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Chloroplast haplotypes suggest preglacial differentiation and separate postglacial migration paths for the threatened North American forest tree *Juglans cinerea* L.

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Abstract Postglacial migration paths for most of the tree species of Eastern North America remain unknown. The presence of no-analogue forest communities prior to the last glacial advance suggests that individual trees species in Eastern North America may respond differently as climate changes and human impacts increase. In this study, we examined chloroplast haplotypes from natural populations of *Juglans cinerea* L., a North American forest tree, to infer postglacial migration patterns. Sequences from eight different regions of the chloroplast genome in 197 trees distributed across the range revealed 10 haplotypes. A minimum spanning network, phylogenetic analysis and haplotype distributions revealed that the three most common haplotypes were geographically disjunct and not closely related. Haplotype 6 (73 trees) occurred only in western populations, haplotype 10 (83 trees) occurred only in eastern populations and haplotype 7 (21 trees) occurred only at the southern edge of the native range. The southernmost population contained the most haplotype diversity but included no eastern haplotypes. Haplotype phylogeography suggested geographical differentiation prior to the last glacial advance in eastern populations and separate postglacial migration paths for eastern and western populations. As migration of *J. cinerea* to Atlantic Canada from southern refugia does not appear possible given known seed dispersal mechanisms, the possibility of northern refugia or dispersal by extinct megafauna merits serious consideration. Differences among species in preglacial history, ecological niche preferences and seed dispersal mechanisms suggest that response to long-term climate change and acute human disturbance may be highly species specific.

Keywords Chloroplast haplotypes · *Juglans cinerea* · Postglacial migration

Introduction

The presence of no-analogue forest communities prior to the last glacial advance suggests that individual trees species in Eastern North America may respond differently as climate changes and human impacts increase. The two *Juglans* species native to Eastern North America, *Juglans nigra* L. (eastern black walnut) and *Juglans cinerea* L. (butternut), are of particular concern, given the economic value of the former and the threatened status of the latter. Both species bear hard-shelled nuts encased in thick, indehiscent, toxic husks. As birds cannot crack either nut unaided and do not cache them (Stapanian and Smith 1986), the primary animal agents of seed dispersal are scatter hoarding squirrels (Wall 2001).

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Given the apparent lack of a long distance seed dispersal mechanism, the disparity between where the *Juglans* were, given the Last Glacial Maximum (LGM) pollen distributions and where both species went, appears to be a particularly acute case of Reid's paradox (Clark et al. 1998). Chloroplast phylogeography, not previously available for either species, could provide some insight into how both Eastern North American *Juglans* species migrated in response to the last major climate shift in this region.

As *J. cinerea* L. (butternut) faces extinction from the combined threats of disease and climate change, we have focussed on the chloroplast phylogeography of this species while the data can still be gathered. *J. cinerea* is listed as an endangered tree in Canada and a threatened tree or species of special concern in the USA (Schultz 2003) due to extensive mortality from butternut canker, a disease caused by the non-native fungus *Ophiognomonia clavigignenti-juglandacearum* (Broders and Boland 2011). Predictive models of twentieth century *J. cinerea* distribution based on Paleoclimate modelling and pollen records are in moderate agreement with the observed distributions suggesting ecological niche conservation over this period (Martínez-Meyer and Peterson 2006). While ecological niche preferences provide guidance for the choice of future ex situ sites for conservation, inference of the patterns of postglacial migrations could provide essential information on the rate of unassisted migration and the locations of glacial refugia in a large-seeded, cold-adapted angiosperm forest tree.

The ecological niche preferences of *J. cinerea* result in low population densities and a patchy distribution (Bryan 1965). Wind pollination suggests a capacity for long distance gene flow, but rate of seed dispersal determines the rate of migration. Birds and small rodents do not cache the nuts of this species, while tree squirrels (*Tamiasciurus hudsonicus*, *Sciurus carolinensis* and *Sciurus niger*) cache the nuts a maximum distance of 40–60 m from the tree (Goheen and Swihart 2003; Ivan and Swihart 2000; Moore et al. 2007). As the nut falls while still encased in a buoyant husk, downstream water dispersal is possible but the seed must strand on a suitable regeneration site before the husk becomes waterlogged (the nut itself will sink if viable). These limitations on seed dispersal and the limited number of suitable regeneration sites once dispersed suggest a limited capacity for rapid natural migration. The natural range of *J. cinerea* prior to the introduction of butternut canker extended as far as ~1,000 km northeast of the LGM, indicating an average migration rate of ~100 m/year if refugial populations were located close to the LGM. This is the upper limit of the rate estimated for other Eastern North American forest trees under the same assumption (McLachlan et al. 2005). The postglacial pollen record for *J. cinerea* in central and Eastern North America suggests refugia located north of the contemporary southern range edge, but the record is sparse. The pollen of *J. cinerea* tends to appear in the early stages of postglacial transition from spruce-dominated forests to mesic deciduous hardwoods (Finkelstein et al. 2006; Watts 1975, 1979). In studies in which *J. cinerea* pollen was distinguished from that of *J. nigra*, *J. cinerea* pollen occurs in sediments dated 25,000 years ago (25 ka) at Anderson Pond, Tennessee (Delcourt 1979), 19.5 ka at Lookout Mountain in northern Georgia (Watts 1975), 14.5 ka at Powers Fort Swale in southwestern Missouri (Royall et al. 1991) and 13 ka at Crystal Lake, IL (Gonzales et al. 2009). However, *J. cinerea* tends to have a patchy distribution that could leave little trace in sediment cores, and thus the sparse sediment core evidence does not rule out small northern refugia (Cruzan and Templeton 2000). Climate reconstructions for Eastern North America during the LGM suggest that cool season conditions suitable for *J. cinerea* could have existed as far north as 35° N (Jackson et al. 2000).

