Pleistocene ice ages created new evolutionary lineages, but limited speciation in Northeast Pacific winged kelp

W. Stewart Grant, and Trevor T. Bringloe

From the Genetics Laboratory, Alaska Department of Fish & Game, Anchorage, AK 99618, USA (Grant) and School of BioSciences, University of Melbourne, Parkville Campus, Victoria, 3010, Australia (Bringloe). W. Stewart Grant is now at the College of Fisheries and Ocean Sciences, University of Alaska Fairbanks, 17101 Point Lena Loop Road, Juneau, AK 99801, USA

Address correspondance to W. S. Grant at e-mail: phylogeo@gmail.com.

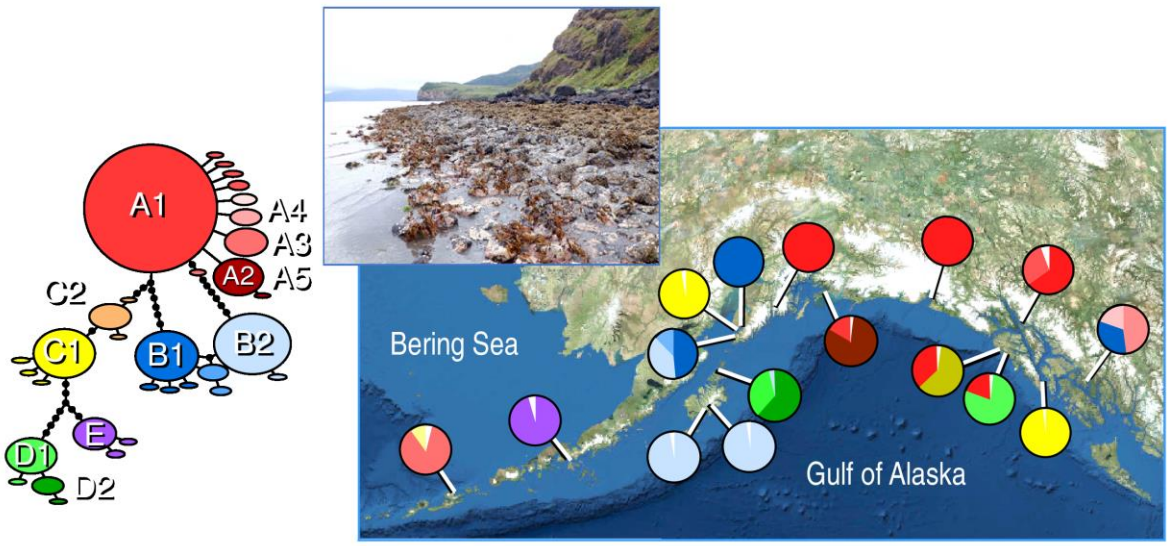
Abstract

The extent that Pleistocene climate variability promoted speciation has been much debated. Here, we surveyed genetic markers in winged kelp *Alaria* in the Gulf of Alaska, Northeast Pacific Ocean to understand how paleoclimates may have influenced diversity in this kelp. The study included wide geographic sampling over 2800 km and large sample sizes compared to previous studies of this kelp. Mitochondrial 5'-*COI* (664 bp), plastid *rbc*L-3' (740 bp) and 8 microsatellite markers in 16 populations resolved 5 well-defined lineages that did not align with described species. *COI-rbc*L haplotypes were distributed chaotically among populations around the Gulf of Alaska. Principal Coordinates Analysis of microsatellite genotypes grouped plants largely by organellar lineage instead of geography, indicating reproductive isolation among lineages. However, microsatellite markers detected hybrids at three sites where lineages co-occurred. Local adaptation on various time scales may be responsible for some genetic differences between populations located along wave-energy and salinity gradients, but the chaotic pattern of variability over hundreds of kilometers is likely due to isolations in northern refugia during Pleistocene ice ages. The range of divergences between populations indicates that episodic glaciations led to the creation of new lineages, but population turnover (local extinctions and recolonizations) limited the formation of new species in the Northeastern Pacific Ocean.

Subject area: Population structure and phylogeography

Keywords: *COI*, genetic population structure, incipient speciation, winged kelp, Pleistocene glacial refugia, Northeastern Pacific Ocean, phylogeography, *rbc*L

Graphical Abstract



A core challenge in evolutionary biology is to unravel mechanisms that both promote and limit evolutionary divergences leading to the emergence of new species. This task can be particularly daunting in the marine realm where species have large dispersal potentials through planktonic spores and larvae, or adult movement, reducing the likelihood of isolation between populations. Nevertheless, genetic differences among marine coastal populations have been documented and attributed to oceanic barriers to gene flow (White et al. 2010; Siegle et al. 2013), isolation by distance (Wright et al. 2015) or adaptation to local environments (Calegario et al. 2019). However, divergences on time scales associated with these processes are unlikely to lead to new species, because ocean habitats change on decadal to millennial time scales, altering barriers to dispersal and promoting mixing between populations (Mantua et al. 1997; Beaugrand et al. 2002; Drinkwater et al. 2003). While rapid speciation can occur in brown algae on short time scales (Bringloe et al. 2020a), divergences in marine organisms leading to new species occur on time scales corresponding to Pleistocene ice ages or longer (Grant and Utter 1984; Buonaccorsi et al. 2002; Hoarau et al. 2007; McGovern et al. 2010; Neiva et al. 2012).

Beginning in the Pleistocene Epoch, large glaciers periodically covered northern Europe (Svendsen et al. 2004) and North America (Barendregt and Irving 1998), displacing terrestrial populations into areas conducive to survival (Hewitt 1999). A widely held model of these refugia, based on phylogeographic patterns of plants and animals across Europe (Taberlet et al. 1998; Hewitt 1999), posits that northern populations were forced into southern ice-free areas and dispersed northward as glaciers retreated. The salient features of this model are phylogeographic radiations from southern refugia and a progressive loss of genetic diversity in poleward populations produced by serial colonizations (Hewitt 2004). However, populations of several species appear to have survived in northern refugia, producing phylogeographic connections that do not radiate from a southern refugium or show a latitudinal diversity gradient (Homburg et al. 2013).

The structure of marine shoreline refugia differ from terrestrial refugia in two regards. First, shorelines are linear, so the extents of southern refugia were limited. Second, not all high-latitude shorelines were covered with the margins of contintental ice sheets. In the Northwest (NW) Pacific Ocean, only a small portion of the shoreline was covered with ice (Nürnberg et al. 2011). In the Northeast (NE) Pacific Ocean, glaciers spilled onto the contintental shelf from Washington to the eastern Aleutian Archipelago, but the extent of the ice was not continuous along the shoreline (Kaufman and Manley 2004; Mann and Hamilton 1995; Carrarea et al. 2007). Several ice-free areas have been identified in SE Alaska that could have served as refugia for intertidal and shallow-water

algae and invertebrates (Carrarea et al. 2007). A handful of studies have postulated northern coastal glacial refugia along NE Pacific shores for species of seaweeds (Lindstrom 2009), invertebrates (Marko and Zslavskaya 2019; Marko et al. 2010) and fishes (Canino et al. 2010).

The focus of this study is on winged kelps in the genus *Alaria*, which are among the most abundant members of rocky intertidal habitats in the NE Pacific and are model organisms for evaluating mechanisms promoting biological diversity in the Gulf of Alaska. *Alaria* features an alternation of heteromorphic life-history stages, consisting of conspicuous rocky intertidal sporophytes (2*n* chromosomal complement) with large blades up to 2–3 meters in length and microscopic filamentous gametophytes (1*n*). Male gametophytes produce gametes with limited planktonic life, which fuse with sessile female oogonia to produce a new generation of macroscopic sporophytes. Populations of *Alaria* tend to turnover annually in southern localities but are perennial at higher latitudes (McConnico and Foster 2005).

