybrid zone. Even though hybrids were phenotypically intermediate to the parental species, there was a discernible fingerprint of the parental genotype within hybrid individuals. Thus, although the pattern of introgression observed suggests geographic limitations to gene flow, it may be reinforced by selection for specific parental traits in the bioclimatically intermediate habitat occupied by the hybrid.
- *Conclusions:* This work uncovered the genetic complexity underlying the hybrid zone of the wild relatives of the cultivated strawberry. It lays the foundation for experimental dissection of the causes of genomic introgression and nuclear–cytoplasmic disassociation, and for understanding other parts of *Fragaria* evolutionary history.

Key words: hybrid zone; introgression; microsatellites; morphology; population genetic structure.

Hybrid zones (Barton and Hewitt, 1985), regions where hybrids and parental species coexist, provide an opportunity to study the ecological forces that influence adaptation and speciation (Buggs, 2007). Although much progress has been made, there is much still to be understood about how patterns of introgression and selection contribute to these processes. For example, introgression in admixed populations can be a direct measure of reproductive isolation (Gompert et al., 2012), and asymmetric introgression can be important in determining the direction of evolutionary change (Bacilieri et al., 1996). Yet we do not know whether asymmetric introgression under these circumstances is a sign of competitive displacement or of higher-frequency backcrossing with one parent (Buggs, 2007). Moreover, the presence of a stable hybrid zone can reflect superiority of hybrid fitness in an ecological (or bioclimatic) niche intermediate to that occupied by either parental taxon (Buggs, 2007). The first step to using these "natural laboratories" for understanding the processes of selection, reinforcement, and speciation is to characterize the degree of introgression and the extent of ecological–phenotypic intermediacy in hybrid populations (Barton and Hewitt, 1985; Rieseberg and Blackman, 2010; Taylor et al., 2012).

Geographic patterns in genomic markers can provide a signature of past hybrid-zone dynamics. Given the differences in inheritance, markers from cytoplasmic genomes (mitochondrial and chloroplast markers) can be compared with nuclear markers to reveal patterns of admixture and whether they derive from maternal or biparental gene flow. Such studies have revealed patterns of extensive mixing among hybrids but isolation from the parents (Zeng et al., 2011) or of little admixture and hybrids associated strongly with one parental taxon (Cruzan and Arnold, 1999; Aboim et al., 2010). Hybrids of species with separate or partially separate sexes (i.e., dioecy and subdioecy) can provide additional insight into the genesis of asymmetry in hybrid zones. Specifically, such hybrids can answer how mating system (selfer vs. outcrosser) or sexual system (separate sexes vs. combined sexes) affects patterns of introgression (e.g., Engel et al., 2005; Wallace et al., 2011). The results of these analyses can be paired with geographic and bioclimatic information to create hypotheses regarding the formation, nature, and stability of the hybrid zone (Buggs, 2007; Wu and Campbell,

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[5]Author for correspondence (e-mail: tia1@pitt.edu)

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2007; Peñaloza-Ramirez et al., 2010; Wallace et al., 2011) and hypotheses can be tested with data on the phenotypic or functional intermediacy of hybrids or the fitness of reciprocal transplants (e.g., Wu and Campbell, 2007).

Fragaria × *ananassa* subsp. *cuneifolia* is the natural hybrid between two sister species of octoploid *Fragaria* (Rosaceae), *F. virginiana* subsp. *platypetala*, and *F. chiloensis* (Hancock and Bringhurst, 1979; Staudt, 1999). The parental species are differentiated by morphology (Staudt, 1999), ecological niche (Hancock and Bringhurst, 1979), sexual system, and, possibly, the particularities of their sex-determining chromosomes (Goldberg et al., 2010; Spigler et al., 2010; Govindarajulu et al., 2012). For instance, *F. virginiana* subsp. *platypetala* inhabits forest margins and woodlands, whereas *F. chiloensis* (sensu latu; Catling and Porebski, 1998a; Hancock et al., 2004) prefers sandy beaches and dunes (Staudt, 1999). Hancock and Bringhurst (1979) suggested that the morphological differences between these species played a major adaptive role in their ecogeographic distribution, especially in regard to water retention and use. It has been suggested that the hybrid might display morphological intermediacy (Catling and Porebski, 1998b; Staudt, 1999) and reside in an intermediate habitat, yet this has not been broadly tested, nor has a bioclimatic examination of the hybrid zone been undertaken. Moreover, although a previous study with limited sampling revealed that either parent can serve as maternal donor of *F.* × *ananassa* subsp. *cuneifolia* (Govindarajulu et al., 2012), the geographic or climatic pattern of introgression throughout the hybrid zone in terms of the nuclear and chloroplast genetic composition is unknown, so questions of the symmetry or parental dominance in the hybrid remain unanswered.

Accordingly, the goal of the present study was to characterize the hybrid zone of *F.* × *ananassa* subsp. *cuneifolia* genetically, bioclimatically, and ecologically. Specifically, we asked the following questions: (1) Is there a maternal species bias or geographic pattern to the nuclear and cytoplasmic genomes of *F.* × *ananassa* subsp. *cuneifolia* or do they show wide admixture? (2) What are the biogeographic characteristics of the *F.* × *ananassa* subsp. *cuneifolia* hybrid zone? (3) Are the hybrids ecologically or phenotypically intermediate? And finally, (4) is there an association between hybrid genotype and habitat or phenotype?

MATERIALS AND METHODS

***Species biology*—**All three octoploid species of *Fragaria* (Rosaceae) (*F.* × *ananassa* subsp. *cuneifolia*, *F. virginiana* subsp. *platypetala*, and *F. chiloensis*) are alloallopolyploids (AAA′A′BBB′B′; Bringhurst, 1990) with disomic inheritance ($2n = 8\times = 56$) (Ashley et al., 2003; Govindarajulu et al., 2012). They are all herbaceous perennial herbs that can reproduce by plantlets on stolons or by seeds on swollen receptacles (Staudt, 1999). *Fragaria chiloensis* is dioecious (males and females), whereas *F. virginiana* subsp. *platypetala* and *F.* × *ananassa* subsp. *cuneifolia* are subdioecious (females, males, and hermaphrodites) (Ashman, 1999; Staudt, 1999; Govindarajulu et al., 2012). Hermaphrodites are self-compatible, pollination occurs via a variety of small bees, flies, and ants (Ashman, 2000), and fruits are animal dispersed (T.-L. Ashman, personal observation).

Fragaria virginiana has a wide range across North America and may have been separated into two vicarious groups as the result of uplifting of the Rocky Mountains (Staudt, 1999). The primary focus of our study, the western subspecies (*Fragaria virginiana* subsp. *platypetala*), ranges from southern California, USA, to southern British Columbia, Canada, reaching from Colorado, USA, to the Pacific coastline; and *F. chiloensis* extends along the Pacific coast from southern California to the farthest west tip of Alaska, USA (Hancock and Bringhurst, 1979; Staudt, 1999). Although they share the same basic vegetative body plan, the leaf morphology of the two species reflects their habitat differences (e.g., the leaves of *F. virginiana* are thin and smooth, generally elongated and narrow, with pointed teeth, whereas those of *F. chiloensis* are short, thick, and coriaceous in texture, with crenate teeth; Staudt, 1999). Moreover, although *F. cascadensis* is a recently described decaploid species that is morphologically similar to *F. virginiana* subsp. *platypetala* (Hummer, 2012), which makes the two species difficult to distinguish in the absence of ploidy information, none of our samples originated from within the range of *F. cascadensis* (above 1000 m in the Oregon Cascades, USA; Hummer, 2012).

***Plastome sequencing and SNP validation*—**To identify single-nucleotide polymorphisms (SNPs) specific to the parental species' chloroplasts, we sequenced and assembled chloroplast genomes from two accessions: a female *F. chiloensis* (accession GP33 [HM1], Goldberg et al., 2010) and a male *F. virginiana* subsp. *virginiana* (accession o477.2; Spigler et al., 2010). Total genomic DNA was extracted in triplicate from fresh frozen leaves using the standard FastDNA extraction protocol (MP Biomedicals, Solon, Ohio, USA), purified with a Qiagen PCR purification kit (Qiagen, Valencia, California, USA), and quantified using the Qubit dsDNA HS assay (Life Technologies/Invitrogen, Carlsbad, California, USA). DNA (~60 ng) from each accession was "tagmented" and amplified by polymerase chain reaction (PCR) in duplicate, with 9 and 12 cycles, according to the Nextera protocols (Epicentre Biotechnologies, Madison, Wisconsin, USA). Enriched samples were electrophoresed on a 2% agarose gel and extracted in six narrow (~30 base pairs [bp]) bands between about 200 and 340 bp using Qiagen Gel Extraction kits. Extraction yields were quantified with the Qubit dsDNA HS assay, and sizes were verified using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). For each accession, three cleaned bands with the highest yields were combined for sequencing. Resulting insert sizes for the libraries were approximately 230–290 bp, and these were submitted to the Oregon State University Center for Genome Research and Biocomputing for 120-bp paired-end (PE) sequencing on the IlluminaGAIIx, and loaded at 10 ρM. The resulting sequence read pools were processed using Illumina's Real Time Analysis version 1.8 and CASAVA version 1.7.