Bayesian cluster analysis of the first range wide genetic diversity and differentiation study in *J. cinerea* showed higher diversity in nuclear microsatellite polymorphisms and more extensive gene flow in the southern parts of the range when compared to the northern range edge, despite severe population losses in the south in the last 50 years due to the fungal disease (Hoban et al. 2010). Evaluation of possible mechanisms for this pattern indicated that lower genetic diversity and higher differentiation along the northern edges of the range was most likely due to founder effects as the range shifted northward during glacial retreat. Although the nuclear data did not clearly show differentiation between eastern and western populations, gene flow in the central and southern parts of the native range may have obscured the original patterns of postglacial migration since the LGM. The *J. cinerea* native range crosses two continental divides, the Eastern and the St. Lawrence (Fig. 1), and includes three major watersheds: the Mississippi, the Atlantic and the St. Lawrence. Migration into the valleys that traverse the continental divide could be slowed by the lack of suitable regeneration sites and seasonal flooding. The nuts of *J. cinerea* fall while tightly encased in a thick, buoyant oblate husk, permitting hydrochorous transport (Middleton 2000; Schultz 2003; Victory et al. 2006). Even if water dispersal is relatively infrequent, flooding could cause a pulse of long-distance migration down river, sending the seeds of upland migrants back into source populations. A study of fine-scale *J. cinerea* differentiation in the Great Smoky Mountains National Park did indicate a small but significant effect of watershed between some but not all populations (Parks et al. 2014).

The fossil and molecular evidence indicates that *J. cinerea* and the other *Juglans* species evolved north of the Arctic circle (66° N) and migrated south, differentiating into separate sections and species during the last 40 million years (Aradhya et al. 2007). Fossils and pollen of a *J. cinerea* ancestor, *Juglans eocinerea*, occur in sediments dated to the early to mid-Miocene (Hills et al. 1974; Whitlock and Dawson 1990;

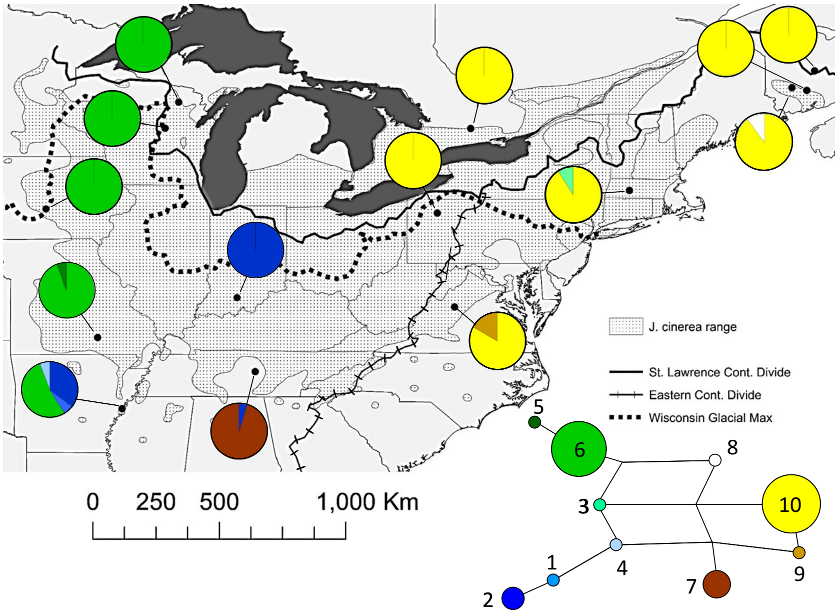

Fig. 1 Locations of butternut sites, haplotypes and relative proportions of haplotypes per site. Grey area is the native range for butternut. Dashed line is the approximate ice margin at the LGM. Inset: haplotype network with the black lines showing the inferred connections between the haplotypes based on parsimonious mutation models. An intersection of lines without a circle represents an unobserved haplotype. Size of circles for haplotypes 2, 6, 7 and 10 is proportional to number of individuals. The remaining haplotypes represent one tree each

Wolfe 1980) in the western Arctic Canadian islands. As the species migrated into Eastern North America, water dispersal and an ecological preference for lower altitudes (<500 m) (Parks et al. 2014) could have resulted in differentiation across the Eastern and St. Lawrence continental divides. In the Atlantic watershed, the Appalachian mesophytic ecoregion could have provided refugia for eastern populations during the Pleistocene glaciations. In the Mississippi watershed, west of the Appalachians, areas amenable to northern refugia are not as evident. Ecologically suitable regions in the Ozark valleys in southern Missouri and northern Arkansas and foothills in southern Tennessee, the contemporary southern edge of the *J. cinerea* range, are already located within 34-35° N. Pollen and macrofossil data from this region indicates the presence of other cool temperate hardwoods during the LGM (Jackson et al. 2000).