Eight or nine species of *Alaria* are currently recognized: one in the North Atlantic Ocean (*A. esculenta*) and several species in the North Pacific Ocean and Bering Sea. Six species are recognized in the NW Pacific (*A. angusta*, *A. crassifolia*, *A. crispa*, *A. ochotensis*, *A. paridisea* and *A. praelonga*), but variously only two species (*A. marginata*, *A. taeniata*) (Gabrielson and Lindstrom 2018), or a single *A. marginata* 'species complex' (Kraan 2020) occur in the NE Pacific. Under the two-species model, previously recognized *Alaria nana* and *A. tenuifolia* are considered to be ecotypic variants of *A. marginata.* NE Pacific taxa variously extend from Central California to the western Aleutian Islands. The morphologies of the NE Pacific taxa are plastic and cannot easily be used to distinguish among species (Widdowson 1971a; Lane et al. 2007). While the taxonomies of NE Pacific *Alaria* are in a state of flux (Kraan 2020; Gabrielson and Lindstrom 2018), we use species names associated with Genbank sequences to anchor the results in recent literature. DNA sequence variability has been interpreted to indicate that the NE Pacific species complex includes partially divergent, but reassociating and interbreeding taxa that have arisen in allopatric isolation in glacial refugia during the Pleistocene Epoch (Lane et al. 2007).

Here, we examine genetic variability among populations of the *Alaria* extending 2800 km from the eastern Aleutian Islands to Southeast Alaska with three marker types, mitochondrial (mt) DNA, plastid (p) DNA, and microsatellites. Uniparental inheritance of organellar markers is a basic assumption of phylogeographic methods (Avise 2000) and provides a means of tracking ancestral relationships among populations. Species of *Alaria* show maternal inheritance of pDNA (Kraan and Guiry 2000a), and mtDNA is likely to be maternally inherited, as it is in other kelps (Li et al. 2016). Furthermore, mtDNA and pDNA evolve independently of each other, providing different

perspectives of the same population history. The use of biparentally inherited microsatellite markers provides yet another view of historical and contemporary population dynamics by providing a means of detecting geographic patterns of hybridization and by tracking the effects of recent population events because of large mutation rates (Tautz and Schlötterer 1994; Nieva et al. 2018).

Our study builds on the phylogenetic work of Lane et al. (2007) by analyzing numerous populations of *Alaria* in the Gulf of Alaska with larger sample sizes and by using multiple genetic markers. We also provide a temporal framework of divergences between the lineages within *Alaria* by using the multi-gene, time calibrated kelp phylogeny of Starko et al. (2019). Our goal is to use the geographic distributions of genetic diversity and time estimates of divergence between lineages to assess whether the origins of incipient species can be traced to environmental changes during the Pleistocene ice ages. Through this analysis, we provide insight into the mechanisms promoting and limiting kelp diversity in the Gulf of Alaska.

Materials and Methods

Laboratory methods

Samples were collected at 16 rocky intertidal sites along the coast of Alaska (Fig. 1a, Table 1). A total of 543 plants (mean sample size = 33.9) were examined for organellar sequence variability (Table 1) and 530 plants (mean sample size = 28) were surveyed for microsatellite variability (Tables 2). A 4 cm^2 piece of frond or sporophyll was damp-dried and placed in a dessicator filled with silica beads soon after collection. DNA was extracted from 10–20 mg of dried tissue with the NucleoSpin® 96 Plant II kit (Macherey-Nagel Inc., Düren, Germany). The standard extraction kit protocol was followed, except dried subsamples were homogenized at room temperature by crushing or chopping on weighing paper with a scalpel. Homogenates were funneled into microtubes on a 96-well plate with PL1 lysis buffer (cetyltrimethylammonium bromide [CTAB] method) and incubated at 65°C overnight.

A segment of the mitochondrial 5'-*COI* gene was amplified with the polymerase chain reaction (PCR), using the forward primer *GazF2* (5' CCAACCAYAAAGATATWGGTAC 3') and reverse primer *GazR2* (5' GGATGACCAAARAACCAAAA 3') (Lane *et al*. 2007). A segment of the chloroplast *rbc*L-3' gene was amplified using the forward primer *rbc*L-*543F* (5' CCWAAATTAGGTCTTTCWGGWAAAAA 3') (Bittner et al. 2008; Silberfeld et al. 2010) and reverse primer *rbc*L-*1381R* (5' ATATCTTTCCATARRTCTAAWGC 3') (Burrowes et al. 2003; Silberfeld et al. 2010). PCR cocktails

consisted of a 50 µL mixture of 2.0 µL template DNA in 1x Colorless GoTaq Flexi buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1µM of forward and reverse primers, and 2.5U GoTaq Flexi DNA polymerase. PCR amplifications were made in ABI 9700 thermocyclers with an initial denaturation at 94° C for 3 min, followed by 35 amplification cycles of 45 s at 94° C, 1 min at 50° C for *COI* and 52° C for *rbc*L, and 1 min 30 s at 72° C, with a final elongation step of 5 mins at 72° C. PCR amplifications were sequenced in the forward and reverse directions by Genewiz Inc. (South Plainfield, NJ), or by the University of Arizona Genetics Core. Forward and reverse-complement sequences were aligned and edited with MEGA 7.0.20 (Kumar et al. 2016), and chromatograms viewed with FINCH TV 1.4.0 (Geospiza Inc.). Unique haplotypes from each of the 96-well plates were re-extracted and re-sequenced for quality control.

Eight microsatellite loci were amplified with primers previously developed for *Alaria marginata*, including *An21, An23*, *An26*, *An27*, *An30*, *An31*, *An38*, and *An29* (Collens 2009). Each 10 µL reaction cocktail consisted of 2 µL template DNA (~0.1µg/µL) in 1x Colorless GoTaq Flexi Buffer (Promega Inc. Madison, WI), 1.5–3.0 mM $MgCl_2$ (Promega Inc. Madison, WI), 0.20 mM of each nucleotide (Applied Biogsytems, Inc.), 0.05–0.25 µM of forward and reverse primers, 0.1 mg/mL of BSA (Sigma Inc. St. Louis, MO), 0.05 U GoTaq Flexi DNA polymerase (Promega Inc. Madison, WI) and deionized water. Optimal thermal cycling profiles varied among loci (Supplemental Table S1). Microsatellites were fractionated by size with electrophoresis in an Applied Biosystems 3730 capillary DNA sequencer, and scored with GENEMAPPER 5.0 (Applied Biosystems) independently by two technicians. A subset of 8% of the samples was re-extracted and re-genotyped by a third technician for quality control.

Statistical methods

For the organellar DNA sequences, ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010) was used to estimate the number of polymorphic nucleotide sites (N_{poly}), the number of observed , and expected haplotypes under neutrality (N_H and N_{EH}, respectively), gene diversity (h), and nucleotide diversity (θ_π). Haplotype divergence between populations was estimated with Φ_{ST} with an appropriate mutation model determined with MEGA 7 (Kumar et al. 2016). We used TCS 1.21 (Clement et al. 2000) to construct a 95% plausible parsimony networks of haplotypes for mtDNA, pDNA and the concatenated sequences. A parsimony network was also constructed for all available *COI* sequences, including those presented in this study and 49 sequences from Genbank (Table S2).

