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A molecular assessment of northeast Pacific *Alaria* species (Laminariales, Phaeophyceae) with reference to the utility of DNA barcoding

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Abstract

Despite their relatively complex morphologies, species in the genus *Alaria* Greville are notoriously difficult to identify with certainty. Morphological characters, often influenced by environmental factors, make individuals in similar habitats artificially appear related. Species identification would, therefore, benefit greatly from the application of molecular tools. We applied DNA barcoding, using the 5′ end of the cytochrome *c* oxidase I (*cox*I-5′) gene from the mitochondrial genome, to define species limits and relationships in northeast Pacific populations of *Alaria*. This emerging technique is being employed to catalogue species diversity worldwide, particularly among animals, and it has been shown to be sensitive enough to discriminate between closely related species. However, the utility of this marker for identifying or categorizing the majority of life remains unclear. We compared the resolution obtained with this marker to two other molecular systems commonly used in algal research: the nuclear internal transcribed spacer (ITS) of the ribosomal cistron, and the plastid Rubisco operon spacer (*rbc*Sp). In agreement with previous results, *Alaria fistulosa* Postels & Ruprecht, with its distinct morphological, ecological and molecular features, stands apart from the other species in the genus and we establish *Druehlia gen. nov.* to accommodate it. For the remaining isolates, distinct mitochondrial haplotypes resolved with the barcode data indicate a period of genetic isolation for at least three incipient species in the northeast Pacific, whereas unexpected levels and patterns of ITS variation, as well as the extreme morphological plasticity found among these isolates, have most probably resulted from a recent collapse in species barriers. The cloning of ITS amplicons revealed multiple ITS copies in several individuals, further supporting this hypothesis.
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Keywords: *Alaria*; *Druehlia*; *cox*I-5′; DNA barcode; ITS; Pacific; Rubisco spacer

1. Introduction

Alaria Greville is a common brown seaweed in the cold temperate regions of the Northern Hemisphere where it can be found in the intertidal and subtidal. *Alaria* is the second largest genus in the Laminariales and the type of the Alariaceae, one of the four "derived" families within this order (Lane et al., 2006). There are currently 12 recognized species (Kraan et al., 2001; Lüning, 1990; Widdowson, 1971a), but 108 specific and subspecific names have been applied to members of this genus (Widdowson, 1971a) since it was described (Greville, 1830). The morphological plasticity common to the kelp has caused considerable taxonomic confusion within *Alaria* despite comprehensive monographs (Widdowson, 1971a; Yendo, 1919), including a statistical attempt to quantify morphological variation in the genus (Widdowson, 1971b).

Widdowson's monograph (1971a) is the most comprehensive treatment of *Alaria* and includes extensive geographic distribution records, as well as a taxonomic key to the 14 species recognized by him, 11 in the North Pacific

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and three reported from both the North Atlantic and Pacific [*A. pylaii* (Bory) Greville and *A. grandifolia* J. Agardh have since been synonymized with *A. esculenta*, see below]. However, the difficulty of actually applying species concepts to specimens in the field was described as follows: "With the exception of *Alaria fistulosa*, all the species vary in all their taxonomically important characteristics", and "...much of this variation appears to be caused by environmental factors, so that different species growing in a similar habitat resemble each other more than do populations of the same species growing in different habitats" (Widdowson, 1971a, p. 45).

The only species of *Alaria* still recognized in the Atlantic Ocean is *A. esculenta*, which has an extensive circumpolar distribution and the largest reported geographic range of any species in the genus (Widdowson, 1971a). Populations of *A. esculenta* can be found as far south as northern Japan in the Pacific, and France and New England in the eastern and western Atlantic, respectively. The other Atlantic species recognized in Widdowson's monograph, *A. grandifolia* and *A. pylaii*, are now considered forms of *A. esculenta* (Lüning, 1990). Subsequently, Kraan et al. (2001) supported Lüning's opinion using hybridization studies and DNA sequences from the Rubisco spacer (*rbc*Sp) to show that *A. esculenta* and *A. grandifolia* are conspecific. Thus, *A. esculenta* is the only representative of the genus in the North Atlantic, whereas 12 species (including *A. esculenta*) are still recognized in the North Pacific.