Paired-end read pools for each accession were combined and searched for chloroplast homology using a perl script (Knaus, 2012) incorporating BLAT (Kent, 2002). From the subsets of reads with chloroplast homology, random draws of 100 000 and 200 000 reads were taken in triplicate using a perl script (B. Knaus and L. Wilhelm, U.S. Department of Agriculture Forest Service, unpublished) implementing the Fisher-Yates Shuffle algorithm (Durstenfeld, 1964). The short-read assembler YASRA (Ratan, 2009), as implemented in Alignreads (Straub et al., 2011), was used for plastome assembly with the *F. vesca* chloroplast genome as a reference (GenBank NC_015206.1, with a single copy of the IR region). Read pools of 100 000 read size were chosen to target chloroplast assemblies at 50–100× coverage depth (Straub et al., 2011); read pools of 200 000 read size were selected to minimize areas of the assemblies affected by stochastic dips in coverage. A cutoff of <5× coverage depth was used for masking positions conserved in relation to the reference. SNPs were masked unless coverage depth was ≥25×, and call proportion was ≥0.8. The resulting assemblies were aligned using default settings in MAFFT version 6.240 (Katoh et al., 2005) and adjusted by hand in BioEdit version 7.0.5.3 (Hall, 1999). A final consensus chloroplast sequence was created for each accession from its six aligned assemblies, using majority rule where discrepancies between assemblies existed. Positions were masked where no majority assembly was present.

To validate the species-specificity of the chloroplast SNPs identified by whole-plastome assembly, we sampled from 10 *F. virginiana* and 5 *F. chiloensis* from locations throughout their ranges (Appendix 1[A] and Appendix S1) and cytotyped them as described below.

Population sampling*—Wide sampling*—To widely assess the distribution of maternal donors of *F.* × *ananassa* subsp. *cuneifolia*, we requested a loan of all herbarium specimens identified as such and any identified as *F. virginiana* subsp. *platypetala* from west of the Cascade foothills or *F. chiloensis* from sites not on dunes from three herbaria: UBC (University of British Columbia, Vancouver, British Columbia), HSU (Humboldt State University, Arcata, California), OSC, and ORE (Oregon State University, Corvallis, Oregon). On the basis of morphological characters, all (54) putative *F.* × *ananassa* subsp. *cuneifolia* specimens (Appendix 1[B] and Appendix S2), including those originally identified as *F. chiloensis* (10) or *F. virginiana* (28), were included in the biogeographic study. Seventeen of these, and two additional single plants from (or from populations identified by) the National Clonal Germplasm Repository (NCGR:http://www.ars.usda.gov/main/site_main.htm?modecode=53-58-15-00), were subject to chloroplast SNP analysis (see below).

Deep sampling—Live plants were collected at ≥2m intervals along transects in 13 populations across the ecogeographic range of *F. virginiana* subsp. *platypetala*, *F. chiloensis*, and *F.* × *ananassa* subsp. *cuneifolia* between 2008 and 2011 (Appendix S3). In addition to populations identified by Staudt (1999) as *F.* × *ananassa* subsp. *cuneifolia* (Fort Stevens [FtS], Wren [WREN], Mary's Peak [MP]), we collected plants from populations that were identified as *F. virginiana* by the Germplasm Resources Information Network (GRIN: http://www.ars-grin.gov/) but phenotypically resembled *F.* × *ananassa* subsp. *cuneifolia* (Fisherman's Bend [FB], Silver Falls [SF]) as well as novel sites in habitats identified as likely locations of *F.* × *ananassa* subsp. *cuneifolia* (Dungeness Campground [DC], Hammond Trail [HAM]) or *F. virginiana* subsp. *platypetala* (Emigrant Springs [ES]). Other populations of *F. virginiana* subsp. *platypetala* (George Hudson Biological Reserve [GH], Kamiak Butte [KB]) were identified by L. Hufford (personal communication). We located sites of *F. chiloensis* using the NCGR (Honeyman State Park [HM]; http://www.ars.usda.gov/main/site_main.htm?modecode=53-58-15-00), previous publications (Salishan Road [SAL]; Goldberg et al., 2010), and Calflora (Eureka [EUR]; http://www.calflora.org).

Ten to 13 plants (genotypes) per population (Appendix 1[C] and Appendix S3) were grown in a 2:1 mix of Fafard no. 4 (Conrad Fafard, Agawam, Massachusetts, USA) and sand in 280-mL pots in the greenhouse at the University of Pittsburgh. In August 2011, two clones from each (except those from HAM, where clones were not available), were planted in 280-mL pots of the same mix. All plants were arranged randomly on benches in January 2012 and exposed to 21°C–16°C day–night conditions with supplemental lighting for three months. The plants received fertilizer and water and were treated for pests as needed.

Genotyping, phenotyping, and environmental characterization—*DNA extraction*—DNA was extracted from 70–100 mg of fresh tissue from 186 plants from the 13 populations (Appendix S3) following Doyle and Doyle (1990), modified to accommodate silica-based spin columns (Epoch Life Sciences, Sugar Land, Texas, USA). One leaflet (15–60 mg dry weight) was collected per plant for 17 herbarium specimens, and DNA was extracted following Jobes et al. (1995).

Cytotyping—We validated two of the SNPs (*pet*D and *ndh*F) identified via whole-plastome sequencing (see Results) that differentiated the chloroplast genomes of *F. virginiana* and *F. chiloensis* in a range-wide panel of 15 samples (Appendix S1) using Sanger sequencing or dCAPS (Neff et al., 1998). We then used these SNPs to determine the maternal donor for the wide and deep sampling of *F.* × *ananassa* subsp. *cuneifolia* and a set of deeply sampled populations of the parental species (Appendix S3) using either direct sequencing or dCAPS.

For all plants in the deep population sampling, either *ndh*F or both *ndh*F and *pet*D were amplified, whereas for the wide sampling both *ndh*F and *pet*D were amplified by PCR following Govindarajulu et al. (2012), with a modified reaction volume of 25 μL consisting of 10× Standard Taq Reaction Buffer (New England Biolabs, Ipswich, Massachusetts, USA), 10 mM dNTP, 0.5 μM each forward and reverse primer, 1 μL 100× Purified BSA (New England Biolabs), 1.5 units *Taq* DNA Polymerase (New England Biolabs), and 1.5 μL DNA. For nine herbarium samples, we used PuReTaq Ready-To-Go PCR beads (GE Healthcare, Pittsburgh, Pennsylvania, USA).

To identify SNPs in the wide samples, the amplified PCR product was Sanger sequenced and aligned using Sequencher version 4.8 (Gene Codes, Ann Arbor, Michigan, USA). For the deep samples, the amplified PCR product was purified using a Qiagen PCR purification kit, and we performed dCAPS following Govindarajulu et al. (2012) to elucidate the SNP genotypes. For this assay 6 μL of purified *ndh*F product from each sample was digested with 5 units of the restriction enzyme MslI for 2 h at 37°C, and 6 μL of purified *pet*D product with 5 units of TaqαI at 65°C for 2 h. The recognition site of MslI includes the bolded variable site (CATTG^AAGT**A**/CATTGAAGT**G**) within *ndh*F, and that of TaqαI includes the bolded variable site (T^C**G**A/TC**A**A) in *pet*D. Products were assayed on agarose gels and species-specific cytotypes identified as follows: (1) *ndh*F locus, a 500-bp (uncut) product, identified *F. virginiana*, whereas two fragments (336 and 166 bp product) corresponded to the *F. chiloensis* cytotype; (2) two *pet*D fragments (56 and 44 bp) differentiated the *F. chiloensis* cytotype from the *F. virginiana* (uncut) cytotype. In sum, both genes were scored in 134 of the 212 total samples, verifying parental assignations.