Mean sequence divergences between *COI* lineages from the Gulf of Alaska and sequences of species of *Alaria* from Genbank (Table S2) were estimated with the Tamura-Nei (1993) (TN93) substitution model determined with MEGA. A Bayes tree of available, overlapping *COI* sequences

(634 bp; Table S2) for species of *Alaria* in the North Pacific and North Atlantic ocean was constructed with BEAST 1.6 using the TN93 model, a strict clock, Yule model of speciation, and *Druehlia* (*Eualaria*) *fistulosa* as an outgroup (Lane et al. 2007). The tree was time-calibrated with a time since divergence of 5.8 million years (Ma) between *A. marginata* and *A. esculenta*, as indicated in Fig. 2 of Starko et al. (2019), with a normal distribution and standard deviation of 0.6 that yielded an approximate node prior interval of 6.8–4.8 Ma. The earlier date approximates the timing of the earliest opening of Bering Strait at 7 Ma (Marincovich and Gladenkov 2001; Gladenkov et al. 2002) that allowed dispersal from the N Pacific Ocean into the N Atlantic Ocean. Ten million MCMC steps produced an effective sample sizes of the various components of well over 200.

An initial analysis of microsatellite genotypic data was made with GENEPOP 4.6 (Rousset 2008) to search for null alleles. When these results indicated the presence of null alleles, we used MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) to further confirm the presence of null alleles, and to determine whether allele stuttering or large-allele dropout had affected a dataset. Some loci failed to amplify with PCR in some individuals, even though amplifications were successful at other loci. These PCR failures were assumed to be homozygous genotypes of null alleles.

GENEPOP was used to test for deviations from expected Hardy-Weinberg genotypic proportions, using Markov-chain-Monte-Carlo chains with 10,000 steps and 100 batches. Because of the repeated tests among loci, Bonferroni correction (Rice 1989) of $P = 0.05/8 = 0.006$ was used to control type I error at $\alpha = 0.05$ among multiple tests. GENETIC DATA ANALYSIS (GDA, Lewis and Zaykin 2001) was used to estimate observed and expected heterozyzygosity averaged over loci (H_O and H_E, respectively), to count the number of alleles at each locus, and to estimate the inbreeding coeficient, F_{IS}. FSTAT (Goudet 1995) was used to estimate allelic richness (A_r) over samples for each locus (minimal $N = 256$) and HP-Rare (Kalinowski 2005) was used to estimate A_r within populations (minimal $N = 20$). F_{ST} between populations was estimated with GENEALEX 5.03 (Peakall and Smouse 2012) with 9999 permutations to establish departures from 0.

Principal Coordinate Analysis (PCoA) of microsatellite allele-frequency variability among populations with standardized covariances was used to identify genetic groups. Geographical structure was assessed with the analysis of molecular variation (AMOVA) in ARLEQUIN with 9999 permutations among individuals, populations and regions to establish significance. Missing genotypes for some loci in some plants were estimated with sample averaging for both the PCoA and AMOVA. Results for each locus were summed across loci under the assumption of linkage equilibrium and independence among loci.

IBD 1.52 (Bohonak 2002) was used to test for isolation-by-distance with Mantel's test between difference matrices of linearized [$\Phi_{ST}/(1-\Phi_{ST})$] for organellar divergences and [$F_{ST}/(1-F_{ST})$] for microsatellite genetic distances between pairs of populations and approximate shoreline distances between populations. Both, geographical distances in km and $\log_{10}$ km of distance were tested with 1000 randomizations.

Results

*rbc*L

Four nucleotide polymorphisms in a 740 base pair segment of the chloroplast gene *rbc*L defined five haplotypes in 560 plants (Table S3, Figure 1a). Haplotype diversity (*h*) ranged from 0.0 to 0.512 and averaged 0.127 among populations. Nucleotide diversity (θ_π) ranged from 0.0 to 0.0014 and average 0.0006 (Table S4). Tajima's D was not significant in any of the populations, or in a pooled sample. Φ_{ST} between populations varied widely from 0.0 for populations fixed for the same haplotype to 1.0 between populations not sharing haplotypes (Figure 1b, Table S5) and was 0.830 overall. AMOVA indicated that 83% of sequences variability occurred among populations and 17% was due to variability on average among individuals within populations (Table S6).

COI

COI was much more polymorphic than *rbc*L. Polymorphisms at 36 nucleotide sites in 585 plants defined 27 haplotypes (664 bp) that fell into five lineages (**A**–**E**), each separated by at least 5 mutations (Figure 1c; Tables S7, S8). Φ_{ST} between populations ranged from 0, for populations fixed for the same haplotype, to 1.0, for populations not sharing haplotypes (Figure 1d, Table S9). Twelve of the 16 populations featured private haplotypes. Populations 1, 13, and 14 had a mix of lineages, producing the largest haplotype diversities (*h* = 0.232–0.636) and nucleotide diversities (θ_π = 0.0023–0.0052). Tajima's *D* indicated that haplotype distributions in three populations (5, 12, 15) deviated from neutralilty. AMOVA indicated that 90.4% of the variability was due to differences among populations and 9.6% was due to diversity within populations, on average (Table S10). Overall, Φ_{ST} was 0.904.

A parsimony network of all available *COI* haplotypes (634 bp) shows the relationships among nominal species of *Alaria* (Table S2) and the five lineages in the NE Pacific (Figure 3). Lineage **A** included sequences attributed to *A. marginata*, *A. nana*, *A. taeniata*, and *A. tenuifolia*. Lineage **B**

included only *A. taeniata*. Lineage **C** included *A. nana*, *A. taeniata* and *A. tenuifolia*. Lineage **D** largely included *A. marginata*, but also a few *A. nana*. Plants in lineage **E** were three mutational steps from the central haplotypes of lineag **C** and five mutational steps from NW Pacific *A. paradisea*.

A Bayes tree of relationships among the five NE Pacific lineages and five NW Pacific *Alaria* species appears in Fig. 4. A prior estimate of 5.8 Ma divergence between *A. esculenta* and *A. marginata* yielded a posterior estimate of 5.798 Ma, given the data. Temporal divergences between the 5 NE Pacific lineages ranged from 4.224 to 2.867 Ma, corresponding to sequence divergences of 0.0248 to 0.0117 (Table 3). Notably, divergences between the five NE Pacific lineages and *A. paradisea* ranged from 0.0197 to 0.0106 and were in the range of divergences between the five lineages. Mean sequence divergences between all the taxa of *Alaria* ranged from d = 0.0080 between *A. praelonga* and *A. crispa* to d = 0.0489 between *A. crispa* and lineage **A**.

Concatenated *rbc*L and *COI*

We concatenated the *rbc*L and *COI* sequences (1404 bp) for further analyses of a dataset with 543 plants (Tables 1, S11). A total of 41 nucleotide polymorphisms defined 33 haplotypes (Fig. 1e; Table 1). Gene diversity (h) ranged from 0.0 to 0.636 and averaged 0.290 among populations. Overall, gene diversity was much larger (h = 0.903) because a large portion of the diversity was due to differences among populations. Nucelotide diversity ranged from 0.0 to 0.00264 and averaged 0.061. The number of private alleles ranged from 0 to 4 among populations and averaged 1.8 alleles. Tajima's D was significant in only population 5 (D = -2.206, P = 0.002, Afognak Island) and was not significant overall in the pooled analysis (D = 0.776, P = 0.832).

Sequence divergence between populations ranged from 0.0 between adajcent populations 9 (Cordova) and 11 (Yakutat), which were both fixed for haplotype **A1** (Figure 1f), to 1.0 between populations 8 and 9 and between 8 and 11, which were fixed for different haplotypes (Table S12). No IBD was detected with Mantel's test for a correlation between genetic distance and large-scale shoreline distance between populations (r = 0.074, P = 0.737) (Figure 2a). AMOVA indicated that 98.6% of the variability was due to differences among populations and 1.4% was due to diversity among plants within populations on average (Table S13). Overall, Φ_{ST} was 0.896.