The taxonomy and number of *Alaria* species in the Pacific has also been questioned. Widdowson (1971a) found common misidentifications among herbarium specimens of the poorly defined species *A. angustata* Kjellman, *A. crassifolia* Kjellman and *A. praelonga* Kjellman. Additionally, Widdowson reported that intermediate forms of *A. marginata* Postels & Ruprecht and *A. tenuifolia* Setchell were common along the coast of southern British Columbia. More recently, in their flora of the Commander Islands, Selivanova and Zhigadlova (1997) rejected the record of *A. esculenta* from there without explicitly stating their reason for doing so, but records from other northwest Pacific locations cited by Widdowson (1971a) have yet to be verified, namely Saghalien, the Okhotsk Sea coast of Kamchatka, and the Kurile Islands.

Confusion over species boundaries and evolutionary relationships within *Alaria*, combined with extensive morphological plasticity, prompted us to employ a molecular approach to investigating the systematics of this genus. DNA barcoding is an emerging tool aimed at cataloguing the diversity of life (Hebert et al., 2003a) using the 5′ end of the mitochondrial gene encoding cytochrome *c* oxidase I (*cox*I-5′). Proponents of DNA barcoding suggest that this short region of nucleotide sequence will allow scientists to identify, quickly and inexpensively, species with a high level of accuracy (Hebert et al., 2003a). However, while DNA barcoding has been used to resolve systematic issues in animals (Hogg and Hebert, 2004; Remigio and Hebert, 2003), this method remains untested for the majority of lineages on the tree of life. In a recent study, Saunders (2005) established the utility of this system for florideophyte red algae, but other algal groups have not been investigated.

Alaria is an excellent test case for barcoding in brown algae because there is an established morphology-based systematic scheme for *Alaria* species, but plasticity and morphological convergence appear to be rampant in this genus. If we can resolve distinct molecular groups within *Alaria* we can use this information to re-examine taxonomically important morphological characters to determine if morphology can be used to separate species of *Alaria*. Commonly used intraspecific gene systems from the nucleus [internal transcribed spacer of the ribosomal cistron (ITS)] and chloroplast (*rbc*Sp) were also used to take advantage of published sequences and for comparison against results obtained from *cox*I-5′ data.

2. Methods

2.1. Sample collection and identification

Samples were collected (Table 1) and identified in the field using the dichotomous key in Widdowson (1971a), then pressed for herbarium vouchers (deposited in UBC and UNB). A portion of each thallus was dried on silica gel in the field. Dried material was ground under liquid nitrogen in the lab with a cold mortar and pestle and stored at −20 °C.

2.2. DNA extraction through sequencing

DNA was extracted from 54 samples of *Alaria* according to the protocol of Lane et al. (2006). Oligonucleotide PCR and sequencing primers for the ITS and *rbc*Sp (KR3-KR4), as well as amplification profiles, followed Lane et al. (2006). Two brown algal mitochondrial genomes (Oudot-Le Secq et al., 2001; Oudot-Le Secq et al., 2002) were used to design primers for amplification and sequencing of the *cox*I-5′ barcoding region. They were the forward primer GazF2 (5′ CCAACCAYAAAGA TATWGGTAC 3′) and reverse primer GazR2 (5′ GGAT GACCAAARAACCAAAA 3′). The thermal profile for PCR amplification of the *cox*I-5′ region included: an initial denaturation cycle of 94.0 °C for four minutes, followed by 38 cycles of 94.0 °C for one minute, 50.0 °C for 30 s and 72.0 °C for one minute. A final extension step was performed at 72.0 °C for seven minutes followed by storage at 4.0 °C until the samples were processed. PCR products were purified and sequenced as outlined in Lane et al. (2006). For seven isolates (Anana1749, Amar9797, Ataen10759, Atenu11487, Amar031501, Atenu9790 and Aesc9803), the ITS PCR products were cloned using the Topo TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Four to eight clones were sequenced per isolate using the CEQ Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter,

Table 1
Collection locations or reference, and GenBank numbers for species used in this study