Nuclear marker genotyping—A total of five nuclear microsatellite primer-pairs (nrSSRs) (ARSFL04, ARSFL014, ARSFL022, ARSFL007 [Lewers et al., 2005], and CFVCT017 [Sargent et al., 2009]) were genotyped for 12–15 individuals per population (Appendix S3). The PCR amplification with each primer pair was performed separately following Poor Man's PCR protocol as previously described (Schuelke, 2000; Spigler et al., 2008; Govindarajulu et al., 2012) on a PTC-225 thermal cycler (MJ Research, GMI, Ramsey, Minnesota, USA) in 15 μL volume. Four positive control samples were included on each 96-well plate. We multiplexed PCR products from two or three primers by mixing 1.5-μL aliquots from each reaction with 0.2 μL LIZ500 standard and 10.5 μl Hi-Di formamide (Applied Biosystems, Life Technologies, Carlsbad, California, USA). Fragment analysis and genotyping were conducted using ABI 3730XL DNA analyzer and GeneMapper (Applied Biosystems). Scoring products of SSR primer pairs in polyploids can be challenging because of high levels of heterozygosity and difficulty in assessing which products occur in more than one copy (Arroyo et al., 2010). Thus, we adopted a commonly used approach of treating products as dominant markers and scoring presence or absence of a given product ("allele") size (Arroyo et al., 2010). In total, 81 products (ARSFL04 [10] ARSFL014 [19], ARSFL022 [15], ARSFL007 [18], and CFVCT017 [19]) were scored on 185 individuals from the 13 populations.

Morphological characters—We scored six morphological characters that have been identified as differentiating the two sister species (coriaceousness, vein reticulation, leaflet length, leaflet width, orientation of trichomes on the petiole, and leaf thickness; Catling and Porebski, 1998a; Staudt,1999; Hancock et al., 2004) on 131 genotypes (plants) from the 13 populations grown in a common greenhouse environment. For the majority of genotypes (86%), we scored two clones per genotype and averaged them. The same individuals were both phenotyped and genotyped whenever possible (64% of all data), and leaves were standardized for age. Qualitative measures of leaf coriaceousness and prominence of secondary veins on the underside of the leaf were scored as absence of coriaceousness or reticulation (1), slight presence (2), or strong presence (3). Trichome orientation was scored as parallel/"appressed" (1) or perpendicular/"spreading" (2) to the pedicle. Quantitative measures of length and width (to 0.01 mm) were taken on the middle leaflet of the largest leaf using a digital caliper. Quantitative measures of leaf thickness were obtained from the dry weight (to 0.01 mg) of a 6-mm-diameter circular sample of tissue (after Witkowski and Lamont, 1991).

Environmental characteristics—Habitat characteristics were collected from five to ten 1-m^2 plots/population at 12 of the 13 populations (excluding EUR). We measured photosynthetically active radiation at both the ground level and in full sun and calculated percent full sun at ground level as 100 × (ground/full). Soil was collected, weighed (wet), dried to constant weight, reweighed (dry), and submitted to the Agricultural Analytical Services Laboratory at Pennsylvania State University (http://www.aasl.psu.edu/) for total nitrogen, phosphorus, and potassium analysis. Percent soil moisture was estimated as 100 × [(wet − dry)/wet].

To assess the bioclimatic and geographic distribution of the hybrid, we used location records for *F. virginiana* subsp. *platypetala*, *F. chiloensis*, and *F.* × *ananassa* subsp. *cuneifolia* in North America from the Global Biodiversity Information Facility (GBIF: http://www.gbif.org). We augmented GBIF records with those of our sampled populations and herbarium specimens. In instances where GPS coordinates were not recorded on herbarium sheets, coordinates were extrapolated from Google Earth (http://google.com/earth/index.html) when city and state (or province) were indicated. We assumed that records in GBIF were properly identified, but we acknowledge that this may not be true in some cases because of difficulties in distinguishing between the species. We obtained 571 records (300 *F. virginiana* subsp. *platypetala*; 209 *F. chiloensis*; and 62 *F.* × *ananassa* subsp. *cuneifolia*) and extracted bioclimatic variables from WorldClim data (2.5 × 2.5 min resolution; http://www.worldclim.org/bioclim) using DIVA-GIS (DIVA-GIS version 7.5; http://www.diva-gis.org). We selected altitude, annual mean temperature, and annual precipitation for analysis. We mapped the coordinates from this data set to visualize the distribution pattern of the hybrid and parental species.

Statistical analyses—*Morphometric analysis*—We used principal component analysis (PCA) to reduce the six leaf traits into fewer orthogonal ones and used these to test for intermediacy in leaf morphology of the hybrid species, as well as to determine whether maternal cytotype affects the phenotypic intermediacy or affinity with the parental cytoplasmic donor. The PCA analysis was performed with both qualitative and quantitative data (as in Schmickl and Koch, 2011) using MVSP version 3.131 (Kovach Computing Services, Wales, UK).

We tested for differences among species in PC1 and PC2 with one-way analysis of variance (PROC GLM, SAS version 9.1; SAS Institute, Cary, North Carolina, USA) and for a cytotype effect within *F.* × *ananassa* subsp. *cuneifolia* with a two-sample *t*-test with unequal variances (Welch's *t*-test with Satterthwaite's approximate degrees of freedom) using Analyst in SAS. In addition, we used canonical discriminant function analysis (PROC CANDISC and PROC DISCRIM in SAS) to phenotypically differentiate hybrids (populations and individuals) from the parental species. Raw data used for morphometric analysis were deposited at Dryad (http://dx.doi.org/10.5061/dryad.84t05).

Cytotypic and nuclear marker analysis—To assess maternal-species bias and geographic patterns in chloroplast donor of *F.* × *ananassa* subsp. *cuneifolia*, we performed chi-square analyses on all widely sampled populations plus the majority type from deeply sampled ones. To infer population admixture based on nSSR markers, we performed a Bayesian model-based clustering analysis in Structure version 2.3.3 (Pritchard et al., 2000) with the 178 individuals that had marker data for three to five SSR loci. This approach assigns individuals to a population or to two or more populations (assuming K populations in a model) if their genotypes indicate that they are admixed. We used the admixture ancestry model with independent allele frequencies and predefined population information for a range of K values starting from 1 to 13. We used 10 000 burn-ins and MCMC replicates for each run, with 10 replicate runs conducted for each K value. Finally, we used the online tool Structure Harvester that implements the method from Evanno et al. (2005) to extract a ΔK value that indicates the optimal number of genetic clusters from data. Data used for structure analysis were deposited at Dryad (http://dx.doi.org/10.5061/dryad.84t05).

Geographic and bioclimatic variables—We determined whether geographic or bioclimatic variables varied among the species using one-way analyses of variance (ANOVA) and Tukey's tests to detect significant differences between species (PROC GLM in SAS). Altitude and annual mean temperature were natural log transformed, and mean annual precipitation was square-root transformed to fit model assumptions of normality. We then tested for a significant cytotype effect within *F.* × *ananassa* subsp. *cuneifolia* populations using a two-sample *t*-test (Analyst in SAS). Annual mean temperature was not normally distributed, so we tested for a cytotype effect using a Mann-Whitney-Wilcoxon two-sample test (PROC NPAR1WAY in SAS). Across all five environmental characteristics measured in situ, we tested whether *F.* × *ananassa* subsp. *cuneifolia* sites were intermediate between the parental species using a binomial probability test (i.e., intermediate or not).

RESULTS

***Plastome sequencing and SNP validation*—**Plastome assembly replicates were consistent within and between species. In brief, across both species, mean (± SE) coverage depth for 100 000 and 200 000 read assemblies was 81.42× ± 0.07 and 163.09× ± 0.13, and the number of contigs in assemblies was slightly higher for 100 000-bp assemblies than for 200 000-bp assemblies (13.83 ± 1.76 vs. 10.83 ± 1.01). Plastome length for *F. chiloensis* (GenBank JN884816) and *F. virginiana* (GenBank JN884817) assemblies averaged 129 492 ± 66 bp and 129 324 ± 259 bp, respectively, with 7089 ± 43 and 1735 ± 1246 positions masked. Final consensus sequences were 129 659 bp and 129 676 bp for *F. chiloensis* and *F. virginiana*, respectively, with 134 and 136 positions masked. With inclusion of both inverted repeat copies, plastome lengths were 155 604 bp and 155 621 bp for *F. chiloensis* and *F. virginiana*, respectively, again with 134 and 136 positions masked.

A total of 60 SNPs were identified between the aligned final consensus sequences of *F. chiloensis* and *F. virginiana* plastomes (Fig. 1; Appendix S4). The majority (42/60) of polymorphisms were located in intergenic regions, whereas smaller proportions were found in protein-coding (11/60) and intronic (7/60) regions. Twenty-five polymorphisms were classified as substitutions or indels directly associated with mono- or dinucleotide repeats, 26 were substitutions that did not affect repeats, and 10 were associated with complex repetitive units or palindromic repeats.