The genealogy reconstructed from the concatenated sequences resolved additional phylogeographic structure within lineages **B**, **C** and **D**. Lineage **B** consisted of geographically separated **B1** in Kachemak Bay and **B2** around Kodiak Island (3 & 4) and in Kachemak Bay (5) (Figure 1f). Lineage **C** was resolved into **C1** in Kachemak Bay (7) and Little Port Walter (15) and **C2** around Magoun Island (13). **C1** appeared in widely scattered populations 1, 7, and 15. Haplotype **D1**

appeared around Afognak Island (5) in the western Gulf of Alaska and at Watson Point (14) in Southeastern Alaska.

Microsatellites

The genotypes of specimens at four locations had more than two alleles in of 4 of the 8 loci (Table S14). For our analysis, two alleles at these loci were chosen randomly to produce diploid genotypes. The number of alleles at a locus ranged from 16 to 87 and averaged 41.6, but allellic richness (A_r) ranged from 15.6 to 50.0 and averaged 37.5 per locus (Table S15). Observed population heterozygosities (H_E) ranged from 0.057 to 0.548 and averaged 0.346. Expected heterozygosities (H_E) were larger for each locus, ranging from 0.287 to 0.695 and averaging 0.563. The inbreeding coefficients (F_{IS}) were positive and large, ranging from 0.120 to 0.748 and averaging 0.382. Sample sizes for the 16 populations varied from 20.6 to 43.4 and average 28.0 over loci (Table 2). The number of alleles (N_a) ranged from 4.5 to 12.6 and averaged 7.6 across populations. Allelic richness (A_r) varied from 3.39 to 7.79 and averaged 5.13. The number of private alleles (N_{PR}) ranged from 1 to 28 and averaged 9.1 across populations, with average frequencies ranging from 0.018 to 1.0. Observed heterozygosities (H_O) were less than expected heterozygosities (H_E) in all of the populations and this led to positive inbreeding coefficients (F_{IS}), ranging from 0.205 to 0.515.

Allele-frequency divergence between populations measured with F_{ST} ranged from 0.086 to 0.505 (Table S16) and overall was 0.457 ($P < 0.0001$). An AMOVA showed that 46% of the variability was due to differences among populations, and 54% occurred as differences between individuals within populations (Table S17). No correlation between linearlized F_{ST} and shoreline distance appeared between populations, both with ($r = 0.016$, $P = 0.445$) and without ($r = 0.1341$, $P = 0.763$) log transformation of geographical distance (Figure 2b). A PCoA of allelic frequencies showed that some populations grouped by organellar lineage rather than by location. For example, plants in the **A** lineage clustered together (Figure 5c), even though they were geographically widely separated (e.g. populations 1 and 12, 16), as were populations 5 and 15 which consisted of lineage **D1** (Figure 5e).

Three populations included plants carrying divergent organellar lineages. Population 1 included plants from lineages **A** and **C**, population 13 **A** and **C** and population 14 **A** and **D**. In each of these populations, the microsatellite genotypes of individual plants in different lineages clustered together, indicating hybridizations between lineages that led to the sharing of microsatellite alleles between lineages.

Discussion

The results of our study extend the conclusions of Lane et al. (2007) by providing greater genetic resolution of the evolutionary components of the *Alaria* complex in the Gulf of Alaska. Our survey of genetic variability around the Gulf of Alaska resolved five major organellar DNA lineages that do not entirely correspond to historical species' delineations. These lineages form a geographic mosaic that most likely reflects divergences during numerous isolations in local refugia during Pleistocene glaciations. Hybridizations between lineages revealed by nuclear markers indicate that reproductive isolation between some of the incipient species is incomplete even after long periods of separation. Our results support the conclusion of Lane et al. (2007) that *Alaria* in the NE Pacific encompasses several recently diverged lineages, but additionally indicate that the term 'recent' encompasses divergences originating over the past 1–4 million years.

Genetic variability in the Gulf of Alaska

A key feature of *Alaria* populations is a chaotic geographic distribution of the genetic lineages. Some lineages (**E** for example) appear to be limited to small areas, but other lineages are wide-spread. For example, lineage **A** is most abundant in south central and southeastern Alaska, but also appears several hundreds of kms away at the western edge of the Gulf of Alaska in the eastern Aleutian Islands (Figure 1f). Geographic transitions between populations of different lineages were abrupt in most cases. In Kachemak Bay for example, populations separated by only about 10 km were fixed for divergent lineages **C** and **B**. Kasitsna Bay (location 6) and Homer Spit (location 8) habitats in Kachemak Bay were wave-protected, whereas Kayak Beach (location 7) was exposed to a fetch of tens of kilometers that produces considerable wave action during storms. Similar contrasts between populations appeared over distances of tens of kilometres between Kodiak (locations 3 and 4) and Afognak islands (location 5; Figures 1f, 5b, 5e). These small-scale patterns likely indicate the importance of local environmental conditions in shaping the distributions of ecotypes in the *A marginata* complex (McLean et al. 2019).

The chaotic distributions of organellar lineages on large spatial scales are best understood in light of isolations in regional refugia over multiple ice-age cycles that produced large divergences dating to 3.68–2.87 Ma, or to 4.22 Ma, if *A. paradisea* is included in the NE Pacific *Alaria* species complex (Figure 4). However, the maintenance of fixed differences between populations reflects the lack of contemporary gene flow between populations. Gene flow between populations of *Alaria* may be hampered by three mechanisms. One is the restricted spore and gamete dispersal. Even though large numbers of spores are produced by sporophylls, realized spore dispersal for kelps is generally

limited to only a few meters (Dayton 1985; Santelices 1990). Another mechanism limiting gene flow may be adaptive constraints imposed by environmental differences, such as wave exposure, which appears to be an important factor in explaining the distributions of morphological types of *Alaria* in Auke Bay (McLean et al. 2019). Sharp genetic discontinuities may also arise because of priority effects, such as high-density blocking (Waters et al. 2013), which has been invoked to account for sharp genetic discontinuities of seaweed lineages along a shoreline in the absence of physical or environmental barriers to gene flow (Neiva et al. 2012).

Phylogeny of *Alaria* species in the NE Pacific

Another key feature of *Alaria* in the NE Pacific Ocean is the confusion of species' identifications based on morphology, which is made clear in Figure 3. For example, the nominal species *A. marginata* and *A. tenuifolia* along the coast of British Columbia show intermediate forms, and in the NW Pacific, *A. crassifolia*, *A. angusta*, and *A. praelonga* are difficult to distinguish from one another (Widdowson 1971b). In our compilation of available *COI* sequences, some of the lineages were not strictly associated with a particular species. Lineage **A** encompasses sequences from the nominal species *A. marginata*, *A. nana*, *A. taeniata*, and *A. tenuifolia*. Lineage **B** includes only sequences identified as *A. taeniata*, whereas lineage ***C*** includes sequences identified as *A. taeniata*, *A. tenuifolia*, and *A. nana*. Lineage **D** includes sequences attributed to *A. marginata* and *A. nana*. Specimens identified as *A. nana* appear in lineages **A**, **C**, and **D**, indicating that this nominal species may be a convergent ecotype of several lineages. *Alaria* in lineage **E** along the Alaska Peninsula do not appear to have previously been examined with molecular markers. *Alaria paradisea* appears to be the sister taxon to the *A. marginata* complex, as previously noted (Klimova et al. 2018; Kraan 2020). While most of the species' names of NE Pacific *Alaria* have been abandoned, except *A. marginata* and *A. taeniata* (Gabrielson and Lindstrom 2018), the lineages depicted in Figure 3 provide starting points for future eco-physiological research and further taxonomic considerations.