Morphological identification	Location	*rbc*Sp	ITS	*cox*I-5′
Alaria crassifolia Kjellman				
Acras	Kraan and Guiry (2000)	**AF109802**	—	—
Acras	Yoon et al. (2001)	**AF318957**	**AF319001**	—
A. crispa Kjellman				
Acrispa1	St. Lawrence Isl., Alaska	EF218947	EF218904	EF218901
A. esculenta (Linnaeus) Greville				
Aesc032601	Mullaghmore Head, Ireland	EF218953	EF218910	EF218859
Aesc033102	Cape Hat, Baffin Island, NVT, Canada	EF218950	EF218907	EF218856
Aesc9804	Prospect Pt., Resolute Bay, Cornwallis Isl., NVT, Canada	EF218949	EF218906	EF218857
Aesc9803	Prospect Pt.	EF218957	EF218929	EF218879
Aesc1253	Grand Manan Isl., NB, Canada	EF218952	EF218908	EF218858
Aesc002	Percé, Quebec, Canada	EF218951	EF218909	EF218860
Aesc(AF318958)	Yoon et al. (2001)	**AF318958**	—	—
Agrand(AF177175)	Kraan and Guiry (2000)	**AF177175**	—	—
A. fistulosa Postels & Ruprecht				
Afist9805	Seldovia Point, Cook Inlet, AK, USA	**AY851536**	**AY857878**	EF218854
Afist11481	Akutan Harbor, Akutan Isl., Alaska	EF218946	EF218903	EF218855
A. marginata Postels & Ruprecht				
Amar01SR	Seal Rock, OR, USA	EF218958	EF218911	EF218890
Amar02SR	Seal Rock	EF218959	ND	ND
Amar03SR	Seal Rock	ND	ND	EF218891
Amar01KP	Bamfield, Vancouver Isl., BC, Canada	ND	EF218913	EF218896
Amar031501	Port Hardy, Vancouver Isl., BC, Canada	EF218954	EF218922	EF218867
Amar031503	Port Hardy	EF218967	EF218923	EF218868
Amar031601	Palmerston Rec. Area, NW Vancouver Isl.	EF218962	EF218914	EF218899
Amar031901	Rennell Sound, Queen Charlotte Isl., BC, Canada	EF218963	ND	EF218893
Amar0409002	Bamfield	EF218968	EF218912	EF218894
Amar9794	Knoll Head, Cook Inlet, Alaska	EF218985	EF218915	EF218892
Amar9796	Gull Isl. Kachemak Bay, Alaska	EF218966	EF218917	EF218870
Amar9797	Gull Isl.	EF218955	EF218918	EF218869
Amar9799	Sunshine Cove, Alaska	ND	EF218933	EF218875
Amar9800	Sunshine Cove	EF218964	EF218935	EF218880
Amar10037	Port Dick, Kenai Peninsula, Alaska	EF218956	ND	EF218895
A. nana Schrader				
Anana1107	Bamfield	EF218974	ND	EF218897
Anana1171	Bamfield	EF218969	EF218916	EF218872
Anana1749	Bamfield	EF218971	EF218921	EF218898
Anana1750	Bamfield	EF218972	EF218920	EF218873
Anana031401	Bamfield	ND	EF218934	EF218871
Anana040801	Palmerston Rec. Area	EF218973	EF218919	EF218900
Anana11570	Akutan Point, Akutan Isl., Alaska	EF218970	EF218936	EF218888
A. praelonga Kjellman				
Aprae7	Hokkaido, Japan	EF218948	EF218905	EF218902
Aprae319004	Yoon et al. (2001)	—	**AF319004**	—
Aprae	Kraan and Guiry (2000)	**AF109801**	—	—
Aprae	Yoon and Boo (1999)	**AF318960**	—	—
A. taeniata Kjellman				
Ataen031701	Rennell Sound	ND	EF218928	EF218877
Ataen9795	Seldovia Point	EF218960	EF218940	EF218861
Ataen9801	Bainbridge Bight, Alaska	ND	EF218925	EF218876
Ataen9802	Bainbridge Bight	EF218979	EF218926	EF218874
Ataen9806	Scott Isl., Cook Inlet, Alaska	ND	EF218937	EF218886
Ataen9974	Turtle Reef, Cook Inlet, Alaska	ND	EF218938	EF218889
Ataen10292	Katmai Bay, Alaska Peninsula, Alaska	EF218980	EF218939	EF218864
Ataen10350	Amalik Bay, Alaska	ND	EF218942	EF218863
Ataen10488	Katmai Bay	EF218961	EF218943	EF218866
Ataen10759	Kiukpalik Isl., Alaska	EF218986	EF218941	EF218881
Ataen11176	Nanwallek Reef, Cook Inlet, Alaska	EF218977	EF218945	EF218862
Ataen11583	Akutan Harbor	EF218976	EF218944	EF218865