In most angiosperms, the chloroplast is inherited only through the egg cell (Zhang et al. 2003) and thus is only dispersed through the seed. When seed dispersal is more limited than pollen dispersal, as is the case with *J. cinerea*, chloroplast haplotypes can provide a more reliable indication of past migrations than nuclear genotypes (Petit et al. 2003). The inference of postglacial migration paths based on geographical distribution of chloroplast haplotypes assumes that founder effects should decrease haplotype richness and increase haplotype differentiation, in the direction of migration (Le Corre and Kremer 1998). Based on this assumption, chloroplast haplotypes from several species of oaks widely distributed across Europe suggest refugia in Iberian, Italian and eastern Balkan refugia with evidence of haplotype exchange during the last or previous glacial cycles (Petit et al. 2002). In Eastern North America, chloroplast haplotype distribution in American beech (*Fagus grandifolia* Ehrh.) and red maple (*Acer rubrum* L.) suggests that some populations colonized northward from refugia closer to the LGM (McLachlan et al. 2005) than the contemporary southern edge of the range. As chloroplast haplotype studies in angiosperm North American trees accumulate (Birchenko et al. 2009; Breen et al. 2012; Magni et al. 2005; Marsico et al. 2009), a complex picture of postglacial migration emerges, in which ecological niche requirements, seed dispersal dynamics, local geography and the intensity of climate instability interact to produce large variations in where tree species find refuge and how long they stay there (Breen et al. 2012; Finkelstein et al. 2006; Soltis et al. 2006; Stewart et al. 2010; Watts 1979). In the wide ranging North American conifer species *Picea mariana* (Mill.) Britton, Sterns & Poggenb. (black spruce) and *Pinus banksiana* Lamb. (jack pine), mitochondrial haplotypes have revealed evidence of multiple refugia, cryptic refugia, population mixing at suture zones and complex patterns of postglacial migration (Godbout et al. 2005; Jaramillo-Correa et al. 2004), suggesting that individual species histories, as well as continental geography, influence forest tree response to long-term climate change. Evidence for forest communities in North America with no modern analogue during the LGM (Gill et al. 2009; Jackson et al. 1997; Williams and Jackson 2007; Williams et al. 2001) and evidence for the extinction of at least one tree species in North America (Jackson and Weng 1999; Liu et al. 2013) during glacial retreat indicate that prediction of the location and species composition of future forests as the climate changes requires more information on the postglacial history of a wider range of North American forest tree species.

As butternut canker has now spread throughout the entire native range of *J. cinerea* (Furnier et al. 1999), the opportunity for studies on the postglacial history of this species based on living descendants is rapidly fading. Although range wide fine-scale geographical sampling in this species is no longer possible due to the local extinctions of southern and central populations, the naturally occurring populations that still remain make a chloroplast haplotype study feasible. Based on evolutionary history, ecological niche preferences and sediment core evidence, we hypothesized that haplotype richness and distribution would be consistent with migration from the contemporary southern range edge in the Mississippi watershed and from Appalachian cryptic refugia close to the last glacial margin in the Atlantic watershed.

Materials and methods

Human and animal subjects

This study did not include or involve the use of human or animal subjects.

Species characteristics and population sampling scheme

The most cold-tolerant species in the *Juglans* genus, *J. cinerea*, occurs in scattered, low density populations in the Eastern United States and in parts of Ontario, Quebec and New Brunswick. *J. cinerea* nuts fall while still fully encased in a thick husk. Tree squirrels are the only animal known to disperse the nuts for winter caching but the oblate green fruits float briefly in water, providing downstream transport in waterways and overland dispersal in seasonal floods (Schultz 2003). Populations for this investigation were selected according to the following criteria: (1) location on a latitudinal gradient to ensure that surviving northern, southern and middle populations were represented; (2) adequate number of trees per population to allow accurate estimation of haplotype richness and frequency (Feng et al. 2008); and (3) inclusion of populations showing a wide range of differentiation for nuclear markers (Hoban et al. 2010). We chose 14 populations with 6–21 individuals from each population. Butternut canker, large-scale agriculture and urban development in the central part of the *J. cinerea* range have resulted in the disappearance of adequately sized naturally occurring populations (>5 trees) and thus this region is unavoidably underrepresented. Many of the trees we sampled in our previous studies (Hoban et al. 2009, 2010) are now dead.

Hybrid exclusion

Japanese walnut (*Juglans ailantifolia* Carr.), imported throughout the *J. cinerea* range in the nineteenth century, hybridizes with *J. cinerea*, producing fertile and vigorous hybrids that can backcross into local populations (Hoban et al. 2012b). All trees included in this study were positively identified as *J. cinerea* (as opposed to *J. ailantifolia* or hybrids) based on 12 nuclear microsatellites (Hoban et al. 2008) and 3*J. cinerea* chloroplast markers (McCleary et al. 2009). Although sympatric in many locations, black walnut and butternut arise from different sections of *Juglans* genus and do not hybridize, eliminating this complication.