Incomplete reproductive isolation between divergent lineages

The haplotype networks of organellar DNA and patterns of divergence for microsatellites depict taxa at various stages of divergence that have not fully developed reproductive isolation from one another. Three populations (1, 13, 14) consisted of a mix of plants from organellar lineages that were 5–15 mutations apart. The clustering of microsatellite genotypes in the PCoA shows convergence of plants in the different organellar lineages at these three localities (Figures 3c, 3d, 3e), providing further evidence of hybridization between taxa of *Alaria* in the Gulf of Alaska. If hybridizations between these divergent lineages were not occurring, Wahlund's effect might

produce elevated inbreed coefficients, but this does not appear to be the case, as values of F_{IS} were not unusually large relative to the other populations. Putative hybridizations appeared between *COI*-*rbc*L haplogroups **A1** and **C1**, **A1** and **C2** and **A1** and **D1** (Figure 1e). In the study by Lane et al. (2007), nuclear ITS genotypes also detected hybrids between organellar lineages corresponding to *COI* haplogroups **A** and **B**, **A** and **C** and **A** and **D** described here (Figure 1c).

Hybridizations between species of *Alaria* have been demonstrated through culture studies of gametophytes to gauge evolutionary relationships among species. Successful hybrids have been produced between species with different morphologies and chromosome numbers (*n*), including *A. tenuifolia* (14*n*) X *A. esculenta* (28*n*), *A. tenuifolia* X *A. praelonga* (22*n*), and *A. marginata* (14*n*) X *A. praelonga* (Kraan and Guiry 2000b). Distantly related *A. esculenta* (28*n*) and *A. praelonga* also produced viable, but slow growing, sporophytes (Kraan and Guiry 2000a). Evidence of polyploidy in a few individuals at four locations in our study was evidenced by the occurrence of more than two microsatellite alleles at a locus (Table S14). The ability of several *Alaria* species to hybridize led Kraan and Guiry (2000b) to conclude that the origins of species diversity in *Alaria* was 'recent'. However, our time-calibrated *COI* phylogeny indicates that 'recent' includes divergences between some species on a scale of a few million years (Figure 4).

Northern glacial refugia

A long-standing model for interpreting patterns of genetic diversity in high-latitude regions of North America and Europe covered by ice sheets during Pleistocene glacial episodes has been the expectation of southern genetic richness and northern poverty (Hewitt 1996, 2004). This model was developed from the observation that the thick ice sheets of Eurasia forced terrestrial plants and animals into southern refugia, and post-glacial colonizations produced north-south gradients in genetic diversity (Taberlet et al. 1998; Hewitt 1999). The margins of the Cordilleran Ice Sheet that blanketed western North America covered coastal areas of the NE Pacific numerous times in the Pleistocene (Thorson 1980, 1989; Ryder and Clague 1989; Mann and Hamilton 1995; Kaufman and Manley 2004; Briner and Kaufman 2008; De Scheeper et al. 2014) and potentially displaced *Alaria* populations to southern unglaciated shores. However, the southern refugium model does not appear to apply to coastal terrestrial species of Northwestern North America (Shafer et al. 2010), or to marine species, which show genetic imprints of survival in northern glacial refugia (Lindstrom 2009; Canino et al. 2010; Marko and Zaslavskaya 2019; Marko et al. 2010; Bringloe et al. 2020b).

Although episodic glaciations on North America began in the early Pleistocene (Prueher and Rea 1998; Haug et al. 2005; De Scheeper et al. 2014), coastal glaciations are best known over the last

Croll-Milankovitch glaciation cycle, which began about 120 kyr ago (Lisiecki and Raymo 2005; Jouzel et al. 2007). The behavior and configurations of these coastal glaciers are assumed to be typical of earlier glaciations. Northern refugia during the last ice age were possible at several locations around the Gulf of Alaska, as the edge of Cordilleran Ice Sheet was not continuous along the coast during glacial maxima. Lobes of the ice sheet reached the continental shelf primarily through fjords betweeen coastal mountains, leaving many shorelines between the glacial lobes ice-free (Mann and Peteet 1994; Mann and Hamiltonn 1995; Kaufman and Manley 2004; Carrarea et al. 2007). Similar ice-free areas occurred along coastal British Columbia (Clague and James 2002). Many of these ice-free shorelines corresponded to nearby terrestrial refugia for plants and animals (Reimchen and Byun 2005; Carrara et al. 2007; Sawyer et al. 2019). The unglaciated, seasonally ice-free southern shores of the Bering Land Bridge (Sancetta 1983; Sancetta et al. 1984) may also have served as a kelp refugium.

Sea surface temperatures (SST) and salinities in these potential northern refugia were well within the physiological tolerances of high-latitude *Alaria* sporophytes and gametophytes (tom Dieck 1993; Karsten 2007; Fredersdorf et al. 2009). SST in coastal areas around the Gulf of Alaska dropped to 2–4° C in summer and 0° C (ice-covered) in winter during glacial maxima (CLIMAP, 1981; Sabin and Pisias 1996; de Vernal and Pedersen 1997), but rose rapidly after coastal glaciers retreated (Barron et al. 2009). The open waters of the Gulf of Alaska were not covered in perennial ice, although icebergs, calved from tidewater glaciers, were common (St. John and Krissek 1999). Surface salinities of coastal waters were generally lower, producing stratification that lessened the mixing of surface waters with nutrient rich deep waters (Zahn et al. 1991; Worne et al. 2019), but were still within the physiological tolerances of *Alaria* (Karsten 2007; Fredersdorf et al. 2009).

Incipient speciation countered by population turnover

The splintering of rocky shore habitats by glacial lobes during Pleistocene glacial maxima isolated populations of *Alaria* and set into motion genetic divergences leading to incipient speciation. However, repeated ice-age cycles also led to population turnover that shortened the longevities of these diverging populations. Support for a population-turnover model can be found in a comparison of taxa in the NW and NE Pacific. While the number of evolutionary lineages is similar between the NW and NE Pacific, lineages in the NE Pacific show shallower divergences (Figure 4). NW Pacific shores were not heavily glaciated by the margins of continental ice sheets (Nürnberg et al. 2011) and hence, populations may not have experienced high rates of turnover. NW Pacific *Alaria crispa* and *A. praelonga* were most divergent from other species of *Alaria* with separation times as long as 9 Ma (95% HPD 12.32–6.37 Ma; Figure 4). *Alaria crassifolia* also showed a deep divergence from other

species of about 6.7 Ma (95% HPD 8.93–4.49 Ma). These deep divergences contrast with separations between the incipient species in the NE Pacific of only 2.9 to 3.7 Ma.

While little is known about the population structures of NW Pacific species of *Alaria*, the chaotic genetic population structure of NE Pacific populations supports a population-turnover model on long time scales that prevents the completion of reproductive isolation. Despite population turnover, average microsatellite heterozygosities among populations (H = 0.563, Table 2), paradoxically, were not greatly reduced relative to other kelps (Neiva et al. 2018). These moderate levels of genetic diversity indicate that glacial refugia around the Gulf of Alaska were most likely large and numerous. The general lack of departures from neutrality expected in populations expanding into newly available habitats after the Last Glacial Maximum 23–18 thousand years ago, together with the chaotic population structure, indicates population turnovers on time scale of hundreds of thousands of years. A similar model was used to explain chaotic population structure of kelps in the North Atlantic (Neiva et al. 2018) and of marine invertebrates in the NE Pacific (Marko et al. 2010).

Conclusions

Fine-scale structure among populations and ecotypes are likely due to environmental variability among rocky intertidal habitats and can best be understood with ecological studies, together with functional genomic datasets. However, large-scale patterns of diversity among *Alaria* populations can only be understood in terms of the Pleistocene glacial history of the North Pacific. The large amount of overall diversity among populations and the lack of departures from neutrality indicate that most contemporary populations in the Gulf of Alaska originated from large local refugia and were unlikely seeded by colonists from a distant southern refuge. The contrast between the NW Pacific, with several deeply separated species, and the NE Pacific, with several incipient species, may be attributable to differences in the severity of the effects of coastal glaciers on population longevity. Deeply divided lineages were able to survive along the shores of the relatively unglaciated NW Pacific, whereas in the NE Pacific, shoreline glaciers led to greater population turnover, arresting the formation of new species.