Regardless of geographic location or subspecies designation, all *F. virginiana* shared the same SNPs in *pet*D (G) and *ndh*F (A) (Appendix S1), and these differed from all *F. chiloensis*, which shared the same SNPs (*pet*D: A; *ndh*F: G).

***Nuclear and cytoplasmic genome structure*—**In total, 212 samples were cytotyped from the three species (Fig. 2A, B). All 31 samples of *F. chiloensis* were found to have the *F. chiloensis* cytotype, and all 32 samples of *F. virginiana* subsp. *platypetala* were found to have the *F. virginiana* cytotype. We found no significant bias in maternal species cytotype across all populations of *F.* × *ananassa* subsp. *cuneifolia,* 42% had *F. virginiana* majority cytotype, and 58% had *F. chiloensis* majority cytotype ($\chi^2 = 0.61$, df = 1, $P > 0.40$, $n = 26$; Fig. 2A). To characterize the longitudinal distribution of cytotypes, we divided the hybrid zone into eastern (toward inland North America) and western (toward the Pacific Ocean) regions using the mean longitude (123.449°W) of the populations. We found that half as many of the populations in the eastern region had the *F. chiloensis* cytotype (3/14) as in the western region (8/12).

Within the deeply sampled populations of *F.* × *ananassa* subsp. *cuneifolia*, we found variation in cytotype composition, with the percentage of the *F. chiloensis* cytotype ranging from 6% to 100% (Fig. 2B; Appendix S5). In the eastern region, one *F.* × *ananassa* subsp. *cuneifolia* population had a majority of the *F. chiloensis* cytotype (MP, 94%) and three had a majority of *F. virginiana* cytotype (SF, 92%; FB, 92%; WREN, 94%). By contrast, in the western region all three sites had a majority of the *F. chiloensis* cytotype (DC, 76%; FtS, 100%; HAM, 100%), again suggesting a geographic bias to maternity.

Our Bayesian analyses of population structure (Fig. 2C) revealed a maximum ΔK value for two clusters, indicating two distinct nuclear genetic groups. Populations of *F. chiloensis* (EUR, HM, SAL) showed a high proportion of membership to cluster 1 (74 ± 14%), whereas those of *F. virginiana* subsp. *platypetala* (ES, GH, KB) to cluster 2 (69 ± 10%), although neither of the parental species displayed exclusive membership to either cluster. On average, populations of *F.* × *ananassa* subsp. *cuneifolia* reflected nearly equivalent membership to each cluster (e.g., cluster 1: 53 ± 12%), but membership varied widely (between 8% and 90%) among populations. For instance, MP (92%), WREN (75%), and FB (72%) showed a high proportion of membership to cluster 2, whereas FtS (90%), HAM (67%), SF (81%), and DC (71%) showed higher values of membership to cluster 1. There was no consistent association between the chloroplast and nuclear genomic affiliations. For instance, MP had a majority of *F. chiloensis* cytotype but showed a high proportion of membership to cluster 2, and SF had a majority of *F. virginiana* cytotype but displayed a higher proportion of individuals with membership to cluster 1. Within populations, individuals with the minority cytotype tended to be representative of their population's nuclear constitution rather than that predicted by their cytotype; for example, the individual with *F. chiloensis* cytotype from FB showed 63% membership to cluster 2. These results suggest a high degree of introgression among both the hybrid and parental populations.

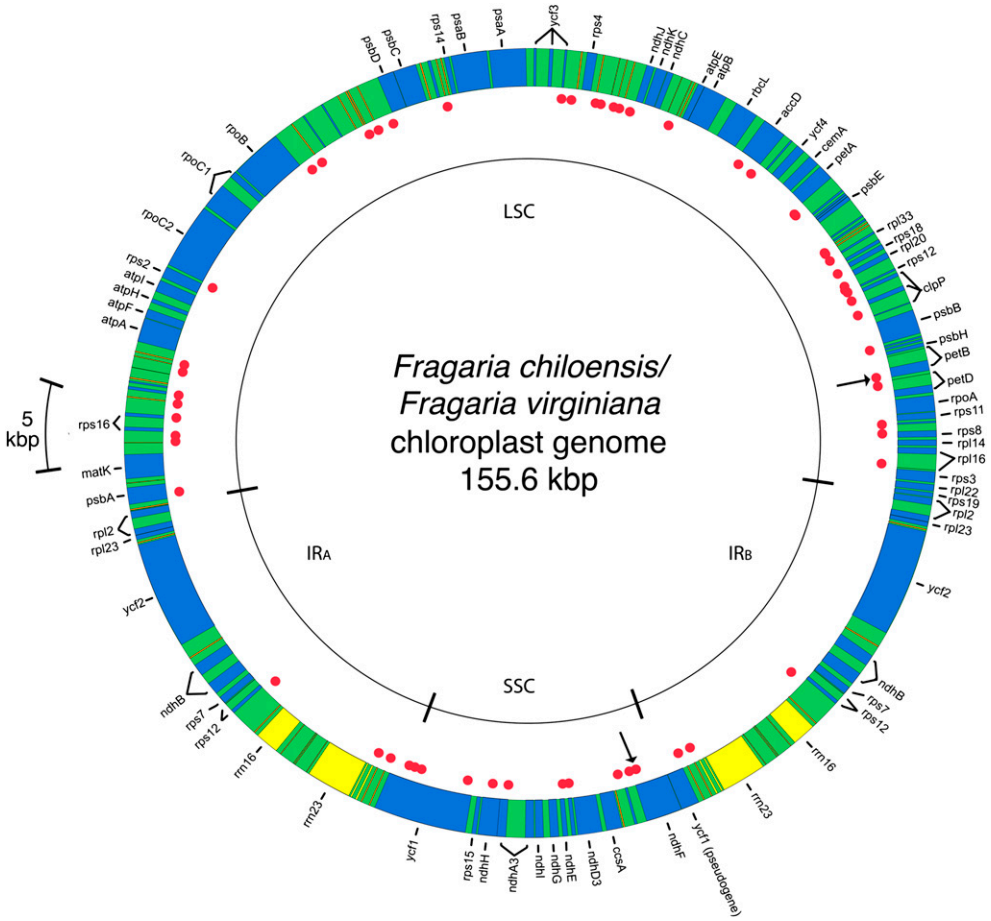


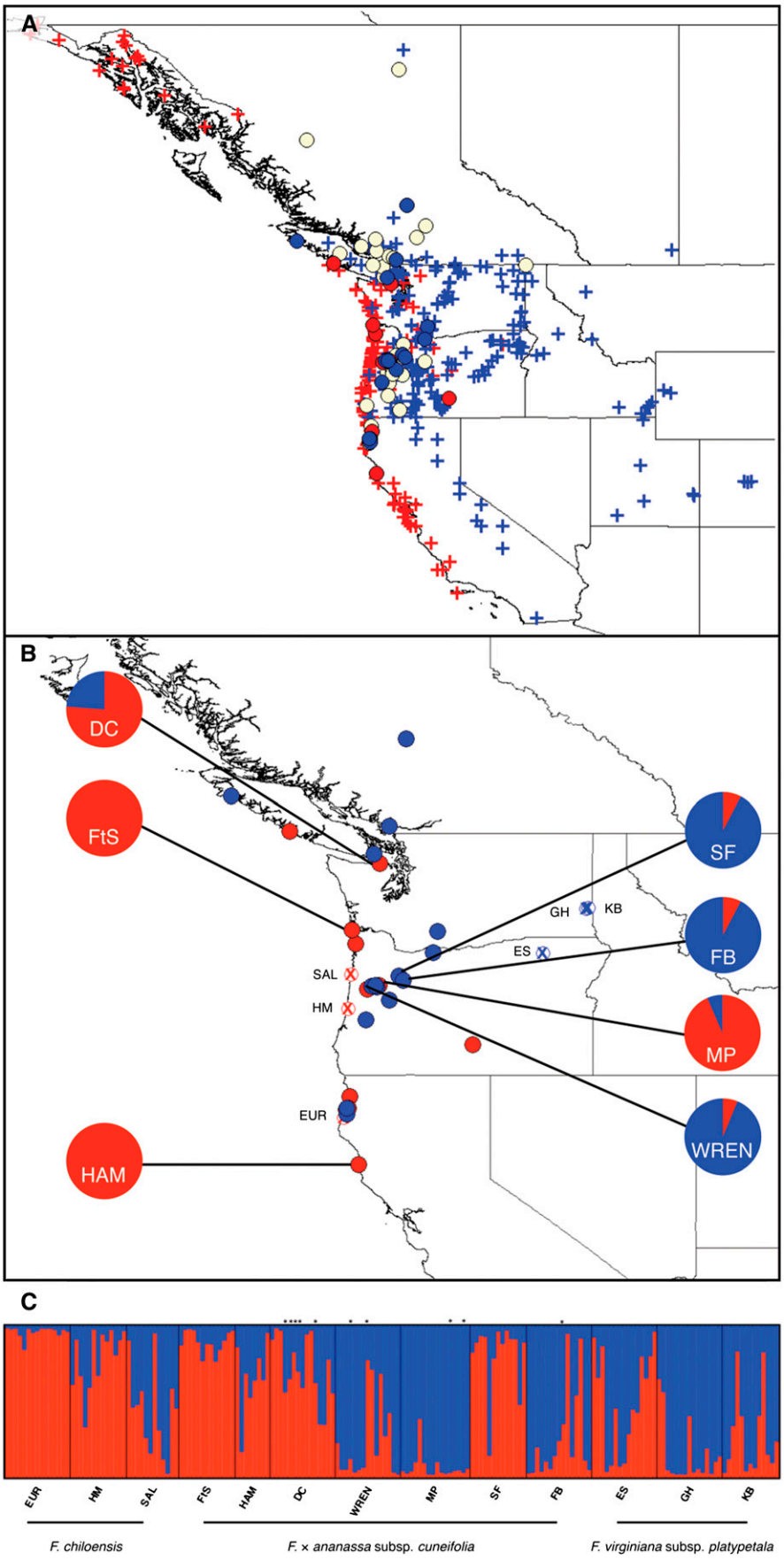
Fig. 1. Map of aligned *Fragaria chiloensis* and *F. virginiana* chloroplast genomes, showing location of interspecific polymorphisms (dots). Arrows indicate SNPs chosen for analysis in this study. Color-coding of plastome map is as follows: blue = protein-coding exons; orange = tRNA loci; yellow = rRNA loci; green = noncoding regions. LSC/SSC = large/small single copy region; IR_A/IR_B = inverted repeat regions.