Table 1 (*continued*)

Morphological identification	Location	*rbc*Sp	ITS	*cox*I-5′
A. tenuifolia Setchell				
Atenu9790	Shrine Isl., Alaska	EF218984	EF218930	EF218882
Atenu9791	Shrine Isl.	EF218983	ND	EF218883
Atenu9792	Pt. Louisa, Alaska	EF218981	EF218931	EF218884
Atenu9793	Pt. Louisa	EF218982	EF218932	EF218885
Atenu9967	Shaman Isl. Alaska	EF218978	EF218927	EF218878
Atenu11487	Akutan Harbor	EF218975	EF218924	EF218887
Agarum clathratum Dumortier	Grand Mannan Is., N.B., Canada	ND	**AY857880**	EF218850
Laminaria digitata Kjellman	Green Pt., Lepreau, N.B., Canada	ND	**AY857886**	ND
Laminaria digitata	Oudot-Le Secq et al. (2002)	—	—	**AJ344328**
Lessonia corrugata Lucas	Gov. Is. Reserve, Tas., Australia	ND	**AY857902**	EF218848
Lessoniopsis littoralis (Farlow and Setchell) Reinke	Frank Island, Uculet, BC, Canada	ND	**AY857874**	EF218851
Pelagophycus porra (Leman) Setchell	San Diego, CA, USA	ND	ND	EF218849
Pelagophycus porra	Yoon et al. (2001)	—	**AF319039**	—
Pleurophycus gardneri Setchell & Saunders	Whiffen Spit, Sooke, BC, Canada	—	**AY857876**	—
Pleurophycus gardneri	Pachena Beach, Bamfield	**AY851534**	—	EF218852
Pterygophora californica Ruprecht	Cape Beale, Bamfield	**AY851539**	**AY857875**	ND
Saccharina lattisima (L.) C.E. Lane, C. Mayes, Druehl and G.W. Saunders	Green Pt.	ND	**AY857893**	ND
S. lattisima	Hannafore Point, Looe, Cornwall, England	ND	ND	EF218847
Saccharina sessile (C. Agardh) C.E. Lane, C. Mayes, Druehl and G. W. Saunders	Whiffen Spit, Sooke, BC, Canada	ND	ND	EF218846
Undaria pinnatifida (Harvey) Suringar	l'Etang de Thau, France	**AY851535**	**AY857873**	EF218853

Data reported on the same line are from the same isolate. Entries in plain type were completed in this study, whereas those in bold were previously published. Cells with (—) indicate no data were available in GenBank for that gene from that particular isolate, whereas ND indicates sequences that were not determined in this study.

Inc., Fullerton, CA, USA) on a Beckman Coulter CEQ8000.

2.3. Data analyses

Overlapping complementary fragments were edited and aligned in Sequencher™ v 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA), and multiple sequence alignments were constructed with MacClade 4.06 (Maddison and Maddison, 2003).

To elucidate the phylogenetic affinity of *A. fistulosa* we used ITS (18 taxa) and *cox*I-5′ (17 taxa, the *cox*I-5′ sequence of *Pterygophora californica* Ruprecht could not be determined) sequences from the major groups of the derived families of the Laminariales (Lane et al., 2006), all genera of the Alariaceae and isolates from the major clades of *Alaria* as determined below. The *A. fistulosa* alignments were subjected to maximum likelihood (ML) analyses in PAUP* v 4.0b10 (Swofford, 2002) using the model parameters (TrN + I + G for the ITS data and K81uf + I + G for the *cox*I-5′ data) estimated in Modeltest v 3.7 (Posada and Crandall, 1998). One hundred random sequence addition replicates were performed under the heuristic search algorithm, and subsequently 100 bootstrap replicates were analyzed with ten random sequence addition replicates. Bayesian analyses were performed with MrBayes v 3.0 (Huelsenbeck and Ronquist, 2003) using default values and the GTR + I + G model with parameters estimated during the analyses. Four independent runs of 1,000,000 generations were performed, and trees were sampled every 100 generations and a "burn-in" value of ~130,000 generations was determined by visual inspection of likelihood values. The first 400,000 generations were discarded to ensure stabilization, and the remaining trees were used to construct the consensus. Finally, the ITS and *cox*I-5′ data sets were combined and analyzed using the K81uf + I model (determined by Modeltest) in ML analyses as described above. Bayesian analyses were carried out as above with each gene as a separate partition and all relevant parameters unlinked.