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Data Availability

DNA sequences: Genbank *COI* Accession nos MT053287–MT053313. *rbc*L Accession nos MT040328–MT040332. Microsatellite DNA genotypes available from the North Pacific Research Board Project 1618 and Figshare doi:10.6084/m9.figshare.11832975

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Figure Captions

[color, one column width]

Figure 1. Networks showing mutational relationships between haplotypes of *Alaria* spp. and pie diagrams for *rbc*L (740 bp) (a, b), *COI* (654 bp) (c, d) and *rbc*L + *COI* combined (1404 bp) (e, f). White wedges indicate frequencies of local private alleles. Haplotype numbers in (a) correspond to the last two digits of Genbank *rbcL* Accession numbers MT040328–MT040332 and numbers in (c) correspond to *COI* Accession numbers MT053287–MT053313.

[black and white, one column width]

Figure 2. Relationship between genetic distance (F_{ST}) and shoreline distance (kilometers) between samples of *Alaria* spp. Mantel's test for isolation by distance were made between linearized F_{ST} and geographical distance and log(geographical distance). (a) Concatenated *rbc*L and *COI* sequences: linear F_{ST} and geographical distance: $r = 0.074$, $P = 0.737$; linear F_{ST} and log(geographical distance): $r = 0.0009$, $P = 0.420$. (b) 8 microsatellite loci: linear F_{ST} and geographical distance: $r = 0.1341$, $P = 0.763$; linear F_{ST} and log(geographical distance): $r = 0.016$, $P = 0.445$.

[color, one column width]

Figure 3. Parsimony network of *COI* haplotypes (634 bp) and corresponding species identifications. Sizes of open ovals are proportional to sample size. Closed ovals represent hypothetical or unsampled haplotypes. See Table S2 for additional information about Genbank sequences.

[grey scale, 1.5 columns wide]

Figure 4. Phylogenetic reconstruction of species of *Alaria* with *Druehlia* (*Eualaria*) *fistulosa* as an outgroup with sequences of *COI* (634 bp). Time scale calibrated with divergence between *A.* '*marginata*' and *A. esculenta* in a kelp phylogeny reconstructed in Starko et al. (2019). Numbers at nodes represent Ma since divergence and 95% Bayesian support for the node. Grey bars represent 95% highest probability density of divergence time [color, one column width]

Figure 5. Principal co-ordinates analysis of allele-frequency variability at 8 microsatellite loci in *Alaria* spp. (a) Total PCO plot. (b-f) PCO plots of individuals from each sample site with numbers corresponding to locations in Table 1. Colors correspond to *rbc*L-*COI* DNA lineages in Figure 1e (g).

Table 1. Estimates of genetic parameters based on contatenated fragments of mitochondrial DNA cytochrome oxidase I (*COI*) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) (1359 base pairs combined) in populations from the Gulf of Alaska and Aleutian Islands. Location number, sample size (N), number of polymorphic nucleotide sites (N_{poly}), number of haplotypes (N_H), expected number of haplotypes under neutrality (N_{EH}), haplotype diversity (h, ±SD: standard deviation), nucleotide diversity (θ_π, SD: standard deviation) and Tajima's D (P: probability of null hypothesis of neutrality)

Location number	Location (Alaska)	N Latitude	W Longitude	N	N_{poly}	N_H	N_{EH}	N_{PH}	h ±SD	θ_π ±SD (%)	D	P
1	Morris Cove, Unalaska Island	53.919	166.438	32	9	3	1.84	2	0.234 ±0.095	0.137 ±0.089	-0.421	0.390
2	Sand Point, Popf Island, Alaska Pen.	55.309	160.513	38	2	3	1.93	3	0.243 ±0.088	0.018 ±0.023	-0.902	0.200
3	Table Island, Kodiak Island	57.188	152.925	28	2	3	1.97	2	0.265 ±0.105	0.020 ±0.024	-0.972	0.190
4	Near Island, Kodiak Island	57.788	152.388	31	1	2	1.41	1	0.125 ±0.077	0.009 ±0.016	-0.774	0.219
5	Black Cape, Afognak Island	58.402	152.882	32	18	5	4.28	4	0.593 ±0.056	0.112 ±0.076	-2.206	0.002
6	Kasitsna Bay, Kachemak Bay	59.468	151.553	39	3	3	4.76	2	0.614 ±0.041	0.089 ±0.064	1.686	0.957
7	Kayak Beach, Kachemak Bay	59.497	151.472	40	1	2	1.17	1	0.050 ±0.047	0.004 ±0.010	-1.124	0.126
8	Homer Spit, Kachemak Bay	59.604	151.418	48	0	1	–	0	0.0	0.0	–	–
9	Lowell Point, Resurrection Bay	60.064	149.443	34	0	1	–	0	0.0	0.0	–	–
10	Cordova, Prince William Sound	60.545	145.768	47	2	3	2.41	2	0.324 ±0.076	0.024 ±0.027	-0.487	0.332
11	Bridge Site, Yakutat	60.056	149.443	32	0	1	–	0	0.0	0.0	–	–
12	Auke Bay, North of Juneau	58.374	134.728	29	4	4	3.80	3	0.554 ±0.064	0.048 ±0.042	-0.874	0.221
13	Magoun Island, W	57.157	135.567	41	7	5	4.02	4	0.545	0.173	1.327	0.910

	Baranof Island								±0.072	±0.011		
14	Watson Point, W Baranof Island	57.070	135.368	31	14	3	2.08	1	0.288 ±0.097	0.264 ±0.152	0.184	0.628
15	Little Port Walter, SE Baranof Island	56.384	134.641	12	1	2	1.42	1	0.167 ±0.134	0.012 ±0.019	-1.141	0.175
16	Tokeen Bay, Prince of Wales Island	55.993	133.464	29	2	3	4.67	2	0.636 ±0.056	0.061 ±0.049	1.414	0.916
Average				33.9	4.1	2.8	2.75	1.8	0.290 ±0.513	0.061 ±0.202	-0.330	–
Pooled				543	41	33	34.3	28	0.903 ±0.006	0.547 ±0.282	0.776	0.832

Table 2. Summary statistics for 8 microsatellite loci in 16 populations from the Gulf of Alaska. N = mean sample size over loci. N_A = mean number of alleles. A_r = allelic richness (with N = 20), N_{PR} = number of private alleles. f(PR) = frequency of private alleles. H_O = observed heterozygosity. H_E = unbiased expected heterozygosity. F_{IS} = inbreeding coefficient.

Location	N	N_A	A_r	N_{PR}	f(PR)	H_O	H_E	F_{IS}
1	24.5	12.6	7.79	28	0.068	0.406	0.767	0.515
2	20.6	6.4	4.80	8	0.115	0.275	0.606	0.510
3	24.3	10.5	7.31	9	0.092	0.414	0.762	0.489
4	26.8	10.5	6.65	11	0.114	0.494	0.680	0.303
5	21.0	4.5	3.45	4	0.037	0.361	0.474	0.275
6	34.8	6.5	4.41	9	0.030	0.386	0.594	0.353
7	36.6	8.5	5.74	5	0.128	0.391	0.655	0.392
8	38.8	5.3	3.53	12	0.109	0.262	0.357	0.205
9	24.5	5.3	4.21	4	0.438	0.253	0.491	0.315
10	43.4	4.9	3.39	1	1.0	0.277	0.396	0.215
11	23.8	5.0	3.80	1	0.018	0.362	0.499	0.305
12	26.5	12.0	7.69	17	0.089	0.488	0.708	0.335
13	36.3	12.0	6.51	11	0.035	0.368	0.604	0.349
14	23.5	7.0	4.96	8	0.160	0.370	0.629	0.401
15	21.0	5.0	3.82	4	0.263	0.207	0.410	0.426
16	22.1	5.4	4.00	13	0.073	0.227	0.381	0.199
Mean	28.0	7.6	5.13	9.1	0.173	0.346	0.563	0.375
Pooled	530	333	–	–	–	0.375	0.882	0.601

Table 3. Mean sequence divergence (TN93) between *COI* lineages in *Alaria* '*marginata*' species complex (A–E) and North Pacific and North Atlantic species of *Alaria*. Distantly related, *Druehlia fistulosa* serves as an outgroup taxon. A total of 32 sequences were used to estimate sequence divergence: 1) all of the unique sequences available for the five lineages in this study and 2) one sequence each of five additional species in Lane et al (2005) that matched the length (645 bp) of the sequences from the Gulf of Alaska.