***Phenotypic intermediacy*—**The PCA on morphological traits identified two main principal components. The first (PC1) accounted for 77% of the observed variation. Leaf coriaceousness and venation were the main contributors to PC1, and both were positively correlated with it ($r = 0.61$ and 0.67, respectively). The second (PC2) accounted for 13% of the variation. Petiole pubescence was positively associated with PC2, but leaf specific mass was negatively associated with it ($r = 0.68$ and −0.66, respectively). One-way ANOVA showed a significant species effect for PC1 and PC2 ($F_{2, 127} = 132.88$; $F_{2, 127} = 122.98$, respectively; $P < 0.0001$ for both; Fig. 3A, B). Post hoc comparisons showed that all three species differed significantly for PC1: *F. chiloensis* had highest values, followed by *F.* × *ananassa* subsp. *cuneifolia* and then *F. virginiana* subsp. *platypetala* (Fig. 3A). For PC2, only *F.* × *ananassa* subsp. *cuneifolia* differed significantly from the parental species, which did not differ from each other (*Fragaria* × *ananassa* subsp. *cuneifolia* vs. *F. virginiana* subsp. *platypetala* or *F. chiloensis*) (Fig. 3B).

Within *F.* × *ananassa* subsp. *cuneifolia*, we found a significant cytotype effect for PC1 ($t_{1, 72.7} = 5.2$, $P = 0.0001$) and PC2 ($t_{1, 70.9} = -2.7$, $P = 0.01$). Chloroplast genotype mirrored parental phenotype values for PC1 but not PC2. Moreover, among individuals, there were significant correlations between PCs and genetic cluster membership. Specifically, PC1 and genetic cluster 1 were positively correlated ($r = 0.42$, $P < 0.0005$, $n = 64$)—that is, plants with greater genetic affiliation to *F. chiloensis* had more coriaceous leaves and pronounced venation; whereas PC2 was negatively correlated with genetic cluster 1 ($r = -0.35$, $P < 0.004$, $n = 64$), which means that individuals with lower genetic affiliation to *F. chiloensis* had greater petiole pubescence but less leaf-specific mass.

Canonical discriminant function analyses produced two canonical variables: the first (CAN1) accounted for 81% of the variation and had high loadings of leaf coriaceousness and petiole pubescence (canonical coefficients of 1.6 and 3.8, respectively) whereas the second (CAN2) accounted for 19% and loaded heavily on leaf-specific mass and petiole pubescence (canonical coefficients of 1.66 and −1.7 respectively). The discriminant function analysis confirmed our classification for the three species overall 93% of the time, with 91% for *F. chiloensis*, 89% for *F.* × *ananassa* subsp. *cuneifolia*, and 100% for *F. virginiana* subsp. *platypetala* (Fig. 4). Of the 11% of *F.* × *ananassa* subsp. *cuneifolia* that were classified as *F. chiloensis* in this analysis, seven individuals were from FtS and one (a minority cytotype) from WREN.

***Environmental characteristics*—**Site characteristics of *F.* × *ananassa* subsp. *cuneifolia* populations were in-between the parental species for all five measured environmental variables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F. chiloensis
F. × ananassa subsp. cuneifolia
F. virginiana subsp. platypetala

(binomial $P = 0.03$; Table 1). Moreover, analysis of bioclimatic data indicated a significant species-effect for all of the variables (altitude: $F_{2, 451} = 126.6$; temperature: $F_{2, 451} = 17.3$, annual precipitation: $F_{2, 451} = 51.8$; all $P < 0.001$; Fig. 5). Locations of *F.* × *ananassa* subsp. *cuneifolia* populations were significantly different from both parental species in altitude and annual precipitation (Fig. 5A,C). With respect to temperature, however, *F.* × *ananassa* subsp. *cuneifolia* sites were similar to *F. chiloensis* sites, and both of these were warmer than *F. virginiana* subsp. *platypetala* sites (Fig. 5B). Within *F.* × *ananassa* subsp. *cuneifolia*, although there were trends that mirrored the habitat of the cytotype donor species, there was no evidence of cytotype differences for any of the bioclimatic variables (Fig. 5A–C; altitude: $t_{1, 22.4} = -0.58$; temperature $U_{11,15} = 151.5$; precipitation: $t_{1, 16.3} = 0.29$; all $P > 0.45$).

DISCUSSION

Our genetic and ecological characterization of the hybrid zone of *F.* × *ananassa* subsp. *cuneifolia* revealed several patterns. First, although there was significant admixture and little overall maternal bias in chloroplast or nuclear genomes within the hybrid, there was wide heterogeneity in the signature of introgression. This plus geographic structure suggests differences in the prevailing direction of gene flow or selection. Second, the hybrid expressed both ecological (i.e., bioclimatic) and phenotypic intermediacy, but there was still a discernible fingerprint of the parental species within the hybrid. That is, there was an association between phenotype and genotype across hybrid individuals, and this was evident from markers in both nuclear and chloroplast genomes. Nonetheless, significant polymorphism within populations is consistent with ongoing introgression in this hybrid.

Evidence of pattern of hybridization from nuclear and cytoplasmic genomes—Consideration of both diagnostic chloroplast SNPs and nSSR confirmed the value of multiple genomes to assess hybridization between two closely related taxa (Bacilieri et al., 1996; Aboim et al., 2010). Even though *F. chiloensis* and *F. virginiana* diverged only 0.19–0.86 million years ago (Njuguna et al., 2013) whole-plastome sequencing and assembly identified 60 species-specific SNPs, and we were able to finely characterize the maternal contributions to the hybrid zone using two of these. Analysis of both widely and deeply sampled populations revealed complex patterns of intermixing. The wide sample of populations indicated that hybrids between *F. chiloensis* and *F. virginiana* are formed in both directions, with no evidence of a maternal bias. Although all populations had a clear majority cytotype, both cytotypes were evident in 5 out of 7 deeply sampled *F.* × *ananassa* subsp. *cuneifolia* populations, indicating continued gene (seed) flow or incomplete cytotype exclusion, because paternal leakage (i.e., nonmaternal inheritance; McCauley et al., 2007) of the chloroplast has not been detected in hybrid crosses of *F.* × *ananassa* subsp. *cuneifolia* (Govindarajulu et al., 2012) or other *Fragaria* species (Davis et al., 2010). Admixed individuals were identified in all hybrid populations (i.e., proportional membership scores ranged from 2% to 99% cluster 1), and some populations showed a distinct incongruity between nuclear and cytotype affiliation (Fig. 2B, C). Even in the case of populations that showed little admixture within individuals, the majority nuclear and cytotypic affinities revealed introgression (i.e., MP was highly associated with cluster 2 but had *F. chiloensis* as the majority cytotype, whereas SF was highly associated with cluster 1 but had *F. virginiana* majority cytotype). That is not to say, however, that all populations of *F.* × *ananassa* subsp. *cuneifolia* showed a cyto–nuclear incongruity; cytotypic and nuclear affiliations corresponded to the same parental species at FtS and FB.