DNA extraction, amplification and sequencing

DNA extraction and amplification were performed as previously described (McCleary et al. 2013), using annealing temperatures optimized for the primers used in this study (Table 1). Amplicons were purified and then sequenced on a ABI 3730xl DNA Analyser as previously reported (McCleary et al. 2009). As our preliminary data suggested that sequence polymorphism in the *J. cinerea* chloroplast was low, we sequenced 16 regions of the chloroplast genome, including 10 regions in which our previous study detected polymorphism in northern red oak (*Quercus rubra* L.). Primers first reported in our previous study for six of these regions, *atp*1-*rps2*, *rpoC2-rpoC1*, *rpoB-rpoC1*, *ndhK-ndhC*, *psbH-petB*, *petB-petD*, were identified as universal in that study based on successful amplification across 11 tracheophyte clades (Borkowski et al. 2014). The primers we used for two of these regions, *trnC-trnD* (Demesure et al. 1995) and *trnV-rbcL* (Dumolin-Lapegue et al. 1997), were as reported by the original authors (Table 1). The primers we used for the *psaA-trnS* region were different than those previously reported for the same region (Demesure et al. 1995). The reverse primer we used for *trnH-trnK* was the same as previously reported (Demesure et al. 1995) but the forward primer was different. We also included *petG-trnP* and *trnT-trnL* (Huang et al. 2002), *psbB-psbF* and *rpl20-rps12* (Hamilton 1999), and *trnL*(UAG)-*rpl32* and *trnQ*(UUG)-5'*rps16* (Shaw et al. 2007). We screened these 16 regions in eight individuals each from populations in Waupaca, WI; Keswick, NB; Gilbert Island, NB; Alley Spring State Park, MO; the Allegheny National Forest, PA; and the St. Francis National Forest, AR. Only 8 of the 16 regions we sequenced in the population subsets were polymorphic in *J. cinerea* (Table 1). We then sequenced 197 trees in 14 populations for all eight regions.

Sequence analysis

We used SEQUENCHER ver. 4.10.1 (Gene Codes Corporation, Ann Arbor, MI) to detect single nucleotide polymorphisms (SNPs) and indels. We generated the geographical distributions of haplotypes in ARCGIS v 9.3 (Environmental Systems

Table 1 Eight polymorphic chloroplast regions in *J. cinerea*

Region[a]	bp[b]	T_m, °C	Forward	Reverse
*atp*I-*rps*2[c]	588	62	CCGTATTCTTCGCCAATCTG	TGGGAATTCCAACGATTTGT
*ndh*K-*ndh*C[c]	331	62	CAGCCCATAACGGTCAAAGT	GTGATGCTTGGCTCCAATTT
*psa*A-*trn*S[d]	216	62	CCTACGAAATACCGGACGAA	CCAAATTAATGAATCATCACAGG
*rpo*B-*rpo*C1[c]	393	62	ATTTCCACAAGCGCAAATCC	AGAGGAAGGGCTAAGCAAGG
*trn*C-*trn*D[e]	330	62	CCAGTTCAAATCTGGGTGTC	GGGATTGTAGTTCAATTGGT
*trn*H-*trn*K[f]	614	66	GCTGGTTATCCAGTTACAGAAGC	CAACGGTAGAGTACTCGGCTTTTA
*trn*L(UAG)-*rpl*32[g]	553	62	CTGCTTCCTAAGAGCAGCGT	CAGTTCCAAAAAAACGTACTTC
*trn*V-*rbc*L[h]	112	62	CGAACCGTAGACCTTCTCGG	GCTTTAGTCTCTGTTTGTGG

[a] Accession numbers by haplotype in Table S2

[b] Trimmed sequence base pairs, amplicon is longer

[c] Borkowski et al. (2014)

[d] Different primers than reported previously for this region (Demesure et al. 1995)

[e] Demesure et al. (1995)

[f] Forward primer different than reported previously for this region, reverse primer the same as for trnK-trnK (Demesure et al. 1995)

[g] Shaw et al. (2007)

[h] Dumolin-Lapegue et al. (1997)

Research Institute Inc., Redlands, CA). A phylogenetic tree was generated using MRBAYES 3.2 (Ronquist et al. 2012) with HKY model of DNA evolution, gamma-distributed rate variation among sites, some sites invariant and the number of generations=410,000. The number of generations was empirically determined by adding generations to the run until the standard deviation of the split frequencies stabilized at values below 0.01 and the potential scale reduction factors were 1± 0.002 for all parameters. The best fit DNA evolution model was chosen based using MODELTEST (Posada and Crandall 1998), as implemented in MEGA5 (Tamura et al. 2011). We used NETWORK v 4.6.1 (Fluxus Technology Ltd) to generate a minimum spanning tree using median-joining (Bandelt et al. 1999). For calculating the minimum spanning tree, epsilon (a weighting factor for the depth of the search) was set to 0 (default value) and deletions were weighted twice (non-default value) as recommended in the manual for the software. The Connection Cost and Greedy FHP algorithms yielded identical networks.

As our sample numbers varied from 6 to 21 individuals, we used rarefaction to six individuals to calculate haplotype richness as implemented in CONTRIB (Petit et al. 1998). We tested for phylogeographic structure using the method implemented in PERMUT&CPSSR (Bordacs et al. 2002; Pons and Petit 1996). Differentiation was calculated for unordered (G_{ST}) and ordered (N_{ST}) alleles then 10,000 haplotype permutations among populations were generated to test if N_{ST} was significantly greater than G_{ST}. When more closely related haplotypes occur more often in the same population than distant populations, N_{ST} will be greater than G_{ST} (Pons and Petit 1996). CONTRIB and PERMUT&CPSSR are available at http://www.pierroton.inra.fr/genetics/labo/Software/.