B	0.0146									
C	0.0108	0.0180								
D	0.0205	0.0248	0.0153							
E	0.0170	0.0211	0.0117	0.0156						
A. paradisea	0.0156	0.0197	0.0106	0.0177	0.0138					
A. esculenta	0.0285	0.0318	0.0228	0.0298	0.0267	0.0173				
A. crassifolia	0.0417	0.0412	0.0360	0.0380	0.0403	0.0324	0.0328			
A. praelonga	0.0451	0.0363	0.0398	0.0457	0.0385	0.0358	0.0325	0.0409		
A. crispa	0.0489	0.0397	0.0427	0.0493	0.0420	0.0392	0.0359	0.0409	0.0080	
Druehlia fustulosa	0.0765	0.0716	0.0766	0.0858	0.0767	0.0758	0.0818	0.0819	0.0708	0.0729
	A	B	C	D	E	par	esc	cra	pra	cri

Figure 1

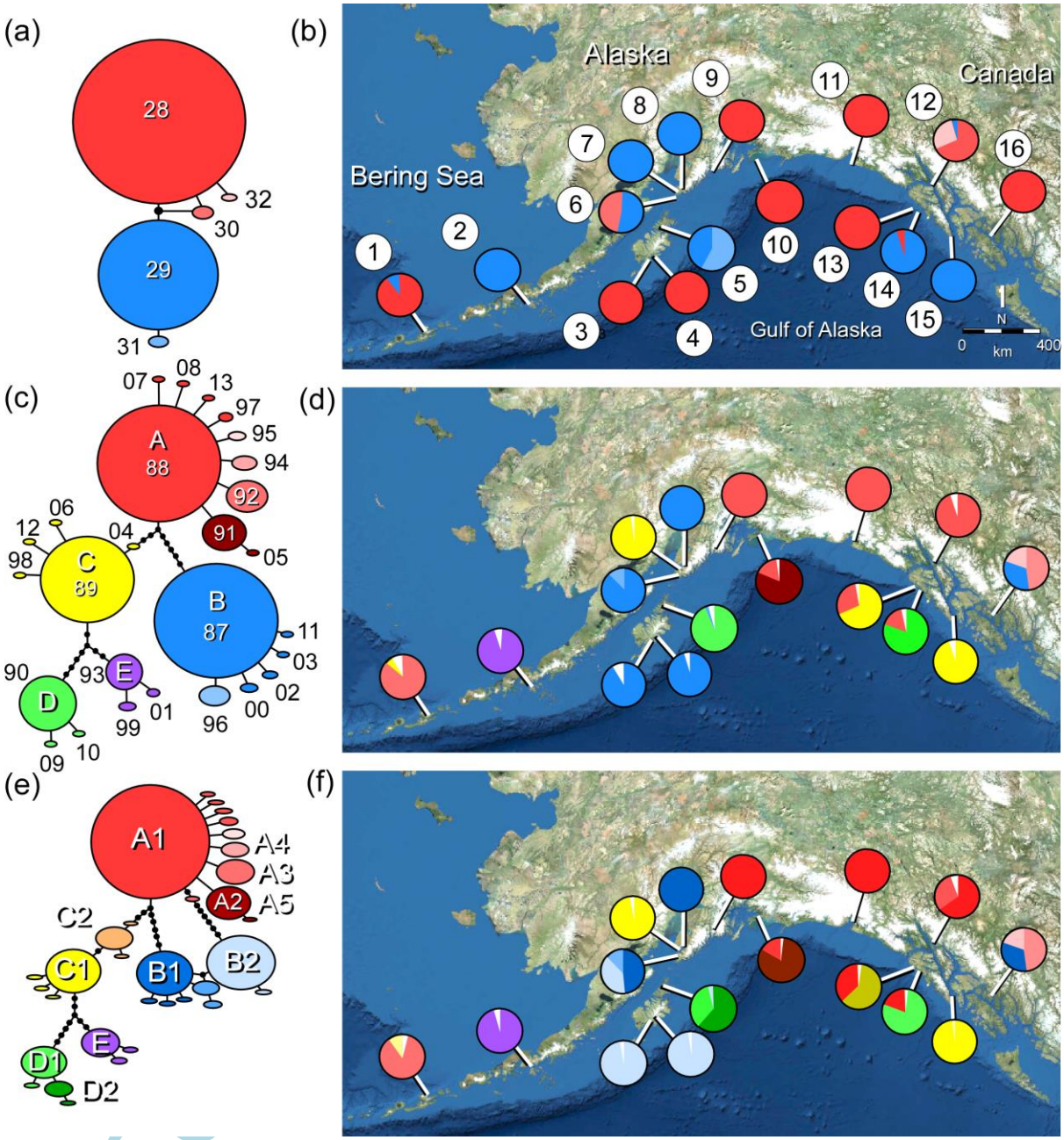

Figure 2

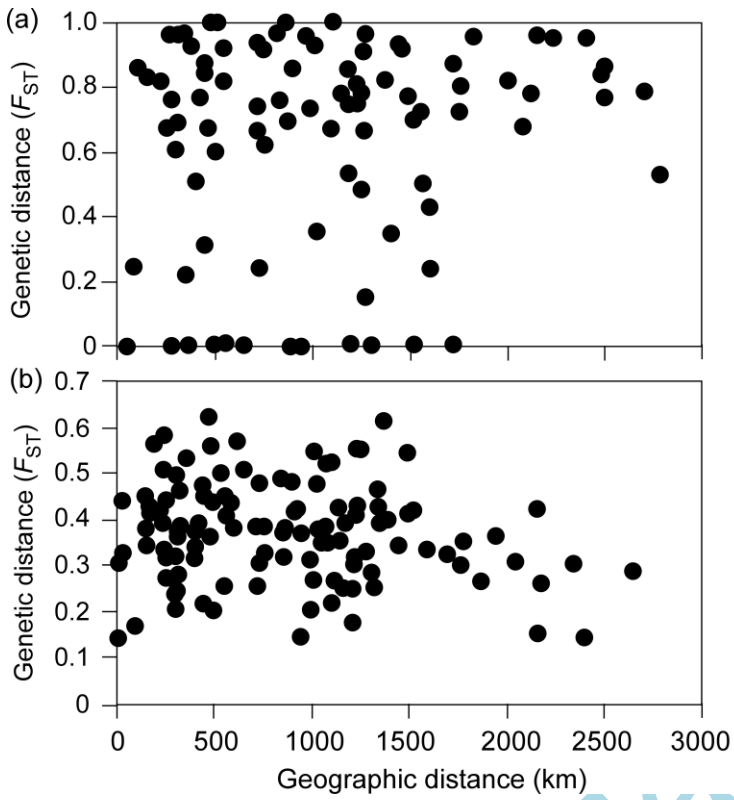

Figure 3

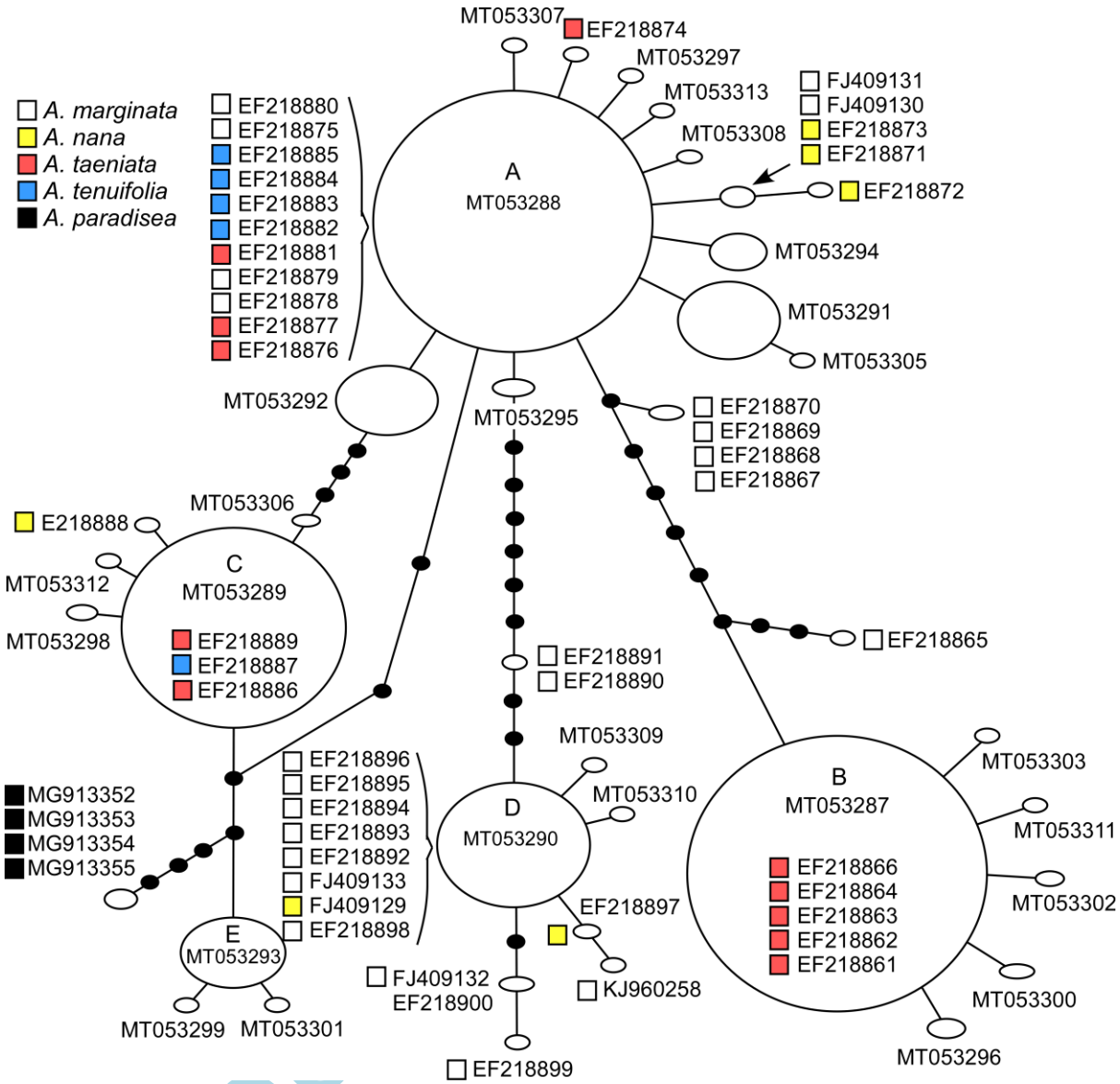

Figure 4

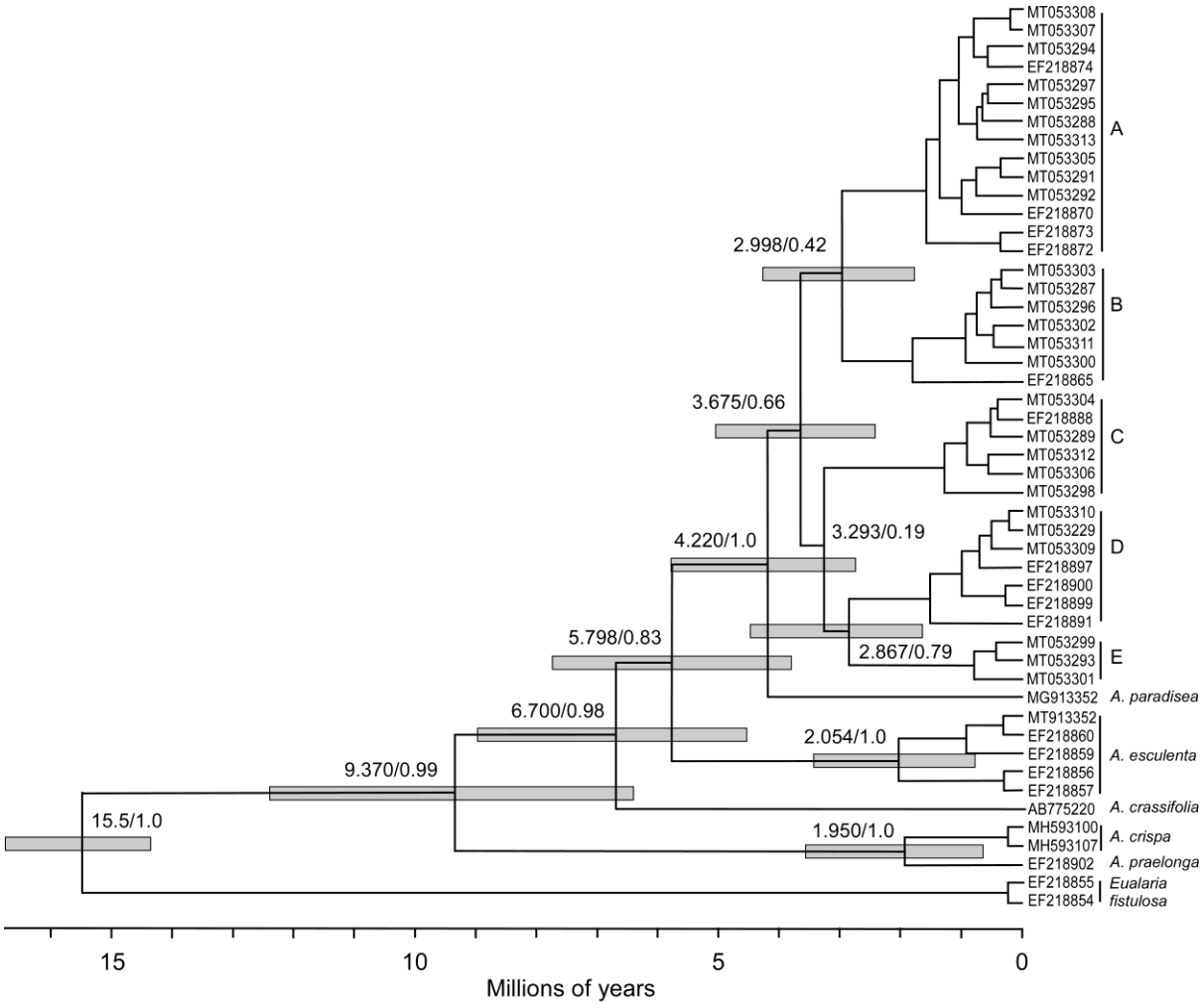

Figure 5