The *cox*I-5′ sequence alignment of *Alaria* spp. was subjected to parsimony, neighbor-joining (NJ) and bootstrap analyses in PAUP*, and Bayesian analyses were performed as above. Fifty random addition replicates under the heuristic search method, using TBR branch swapping, were completed under unweighted parsimony with gaps treated as missing data. Model parameters used in NJ analyses were estimated in Modeltest (Posada and Crandall, 1998). Bootstrap analyses (1000 replicates) were calculated under NJ and with 10 random additions in parsimony. For bootstrap analyses, only a single taxon representing isolates with identical sequences was included. Due to the

large number of nearly identical sequences, the analysis of the ITS and *rbc*Sp data sets was limited to NJ and Bayesian. For Bayesian analyses, four independent runs of one million generations were performed as above, with "burn-in" values of 110,000 (*rbc*Sp), 100,000 (*cox*I-5′) and 160,000 (ITS) generations determined by visual inspection. The first 400,000 generations were discarded and consensus trees were constructed in PAUP*.

For both the *cox*I-5′ and *rbc*Sp analyses, sequences from all genera of the Alariaceae, as well as *A. fistulosa* alone, were used as outgroups in two independent series of analyses. Owing to the limited phylogenetic signal in the ITS data set (see results), the *A. esculenta* clade was used as an outgroup because of its sister relationship to the northeastern Pacific isolates to reduce potential attraction artifacts (see Holland et al., 2003).

Cloned sequences from isolate Anana1749 were added to a reduced alignment, including representatives from other *Alaria* ITS clades, and examined for distinct signatures from multiple clades. Autapomorphies were removed from the cloned sequences as probable PCR errors, and a NJ tree was constructed as described above.

3. Results

3.1. Phylogenetic placement of *Alaria fistulosa*

Both ML and Bayesian analyses of *cox*I-5′ and ITS data from members of the derived families of the Laminariales resulted in nearly identical tree topologies but with moderate to low levels of bootstrap support and few significant posterior probability scores (data not shown). Because both data sets resulted in nearly identical topologies, we combined the data and analyzed them as a single data set (separate unlinked partitions in Bayesian analysis). Again, the same topology was resolved by both methods of analysis, but with much stronger support than for the individual gene alignments (Fig. 1). These data indicate that *A. fistulosa* is sister to the other members of the Alariaceae, with affinities as close or closer to other genera in the family than to *Alaria sensu stricto*.