Results

Out of the 16 chloroplast intergenic regions sequenced, only 8 showed polymorphisms. Seven of the polymorphic regions (*atp*I-*rps*2, *ndh*K-*ndh*C, *psa*A-*trn*S, *rpo*B-*rpo*C1, *trn*C-*trn*D and *trn*H-*trn*K) are also polymorphic in *Q. rubra* (Fagaceae, Fagales) and have demonstrated utility as universal primers. The region *trn*L(UAG)-*rpl*32, originally selected by Shaw and colleagues (2007) for investigations at low taxonomic levels, proved informative in this one. Sequences for all eight regions in 197 individuals across 14 populations revealed 11 polymorphic sites: 10 SNPs and 1 indel. Two of the SNPs were located in *trn*L(UAG)-rpl32, in the small single copy region of the chloroplast, a region not queried in most chloroplast haplotype studies. Eight of the 10 SNPs detected were transversions (Table 2).

The 10 haplotypes detected (Table 2) were highly differentiated among sites (G_{ST}=0.842 se=0.069) and haplotypes richness was low across the range (Table 3). The value of N_{ST} (0.91, se=0.053), a measure of differentiation for ordered alleles, was significantly greater (P=0.01) than the measure of differentiation for unordered alleles (G_{ST}=0.842 se=0.069), based on 10,000 permutations (P=0.01), indicating high phylogeographic structure. Over 80 % of the trees genotyped (158) had one of two haplotypes (6 or 10), with haplotype 6 present only in western populations and haplotype 10 present only in eastern populations (Table 3, Fig. 1). Haplotypes 6 and 10 differed at 7 of the 11 polymorphic sites detected (Table 2): four A-C transversions, two G-T transversions and one C-T transition.

The southernmost population (St. Francis National Forest, AK) contained the highest haplotype diversity. Eight populations were monomorphic. Populations in the northeastern part

Table 2 SNP polymorphisms of the ten chloroplast haplotypes detected in J. cinerea

Haplotype	*trn*L(UAG)-*rpl*32_1	*trn*L(UAG)-*rpl*32_2	*psa*A-*trn*S	*ndh*K-*ndh*C_1[a]	*ndh*K-*ndh*C_2	*atp*I-*rps*2	*rpo*B-*rpo*C1	*trn*H-*trn*K_1	*trn*H-*trn*K_2	*trn*C-*trn*D	*trn*V-*rbc*L
1	T	T	C	–	G	C	T	A	C	A	A
2	T	T	C	–	G	C	T	A	C	C	A
3	T	T	C	T	T	C	T	A	C	A	G
4	T	T	C	T	T	C	T	A	C	A	A
5	T	T	A	T	T	A	C	A	C	A	G
6	T	T	A	T	T	A	T	A	C	A	G
7	G	G	C	T	T	C	T	A	C	C	A
8	G	G	A	T	T	C	T	A	C	A	G
9	G	G	C	T	T	C	T	C	T	A	A
10	G	G	C	T	T	C	T	C	T	A	G

[a] The en dash indicates a single base pair deletion

of the range contained haplotypes 8 and 9, in addition to 10; none of which were found in the southern or western populations. Haplotype pairs 1-2, 5-6, 9-10 and 3-4 differed from each other by a single SNP. The pairs 1-2, 5-6 and 9-10 occurred together but haplotypes 3 and 4, each represented by a single tree, occurred at opposite sides of range (Fig. 1, Table 3).

The minimum spanning tree (Fig. 1), based on a parsimony model, and the maximum likelihood phylogeny (Fig. 2), based on a DNA evolution model of different rates of transitions and tranversions, reveal at least two lineages. Under the simple two-lineage interpretation, lineage A (haplotypes 1–6) and lineage B (haplotypes 7–10) are largely but not completely geographically disjunct. However, the minimum spanning tree and the phylogenetic analysis indicate that this interpretation is too simple. Haplotype 10 is the predominant haplotype in all of the populations in the Atlantic and the St. Lawrence watersheds. Haplotype 6 occurred only in populations in the Mississippi watershed. The five westernmost locations, distributed across a latitudinal gradient spanning nearly 11°, share related haplotypes and display a pattern of haplotype loss northward as predicted by the stepping stone model for postglacial migration. The seven northeast locations span ~8.5° of latitude but are nearly monomorphic. Haplotype 8, not closely related to haplotype 10, occurred in one tree at one of the New Brunswick sites, at the extreme