It is also interesting to note that Structure analysis detected levels of nuclear admixture in some of the parental species' populations (e.g., SAL and ES; mean cluster 1: 47% and 51%, respectively) that were equivalent to or greater than the average level of nuclear admixture across *F.* × *ananassa* subsp. *cuneifolia* populations (mean cluster 1: 53%). Populations like these (and the "misclassified" *F. chiloensis* individuals at FtS; Fig. 4) could reflect ongoing backcrossing between the hybrid and the parental species, as suggested by Staudt (1999) on the basis of phenotypic observations and habitat disturbance level. By contrast, however, we found no evidence of admixture in chloroplast genomes in the six parental species populations that were deeply sampled (Appendix S3), which suggests that the observed admixture in nuclear genome of SAL and ES could simply reflect the recent divergence of the parental species (Njuguna et al., 2013) and lack of private alleles at the neutral nSSR loci (Beaumont et al., 2001; Mirol et al., 2010). Additional data from highly variable nuclear loci would help resolve this issue, because the high assignment probabilities based on phenotypic data support the dissimilarity of the parental species (Fig. 4).

Although, on average, both parental species contributed equally to the genomic constitution of *F.* × *ananassa* subsp. *cuneifolia*, populations of this hybrid species showed geographic structure. The *F. chiloensis* type dominated in the western region, and the *F. virginiana* type was more common in the inland eastern region. This pattern was seen most strongly in the maternally inherited chloroplast, which could suggest a geographic limitation to gene flow via seeds, perhaps

←

Fig. 2. (A) Geographic representation of *Fragaria chiloensis* (red +), *F. virginiana* subsp. *platypetala* (blue +), and *F.* × *ananassa* subsp. *cuneifolia* (circles) populations from GBIF records, wide samples, and deep sampled populations. *Fragaria* × *ananassa* subsp. *cuneifolia* records are broken down by cytotype (red circle = *F. chiloensis*; blue circle = *F. virginiana*; yellow circle = unknown cytotype). Note: 47 *F. chiloensis* sites along the Pacific coastline to Alaska are not pictured. (B) Geographic representation of cytotype of *F.* × *ananassa* subsp. *cuneifolia* populations in the hybrid zone (red circle = *F. chiloensis*; blue circle = *F. virginiana*). Pie-chart inserts show the breakdown of maternity within deeply sampled populations (red = *F. chiloensis*; blue = *F. virginiana*). Sites marked by (×) are locations of *F. chiloensis* (red) and *F. virginiana* subsp. *platypetala* (blue) populations that were deeply sampled. (C) Bar plot of individual assignment probability for each individuation of deeply sampled populations of *F. chiloensis*, *F.* × *ananassa* subsp. *cuneifolia*, and *F. virginiana* subsp. *platypetala* produced by Structure. Populations are organized in order of increasing longitude within each species. Red = cluster 1, high values of membership held by *F. chiloensis*; blue = cluster 2, high values of membership held by *F. virginiana* subsp. *platypetala*. The highlighted asterisks on top indicate individuals with a minority cytotype within a given deeply sampled population.

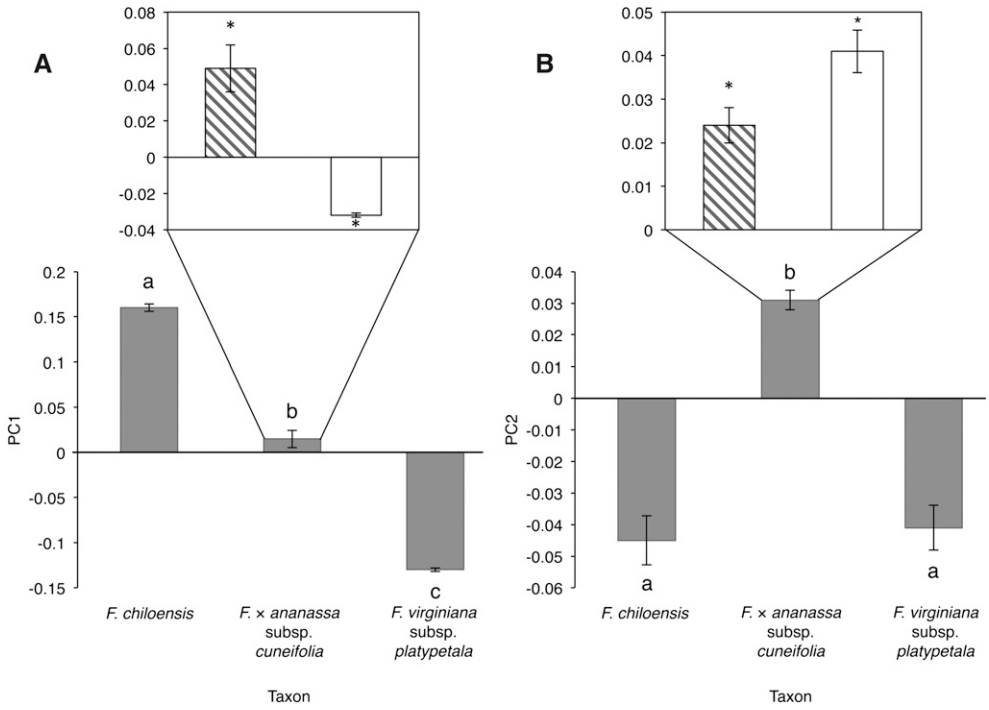

Fig. 3. (A) PC1 and (B) PC2 from a principal component analysis based on six morphological traits. Mean ± SE are shown for *Fragaria chiloensis*, *F.* × *ananassa* subsp. *cuneifolia*, and *F. virginiana* subsp. *platypetala* with inserts reflecting the subset of *F.* × *ananassa* subsp. *cuneifolia* with *F. chiloensis* (striped bars) or *F. virginiana* (white bars) cytotype.

because of limits imposed by dispersal of diaspores via birds or other small mammals (Aguinagalde et al., 2005; Worth et al., 2010). Alternatively, the geographic pattern could reflect strong habitat-based patterns of natural selection (Nakazato et al., 2010).

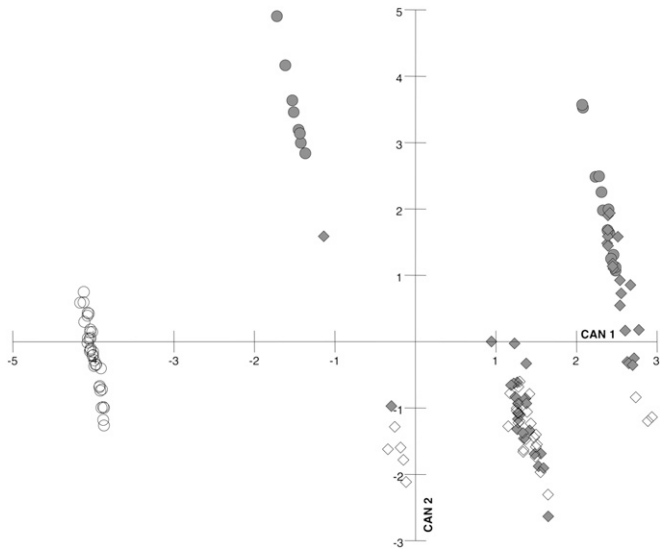

Fig. 4. Scatter plot of canonical discriminant scores (CAN1 and CAN2) for each plant (genotype) in the deep population sampling. Circles are the parental species (*F. chiloensis* [filled] and *F. virginiana* subsp. *platypetala* [open]). Diamonds are *F.* × *ananassa* subsp. *cuneifolia* with *F. chiloensis* cytotype (filled) or *F. virginiana* subsp. *platypetala* cytotype (open).