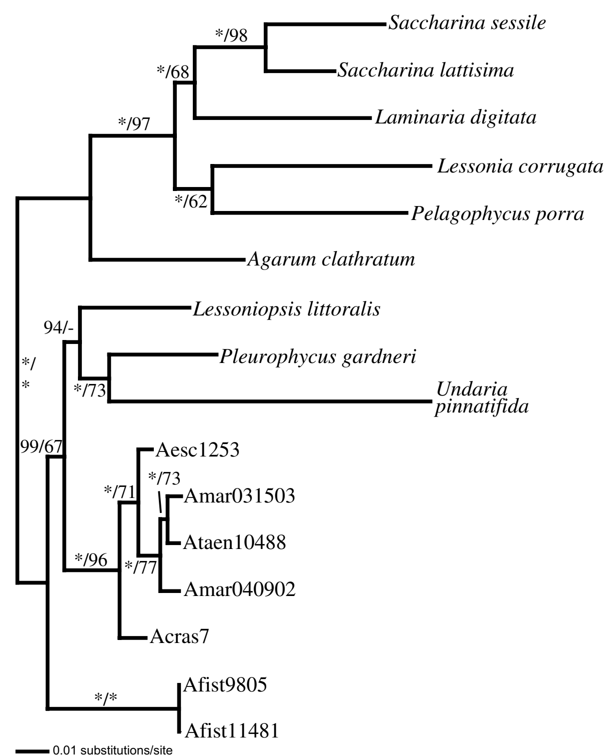


Fig. 1. Bayesian tree for the Alariaceae calculated from combined ITS and *cox*I-5′ data. The topology shown is nearly identical to that found using maximum likelihood, with the grouping of *Lessoniopsis* with *Pleurophycus* and *Undaria* the only difference between them. In our analyses, *Alaria fistulosa* fails to group with other *Alaria* isolates. Abbreviated taxon names are as in Table 1. Support values are bayesian posterior probabilities and ML bootstrap, respectively. "*" represents 100% support, whereas "-" means <50 % support for an analysis.

3.2. *Alaria* spp. in the northeast Pacific: chloroplast marker

The *rbc*Sp and flanking coding regions yielded a data set of 328 bp from 51 taxa. Variability was moderate within *Alaria*, with 61 variable characters, 38 (11.6%) of which were parsimony informative. Modeltest indicated that the K81uf + G model best fit the data. The only gaps in the alignment between species of *Alaria* were a three bp region in *A. fistulosa* and a 10 bp deletion at the beginning of the spacer shared by *A. crispa*, *A. praelonga* and all but seven of our northeast Pacific samples, which were from two distant geographical locations: five samples from Cook Inlet, Alaska, and two from Seal Rock, Oregon (Table 1).

Analysis of the *rbc*Sp data indicated that this region was not variable enough to resolve phylogenetic relationships among northeast Pacific samples of *Alaria*. Excluding the 10 bp gap, there were only four or fewer nucleotide changes among all of our samples from the northeast Pacific (not including the distinct *A. fistulosa*), resulting in no resolution among these taxa (Fig. 2). However, the *rbc*Sp indicated a large divergence (18–26 changes) between *A. fistulosa* and other species of *Alaria*, similar to other data sets. An *A. esculenta* clade as well as an *A. crispa* and *A. praelonga* group were also well resolved from the *rbc*Sp data, and all of the northeast Pacific samples formed a moderately supported clade.

3.3. *Alaria* species in the northeast Pacific: mitochondrial marker

Sequencing of the *cox*I-5′ resulted in 653 bp of data from 49 samples with 118 variable sites, 80 of which were parsimony informative. Modeltest found the TIM model, with invariable sites, to be the best fit to the data. Bayesian, maximum likelihood, parsimony and neighbor-joining analyses all resulted in trees with identical topologies, which did not reflect morphological species designations (Fig. 3).

Six mitochondrial haplotype groups (herein referred to as mitotypes) were resolved among the 49 individuals of