Table 3 Descriptive statistics for sites and haplotypes

Site	Latitude	Longitude	Sample size	Haplotypes										Haplotype number	Haplotype richness[a]
				1	2	3	4	5	6	7	8	9	10		
Polk, IA	41.6404677	−93.3833830	8	–	–	–	–	–	8	–	–	–	–	1	0
Alley Spring State Park, MO	37.1248828	−91.5100371	17	–	–	–	–	1	16	–	–	–	–	2	0.353
St. Francis National Forest, AR	34.5971422	−90.6427251	17	1	6	–	1	–	9	–	–	–	–	4	1.666
Waupaca, WI	44.5456027	−89.1124031	19	–	–	–	–	–	19	–	–	–	–	1	0
Chequamegon–Nicolet National Forest, WI	45.4713089	−88.6286044	21	–	–	–	–	–	21	–	–	–	–	1	0
Hoosier National Forest, IN	38.4760943	−86.5400731	6	–	6	–	–	–	–	–	–	–	–	1	0
Butternut Valley, TN	35.9119765	−85.9091913	21	–	1	–	–	–	–	20	–	–	–	2	0.286
Shenandoah National Park, VA	38.0930315	−84.7821816	6	–	–	–	–	–	–	–	–	1	5	2	1
Allegheny National Forest, PA	41.5337637	−79.4356876	20	–	–	–	–	–	–	–	–	–	20	1	0
Bobcaygeon, Ontario	44.6041498	−78.5269692	14	–	–	–	–	–	–	–	–	–	14	1	0
Hadley, MA	42.3376465	−72.6080057	11	–	–	1	–	–	–	–	–	–	10	2	0.545
Keswick, New Brunswick	45.9854034	−66.8314974	10	–	–	–	–	–	–	–	1	–	9	2	0.6
Gilbert Island, New Brunswick	45.8870335	−66.3039826	16	–	–	–	–	–	–	–	–	–	16	1	0
Blackville, New Brunswick	46.5813304	−66.0259166	11	–	–	–	–	–	–	–	–	–	11	1	0
Total			197	1	13	1	1	1	73	20	1	1	85		

[a] Rarefaction to six individuals

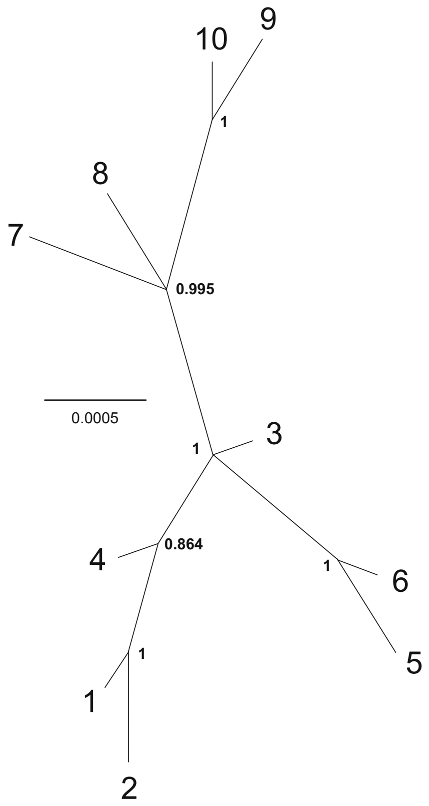

Fig. 2 Unrooted phylogeny of *J. cinerea* chloroplast haplotypes. Numbers in bold next to branch points indicate the probability of the partition. Numbers at the tips indicate the haplotype. Scale bar indicates branch length for an average substitution rate of 0.0005

northeast corner of the range, where haplotype 10 predominates. Haplotype 7, not directly related to either haplotype 6 or haplotype 10, was the predominant haplotype in the Tennessee population.

Discussion

We queried 16 different intergenic regions of the J. cinerea chloroplast (>7,000 bp) and found polymorphism in only eight of these regions. The SNPs detected included more transversions than transitions, a result also noted in the sequence of the trnT−trnF and atpB−rbcL chloroplast regions in 17 taxa of the *Juglans* genus (Aradhya et al. 2007), including *J. cinerea*. Ultimately, we detected 11 polymorphisms comprising 10 haplotypes in populations preselected to represent a wide range of geographical and genetic diversity within the limitations imposed by local extinctions. These 10 haplotypes show high geographical differentiation, with little or no haplotype richness within northern populations. Interpretation of this pattern requires consideration of Pleistocene glacial dynamics, geography and seed dispersal mechanisms.

Assuming that the three most common haplotypes existed prior to the Holocene and taking the phylogeography into consideration, we argue that our data are consistent with multiple glacial refugia. Haplotype phylogeography in the west suggests source populations close to or south of the current southwestern edge of the contemporary range. Sediment cores reveal black walnut pollen (*J. nigra*) at Nonconnah Creek, Mississippi (35.083° N, −89.916° W) during the LGM (Delcourt et al. 1980), suggesting that *J. cinerea*, the most cold-tolerant species in the *Juglans* genus, could have existed in this region and further north. The St. Francis National Forest site, the only site in which we detected four haplotypes, occurs almost at the same latitude, in the Central Mississippi Alluvial Valley. *Juglans* pollen (species not specified) is just detectable in sediment cores dated to ~16 ka at the Fort Powers Swale, MO, site (36.58° N, −90.56° W), but in the interval 14.5 to 9.5 ka, the authors report both J. cinerea and J. nigra pollen at this site.