Phenotypic and bioclimatic patterns of hybridization— Hybrids occupied sites bioclimatically and ecologically intermediate between the parental species (Table 1; Fig. 5), and those with *F. chiloensis* cytotype tended toward sites more like the *F. chiloensis* habitat (similarly for *F. virginiana*). Such a pattern could reflect selection for coordination between the nuclear and chloroplast genomes for physiological competency (especially photosynthesis and WUE) and high performance in parent-like sites (Wu and Campbell, 2007). Consistent with this hypothesis, we found that greenhouse-grown hybrids with *F. chiloensis* cytotype were more similar to *F. chiloensis* in PC1 (leaf coriaceousness and reticulation of venation) and that there was a significant correlation between PC1 and nuclear affinity to *F. chiloensis*) (e.g., genetic cluster 1). In addition, Hancock et al. (1989) found a positive relation between photosynthetic rate and proportion *F. chiloensis* cultivated hybrids. In sum, these data support an ecologically based selective hypothesis for the hybrid-zone heterogeneity, but experiments with genetically characterized natural hybrids or experimentally produced ones grown and phenotyped under field conditions would be needed to confirm this (Wu and Campbell, 2007).

Two populations were fixed for one species' chloroplast genome, but with strong nuclear affinity to the other species (MP and SF: 7% and 81% *F. chiloensis*, respectively), provide an interesting contradiction to the cyto–nuclear coordination hypothesis. These two populations are in the meadows of the Coast and Cascades mountain ranges and could reflect long-distance bird-mediated seed dispersal followed by widespread pollen swamping. Such patterns have been proposed in other species whereby

TABLE 1. Habitat characteristics (mean ± SE: sun exposure, soil moisture, and soil nutrient content: nitrogen [N]; phosphorus [P]; potassium [K]) for *Fragaria chiloensis*, *F.* × *ananassa* subsp. *cuneifolia*, and *F. virginiana* subsp. *platypetala* populations.

Species	Percent full sun	Percent moisture	N (%)	P (ppm)	K (ppm)
F. chiloensis	71.9 ± 19.9	3.6 ± 1.4	0.04 ± 0.03	16.5 ± 3.5	39.1 ± 0
F. × *ananassa* subsp. *cuneifolia*	29.2 ± 7.2	16.9 ± 5.1	0.23 ± 0.09	54.1 ± 17.7	162.0 ± 21.6
F. virginiana subsp. *platypetala*	23.2 ± 6.8	27.7 ± 6.2	0.34 ± 0.11	66.7 ± 35.7	404.0 ± 175.3

long-distance dispersal leads to a cytotypic bottleneck (e.g., Le Corre et al., 1997), and unidirectional pollen swamping can lead to the extinction of one nuclear gene pool over time (Beatty et al., 2010). In addition, similar to asymmetries derived from the mating system or pollination (Wallace et al., 2011), one might predict that loss of one parental nuclear genome may occur faster when hybridizing species are dioecious (separate sexes) because half of the propagules do not produce pollen (females) and, thus, may be more likely to be pollinated by resident individuals (i.e., the other species) than by comigrants. Engel et al. (2005) proposed a similar idea when explaining the asymmetric introgression between hermaphrodite *Fucus spiralis* and dioecious *F. vesiculosus*: lower introgression of *F. spiralis* into *F. vesiculosus* was attributed to an asymmetry in sperm production. Alternatively, because the two hybridizing species may have different sex-determining regions (Goldberg et al., 2010; Govindarajulu et al., 2012), it is possible that sterility–inviability that results from hybridization (Haldane's rule; Orr, 1997) could lead to asymmetries in reproduction or survival that are reflected in the incongruity of genomes (R. Wang, unpublished data). These ideas can be effectively explored by comparing introgression of markers linked to the sex-determining region with others elsewhere in the genome (Payseur, 2010). Integrating genetic linkage maps (Goldberg et al., 2010; Govindarajulu et al., 2012) and the genome sequence of *Fragaria vesca* (Shulaev et al., 2011) will help facilitate this endeavor.

***Conclusions*—**Despite the heterogeneity among populations, the genetic results as a whole suggest a high degree of interfertility between the two parental and the hybrid species and point to a long history of backcrossing and genetic mixing in the hybrid zone. Our results also lend support to conjectures made by previous authors that *F. virginiana* subsp. *platypetala* is morphologically and genetically more similar to *F. chiloensis* than to *F. virginiana* (Harrison et al., 1997), although this appears more clearly in the phenotype than in the genotype. The present work is novel in that it uncovers the genetic complexity that underlies the hybrid zone first hypothesized by Staudt (1999) and provides a geographic fingerprint of introgression that reflects distance to the parental species' range margins. Whether this is due to gene flow and/or reinforced by selection for specific parental traits in the bioclimatically intermediate habitat occupied by the hybrid will require additional work to dissect the causes of genomic introgression and nuclear–cytoplasmic disassociation. Nonetheless, the present work provides evidence of gene flow between *F. virginiana* and *F. chiloensis* and suggests that this hybrid zone may provide an exciting ecological

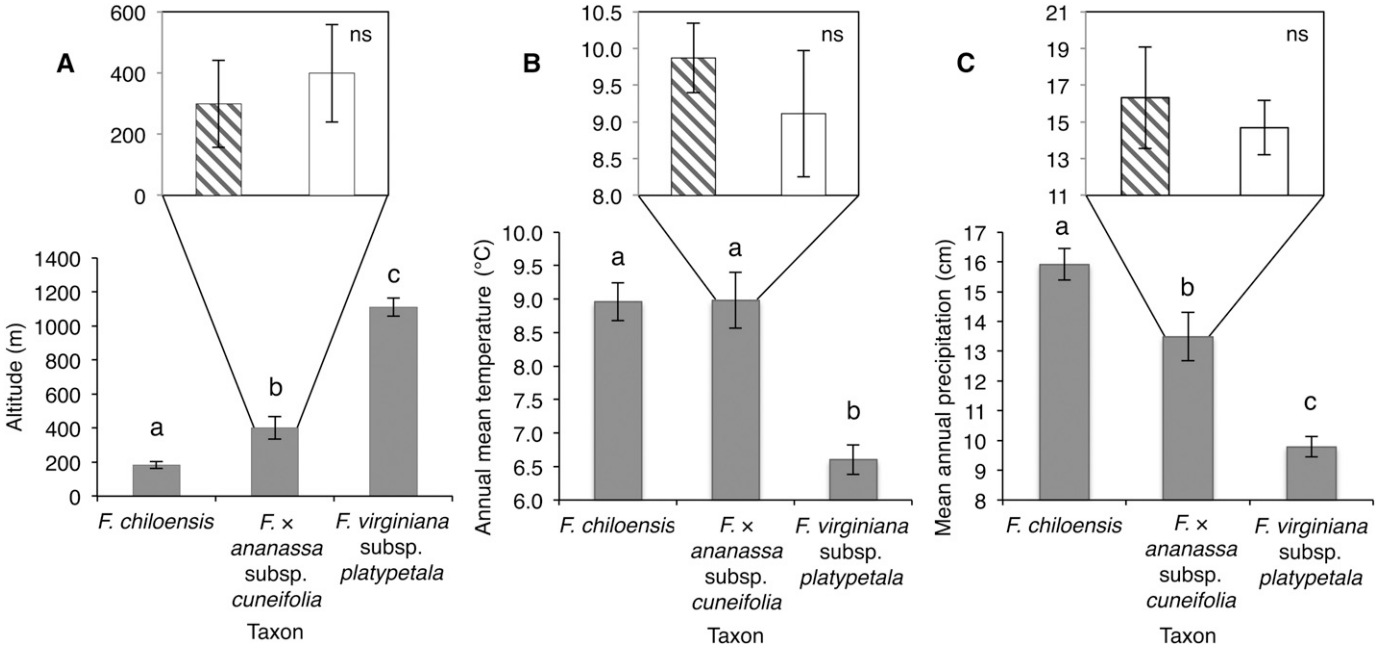

Fig. 5. Mean ± SE (A) altitude, (B) annual temperature, and (C) annual precipitation for *F. chiloensis*, *F.* × *ananassa* subsp. *cuneifolia*, and *F. virginiana* subsp. *platypetala*, with inserts reflecting the subset of *F.* × *ananassa* subsp. *cuneifolia* with *F. chiloensis* (striped bars) or *F. virginiana* (white bars) cytotype.

context for elucidating other parts of the evolutionary history of octoploid *Fragaria*, such as the dynamic nature of sex-chromosome evolution in these species (Govindarajulu et al., 2012).

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APPENDIX 1. Plant material used for phenotype and genotype analyses. Accession numbers with location and GenBank accession numbers are provided for samples that were sequenced to validate SNP in *pet*D and *ndh*F. Voucher information for herbarium samples loaned from four herbaria HSU, OSC, UBC, and ORE for wide sampling of *F.* × *ananassa* subsp. *cuneifolia* are provided with collector name, voucher number, and locality with GPS location. Two voucher samples from NCGR were fresh tissue samples obtained from their live collections. Samples for deep sampling were obtained from the University of Pittsburgh in-house plant collections from different populations.