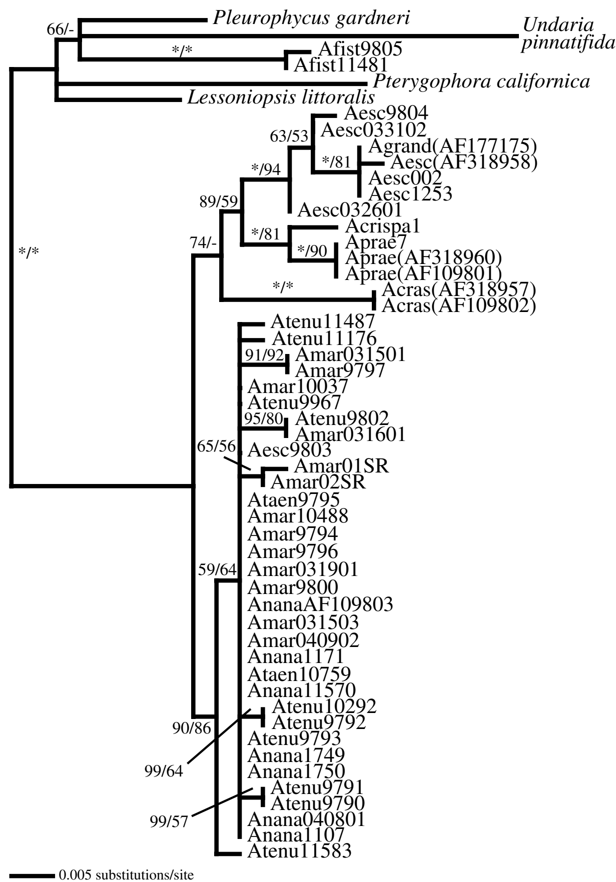


Fig. 2. Bayesian tree of Rubisco spacer sequences from isolates of *Alaria*. Whereas the Rubisco spacer was able to separate the genera of the Alariaceae and provided some support with *Alaria*, it was not variable enough to resolve relationships among northeast Pacific isolates of *Alaria*. Abbreviations for taxa are as in Table 1. GenBank Accession Numbers for data generated elsewhere are in parentheses. Values along branches indicate posterior probability and neighbor-joining bootstrap support, respectively. Branches lacking values, or support values denoted with "-" received <50 % support.

Alaria, most with strong bootstrap and significant posterior probability support (Fig. 3). Mitotypes A, B and C are distinct, with relatively large numbers of nucleotide differences between them (Fig. 3). Mitotypes D, E and F are more closely related to one another, as suggested by their relatively low degree of nucleotide divergence. Mitotype D was only collected along the Pacific coast of Alaska from Cook Inlet westward (Fig. 4). Mitotype E was also found throughout our northern collections and as far south as Bamfield, British Columbia. In contrast, mitotype F was mainly sequenced from southern isolates, including samples from Seal Rock, Oregon, but was also found in two Alaskan isolates (Fig. 4).

3.4. *Alaria* species in the northeast Pacific: nuclear marker

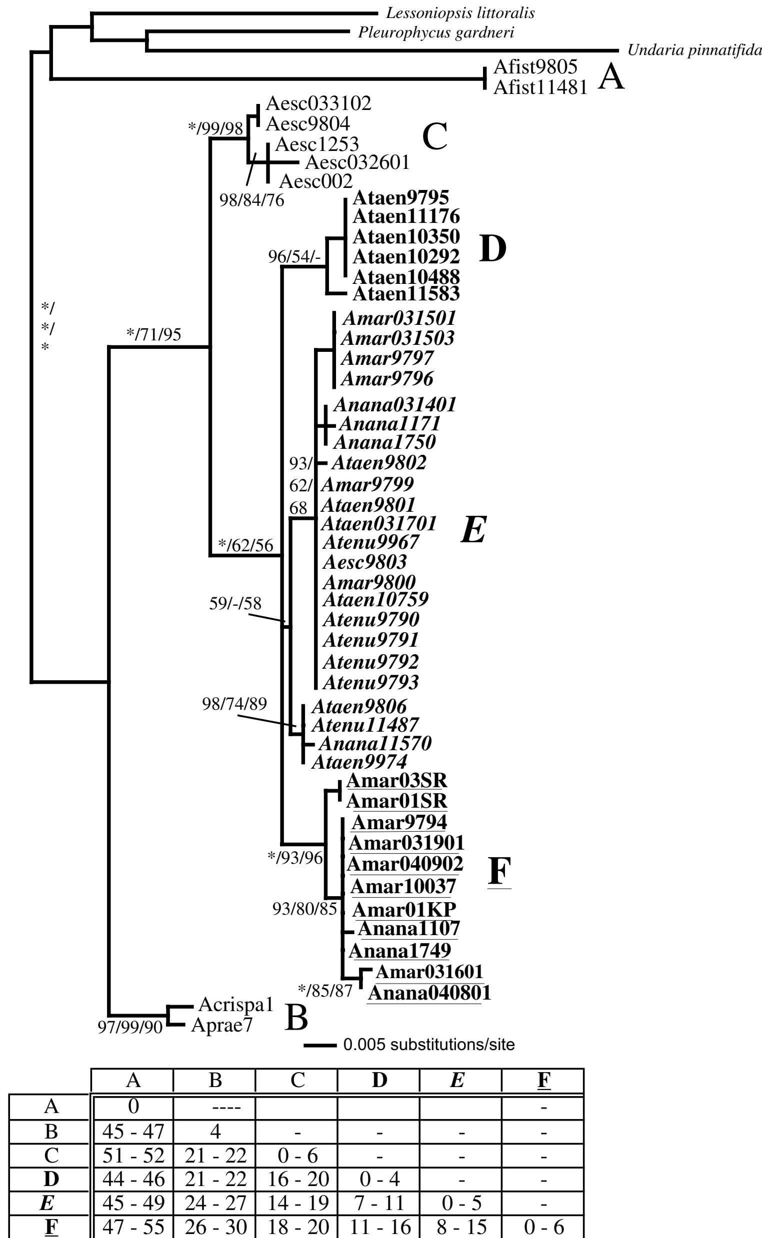
Internal transcribed spacer (ITS) data resulted in an alignment of 735 bp from 46 taxa, with the TrN + I + G model recovered as the best fit. The variability in the ITS alignment may be underestimated due to a long string of C followed by G nucleotides, which may have led to compression artifacts. In most cases, the sequencing polymerase was unable to read cleanly through this region. Unfortunately, the area affected by these artifacts is the most variable portion of the ITS and may provide additional signal at the species level. Because of the artifacts, 27 alignment positions were removed from analyses to avoid noise in the data, making the number of changes reported here only minimum estimates among our *Alaria* isolates (Fig. 5). When the 27 problematic characters were removed, there were 63 variable characters, 50 (7.1%) of which were parsimony informative. However, with gaps included as a fifth state, there were 114 (16.1%) parsimony informative characters out of 134 variable characters.