The eastern haplotypes could be interpreted as the result of a migration out of a southern refugium east of the Mississippi embayment, in southern Georgia and Florida. Juglans pollen is never abundant but it is present in sediment cores in southern Georgia and Florida during the LGM (Jackson et al. 1997, 2000). Although most pollen records do not distinguish *J. nigra* from *J. cinerea* pollen, the latter is more cold tolerant and thus it is reasonable to suppose that *J. cinerea* was present. The major difficulty with one southeastern refugium is the lack of seed dispersal mechanisms that could have moved *J. cinerea* from southern Georgia to New Brunswick, a distance of over 2,300 km, in 18–20,000 years. Tree squirrels are the only known animal dispersal agent. Johnson and Webb, in their discussion of bird-dispersed tree seeds, do not consider the extinct passenger pigeon (*Ectopistes migratorius*) a viable seed dispersal agent, as the birds ate beechnuts and acorns but did not cache them (Johnson and Thompson Webb 1989). Even assuming that delayed digestion could have resulted in occasional transport of viable, intact seed, *J. cinerea* nuts are large (~3 × 5 cm) and have deeply ridged shells much harder and thicker than the shells of pecan (*Carya illinoensis* (Wangenh.) K.Koch) and *Juglans regia* L., the cultivated Persian walnut. Birds cannot crack the nuts unaided. Crows (*Corvus brachyrhynchos*) crack the nuts of *J. regia* and Northern California walnut (*Juglans hindsii* (Jeps.) Jeps. ex R.E. Sm.) by dropping the nuts from a height (Cristol and Switzer 1999), but there are no reports of any bird attempting to do this with the large, sharp-edged nuts of *J. cinerea*.

Glacial refugia further north could have made the migration to Atlantic Canada possible. Possibilities include eastern refugia further north and a northeastern coastal refugium on the now submerged continental shelf. Many investigators have proposed cryptic northern refugia as a possible solution to Reid's paradox, the disparity between the predicated rate of tree migration assuming known seed dispersal mechanisms and the apparent rate, as inferred from pollen and fossil evidence (Clark et al. 1998). Although *J. cinerea* can persist in southern sites where water is abundant, this species as a rule prefers cool, moist conditions and could have survived even further north than the 34-35° N suggested for the northern limit of cold-tolerant temperate hardwoods during the LGM (Jackson et al. 2000) if the periglacial climate was more moderate than previously thought (Loehle 2007). Although our data give little indication of the location of possible northern refugia in the east due to lack of polymorphism, the geological, pollen and fossil evidence suggests a warm thermal enclave between the Southern Appalachians and the Atlantic Ocean, extending as far north as Cape Hatteras (35.2053° N, −75.552° W) and east onto what is now submerged continental shelf (Royall et al. 1991). However, even if butternut has persisted in this region, the distance to Atlantic Canada still exceeds 1,700 km.

Nuclear microsatellite genotypes have revealed that *J. cinerea* populations in New Brunswick (Hoban et al. 2010) and the red pine (*Pinus resinosa* Ait.) populations of Newfoundland and New Brunswick (Boys et al. 2005) are genetically distinct from conspecifics further south and west. *P. banksiana* populations in New Brunswick, Nova Scotia and Prince Edward Island are nearly monomorphic for a mitochondrial haplotype not found anywhere else in a native range that extends to Alberta (Godbout et al. 2005). These results, as well as other data from coastal plain flora and fauna in this region, suggest the possibility of a now submerged coastal glacial refugium in northeastern North America (Godbout et al. 2010; Jaramillo-Correa et al. 2004). As speculative as this interpretation is, given the absence of fossil evidence, it is difficult to explain the data otherwise.

J. cinerea has migrated long distances before, but over much longer time scales. Fossil remains of the species inferred as the ancestor of *J. cinerea*, *J. eocinerea* n.sp., occur at many sites in Arctic Canada (Fyles et al. 1994; Hickey et al. 1988; Hills et al. 1974; Whitlock and Dawson 1990) dated to the early Miocene epoch (~22 Ma). As the climate cooled and the deciduous forest migrated south, *J. cinerea* appeared and eventually populated Eastern North America well before the Pleistocene glaciations. We have shown in other studies that seed dispersal distances in *J. cinerea* are small (<100 m) and population sizes are limited by the limited availability of favourable regeneration sites (Hoban et al. 2012a). Assuming the ecological preferences of butternut have not changed and that no dispersal agents existed other than squirrels, water remains the least speculative option for long distance seed dispersal. Seed from *J. cinerea* populations located along rivers could easily be transported further than 100 m downstream or overland during seasonal floods. During the northward retreat of the glacial margin 18–20 ka, butternut could have migrated slowly west and north from eastern coastal refugia. Once northern migrants got across the Laurentian divide, water transport to open sites northward could have provided an opportunity for bursts of seedling regeneration, unlike the resource restricted sites provided by gaps in upland forests (Hoban et al. 2014). Further south, migration westward and north would have been slow, but water transport would have hastened migration in the south east direction. This patchy, uncertain pattern of regeneration would have resulted in repeated founder effects during recolonization.

If haplotype diversity in eastern glacial refugia, regardless of where they were, was high, this proposed regeneration pattern should have resulted in inland populations nearly fixed for different haplotypes. Instead, we see a near monomorphism for haplotype 10, with two divergent haplotypes (3 and 8) in different populations in the northeast. This pattern suggests that the haplotype 10 was already predominant prior to the last glacial advance. The highly divergent nuclear genotypes of the New Brunswick butternuts in the absence of unique chloroplast haplotypes also suggests that this lack of chloroplast diversity existed before the last glacial advance. The individuals having haplotypes 3 and 8 were sequenced twice with the same result, so cannot be dismissed as sequencing errors. Haplotypes 3 and 8 may represent secondary contact during previous glacial episodes but now are nearly lost through genetic drift.