(A) SNP validation—*pet*D and *ndh*F

***Taxon*,** accession number—locality—GenBank number.

***F. virginiana* subsp. *virginiana* Duchesne,** PI 612492 - Ontario, Canada - JX117908. PI 612494 - South Dakota, USA - 42.567N, 81.567W—JX064444, JX064459. O477 - Pennsylvania, USA - 41.633N, 80.340W. Y33b2 - Pennsylvania, USA - 41.645N, 113.750W—JX064441, JX064461. ***F. virginiana* subsp. *glauca* (S.Watson) Staudt,** BOX5 - Idaho, USA - 44.407N, 111.391W—JX064443, JX064458. BOX10 - Idaho, USA - 44.407N, 111.391W—JX064442, JX064457. ***F. virginiana* subsp. *platypetala* (Rydb.) Staudt,** ES17 - Oregon, USA - 45.543N, 118.464W. GH10 - Washington, USA - 46.828N, 117.212W. KB17 - Washington, USA - 46.851N, 117.153W. ***F. chiloensis* subsp. *chiloensis* (L.) Miller,** PI 612318 - Tungurahua, Ecuador - 1.283 S, 78.633W. ***F. chiloensis* subsp. *lucida* (E. Vilm. ex J. Gay) Staudt,** SAL8 - Oregon, USA - 44.916N, 124.027W. SAL3 - Oregon, USA - 44.916N, 124.027W. PI 551459 - Oregon, USA - 43.098N, 124.430W—JX064430, JX064446. ***F. chiloensis subsp. pacifica* Staudt,** PI 612489 - Oregon, USA - 43.932N, 124.111W—JX064432, JX064448. ***F. virginiana* subsp. *grayana* (E. Vilm. ex Gay),** PI 612569 - Mississippi, USA - 33.885N, 88.539W—JX064445, JX064460.

(B) Wide sampling

***Taxon*,** collector name, voucher number—locality—GPS location.

***F.* × *ananassa* subsp. *cuneifolia* (Nutt. ex Howell) Staudt**. **HSU** - Linda M. Barker, 40975 - California, USA - 40.867N, 124.14W. Linda M. Barker, 39972 - California, USA - 40.867N, 124.14W. J. D. Ackerman, A. M. Montalvo, 27562 - California, USA - 40.873N, 124.158W. C. O. Baker, 18618 - California, USA - 41.04N, 124.115W. J. P. Smith, T. W. Nelson, 46955 - California, USA - 40.823N, 124.184W. P. A. Daly, 89431 - California, USA - 41.052N, 124.151W. Clifton Raymond Chesbrough, 27887 - California, USA - 41.375N, 124.061W. E. W. Styskel, 18637 - Washington, USA - 46.174N, 121.518W. Clifton Raymond Chesbrough, 26889 - Oregon, USA - 45.335N, 122.597W. Marc Andre Baker, 60056 - California, USA - 41.602N, 124.1W. **OSC** - F. K. Greene, 16176 - Oregon, USA - 43.565N, 123.279W. W. E. Lawrence, 62212 - Oregon, USA - 44.51N, 122.969W. H. C. Gilbert, 12973 - Oregon, USA - 44.555N, 123.278W. M. Bridge Cook, 37945 - Oregon, USA - 44.588N, 123.427W. J. Merkle, 68354 - Oregon, USA - 44.504N, 123.55W. Fanny Gitty, 5299 - Oregon, USA - 44.628N, 123.386W. L. R. Heckard, 140948 - Oregon, USA - 45.816N, 123.886W. Ruth Hopson, 192814 - Oregon, USA - 44.173N, 122.925W. W. E. Lawrence, 71204 - Oregon, USA - 43.961N, 122.662W. S. Sundberg, 194790 - Oregon, USA - 44.628N, 123.387W. Theodora Gustafson, 39898 - Oregon, USA - 45.561N, 121.638W. C. T. Dyrness, 107157 - Oregon, USA - 42.833N, 120.501W. W. V. Guthrie, 107671 - Oregon, USA - 44.557N, 123.281W. K. C. Swedberg, 106046 - Oregon, USA - 44.558N, 121.624W. **UBC** - K. J. Roller, v83772 - British Columbia, Canada - 49.25N, 123.133W. NA, v218357 - Oregon, USA - 44.609N, 123.215W. Ian Sharpe, v180180 - British Columbia, Canada - 50.257N, 122.003W. D. Brix, M. Benson, v101262 - British Columbia, Canada - 48.438N, 123.522W. Robert K. Scagel, v159909 - British Columbia, Canada - 50.767N, 121.566W. T. R. Ashlee, v765958 - British Columbia, Canada - 48.905N, 123.539W. J. R. Anderson, v80222 - British Columbia, Canada - 48.414N, 123.362W. V. Krajna, v75867 - British Columbia, Canada - 49.374N, 123.274W. Frank Lomer, v201131 - British Columbia, Canada - 49.217N, 122.917W. L. Holm, v78940 - British Columbia, Canada - 49.5N, 125.5W. R. T. Ogilvie, v45,032 - British Columbia, Canada - 49N, 124W. K. Racey, v27,588 - British Columbia, Canada - 49.074N, 125.805W. G. C. Carl, v49702 - British Columbia, Canada - 50.1N, 127.5W. K. I. Beamish, v58412 - British Columbia, Canada - 49.621N, 123.828W. P. Raymer, v71007 - British Columbia, Canada - 49.319N, 123.067W. F. Szy, v79120 - British Columbia, Canada - 49.835N, 124.525W. Bruce Ralston, v179367 - British Columbia, Canada - 51.742N, 122.429W. K. P. Bannister, v218907 - British Columbia, Canada - 57.95N, 122.817W. L. J. Gilmore, v179765 - British Columbia, Canada - 49N, 117W. David A. J. Irwin, v170171 - British Columbia, Canada - 50.167N, 123.867W. Gail Franco, v165961 - British Columbia, Canada - 54.717N, 127.017W. ORE - L. F. Henderson, 46291 - Oregon, USA - 44.831N, 122.935W. Lilla Leach, 46332 - Oregon, USA - 44.052N, 123.087W. L. F. Henderson, 46317 - Oregon, USA - 42.545N, 124.28W. L. E. Detling, 46183 - Oregon, USA - 44.049N, 123.351W. L. E. Detling, 46172 - Oregon, USA - 43.612N, 123.59W. L. E. Detling, 46182 - Oregon, USA - 44.126N, 123.106W. Rayma Brown, 46313 - Oregon, USA - 43.989N, 123.099W. L. E. Detling, 46249 - Oregon, USA - 43.02N, 123.293W. L. E. Detling, 46262 - Oregon, USA - 42.335N, 122.765W. **NCGR** - NA, PI 551711 - McKinleyville, CA - 41.000N, 124.167W. NA, PI 664407 - Oregon, USA - 44.615N, 123.318W .

(C) Deep sampling

Taxon, population name, location—GPS location—number of samples phenotyped, cytotyped, and microsatellite analysis per population.

***F.* × *ananassa* subsp. *cuneifolia* (Nutt. ex Howell) Staudt.** Dungeness Campground (DC), Washington, USA - 48.139 N, 123.194 W - 10,21,15. Fort Steven's State Park (FtS), Oregon, USA - 46.216 N, 124.002 W -10,12,13. Silver Falls (SF), Oregon, USA - 44.876 N, 122.653 W - 10,13,13. Hammond Trail (HAM), California, USA - 39.424 N, 123.817 W -10,25,14. Fisherman's Bend (FB), Oregon, USA - 44.756 N, 122.516 W - 10,15,15. Mary's Peak (MP), Oregon, USA - 44.504 N, 123.550 W - 14,31,16. Wren (WREN), Oregon, USA - 44.588 N, 123.427 W - 12,32,15. ***F. chiloensis* subsp. *lucida* (E. Vilm. ex J. Gay) Staudt.** Eureka (EUR), California, USA - 40.762 N, 124.225 W - 4,10,15. Honeyman State Park (HM), Oregon, USA - 43.930 N, 124.107 W - 10,11,15. Salishan Road (SAL), Oregon, USA - 44.916 N, 124.027 W - 7,10,12. ***F. virginiana* subsp. *platypetala* (Rydb.) Staudt.** George Hudson Reserve (GH), Washington, USA - 46.828 N, 117.212 W - 13,10,15. Kamiak Butte (KB), Washington, USA - 46.851 N, 117.153 W - 10,11,13. Emigrant Springs (ES), Oregon, USA - 45.543 N, 118.464 W - 11,11,15.