Still assuming that the only dispersal mechanisms for this species are squirrels and water, haplotype 7, detected in one southern site and not closely related to haplotype 6 or haplotype 10, may represent a local chloroplast lineage that became separated during the original colonization of the Eastern United States. This population is the result of a documented burst of natural regeneration within the last 50–70 years (Hoban et al. 2012a). A previous study (Hoban et al. 2010) using nuclear microsatellites indicated that this population is divergent from the other populations (although not as divergent as the butternuts in New Brunswick) and consists of related groups of half sib families, a classic case of genetic drift. The haplotype 7 butternuts in Butternut Valley may represent a remnant of the southern glacial refugium east of the Mississippi embayment. The expectation that additional haplotypes not present in the north should be present here cannot be tested now, due to the extensive local extinction of butternut populations in the South.

Furthermore, the assumption that squirrels were the only mammalian animal dispersal agent for butternut should be reconsidered, given the number of herbivorous Pleistocene megafauna that became extinct in Eastern North America as the ice margins receded and the first people arrived. The contemporary distribution of butternut (before butternut canker) shares similarities to another disappearing tree, the Kentucky coffee tree (*Gymnocladus dioicus* (L.) K. Koch), native to the central USA. The only contemporary long-distance seed dispersal mechanism for both species and indeed the only seed dispersal mechanism for Kentucky coffee tree is water (Zaya and Howe 2009). Despite the toxicity of the pods and seeds, Zaya and Howe hypothesize that *G. dioicus* relied on megafaunal fruit dispersal, a mechanism that requires consumption of the fruit, fruit pulp or husk. Based on their argument, three indications of possible megafaunal seed dispersal are large, indehiscent fruits toxic to smaller animals, well-protected seeds that could have withstood megafaunal teeth and fruits that now attract few or no native mammals. Butternut have large, indehiscent toxic fruits (the thick greenish husk) not eaten by extant wild animals or domesticated livestock and a hard, thick nut shell with high, sharp ridges. Eastern North American forest dwelling megafauna existed until 10–12 ka, overlapping with the first people to arrive as the glacier retreated (Boulanger and Lyman 2014). Even if the megafauna usually discarded the nuts, the occasional long distance transport of a few may have sufficed to move the species from midcountry (~35° N) to New England before the extinction. Finally, the first people may have played a role thereafter, as butternut was highly valued for the oily nut and for making dye and medicines (Omar et al. 2000; Schlarbaum et al. 1997).

The decimation and local extinction of southern and central *J. cinerea* populations (Anderson and LaMadelaine 1978; Schlarbaum et al. 1997) due to butternut canker, intensive agriculture and other anthropogenic disturbances creates an unavoidable ascertainment bias in our data. A more complete geographical sampling may have enabled us to speculate less on the migration patterns of this large-seeded species adapted to cool mesic conditions. This ecological preference makes *J. cinerea* an excellent candidate for a tree that could have persisted in the cryptic northern refugia or the eastern coastal refugia proposed by other investigators (McLachlan et al. 2005; Stewart and Lister 2001; Stewart et al. 2010). The evidence in living trees is rapidly fading but *J. cinerea* macrofossils, particularly nut shell fragments and pollen, are distinctive and may lie undiscovered in caves and sediments not yet investigated.

Although our study did not provide a definitive answer to the question of how *J. cinerea* responded to the last glacial retreat in eastern populations, our data does suggest that in Eastern North America, tree migration patterns are the result of complex interactions of climate shifts, regional geography, ecological preferences and seed dispersal mechanisms. The role of watersheds in butternut migration must remain speculative as the species is dying out but this concept could be tested in black walnut, the only other *Juglans* species native in Eastern North America. Black walnut is not threatened, has the same seed dispersal mechanisms and is sympatric with butternut in the southern half of the range.

Finally, chloroplast or mitochondrial haplotyping, paleobotanical sampling and pollen counts in sediment cores may all be insufficient, when used alone, to reconstruct past tree migrations. Pollen records do not provide evidence of absence or define either the southern or northern edge range boundaries under some conditions (Jackson et al. 1997). Present day chloroplast haplotype diversity may not coincide with the paleobotanical evidence. Breen and colleagues (2012) found in their study of chloroplast haplotypes in *Populus balsamifera* a shallow chloroplast genealogy not at all suggestive of the LGM Beringian refuge indicated in the paleobotanical record. The last major climate shift in Eastern North America was accompanied by the appearance of humans, two events whose combined impact resulted in the extinction of Pleistocene megafauna, a consequence that may have had a not fully recognized impact on forest community composition that endures to this day.

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Conflict of interest The authors affirm that they have no conflicts of interest.

Data archiving statement Sequences for haplotypes 2, 5, 6 and 10, which show all the polymorphisms we detected except one, are deposited at NCBI. Accessions numbers are listed in Table S1. The polymorphic region of ndhK-ndhC, being <200 bp and thus too short to deposit, is reported in Table S2.

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