Phylogenetic analysis of the ITS data resulted in the recovery of several clades with virtually no bootstrap support, but most clades had significant posterior probability scores (Fig. 5). In agreement with the previous analyses, the isolates did not group based on their morphology, but the ITS data did reveal a loose geographic pattern for the isolates (Fig. 5). The "Northern clade" contained isolates from the Queen Charlotte Islands to Cook Inlet, Alaska. The only exception to this range was one sample from southern Vancouver Island. Atenu11487 was resolved independently from all of our other samples, but between the Northern and Southern groups. The "Southern clades" encompassed samples from Oregon to the northern tip of Vancouver Island, as well as two isolates from Cook Inlet and one from Prince William Sound, Alaska. There was also a clade of samples from the area around Cook Inlet, but not all of the samples from this region were resolved in this group. One unexpected result from the ITS data was the grouping of *A. crassifolia*, *A. crispa* and *A. praelonga* within the *A. esculenta* group, but this could be an artifact of sample size or the arbitrary rooting of the tree.

Interestingly, when the mitotypes are mapped onto the ITS trees (Fig. 5), an introgression of the "E" mitotype from the north into isolates of the Southern clade#2 is revealed. This weakly indicates asymmetric introgression of the mitochondrion between the two groups. Additionally, a single member of the Cook Inlet clade (Ataen10759) contains an E mitotype, indicating that there is crossing between members of the Northern and Cook Inlet clades as well.

3.5. Cloning of ITS products

Seven isolates were investigated further to determine if multiple variant copies of the ITS existed within an individual. The ITS region was amplified and cloned from three samples in the Southern clade #2 (Amar9797, Anana1749 and Amar031501), the phylogenetically distinct Atenu11487, two isolates from the Northern clade (Atenu9790 and Aesc9803), as well as for one isolate from the Cook Inlet clade (Ataen10759). In most cases, variability among the different clones was modest (zero to three nucleotide changes) and concentrated in the variable C–G stretch



	A	B	C	**D**	***E***	**F**
A	0	----				-
B	45 - 47	4	-	-	-	-
C	51 - 52	21 - 22	0 - 6	-	-	-
D	44 - 46	21 - 22	16 - 20	0 - 4	-	-
E	45 - 49	24 - 27	14 - 19	7 - 11	0 - 5	-
F	47 - 55	26 - 30	18 - 20	11 - 16	8 - 15	0 - 6

Fig. 3. Bayesian tree of *cox*I-5′ sequences from isolates of *Alaria*. Five groups of mitochondrial haplotypes (not including the divergent *A. fistulosa*) are resolved within *Alaria*. Groups E and F include isolates that exhibit morphologies that correspond to more than one recognized species. Northeast Pacific isolates are in bold text, whereas samples collected elsewhere appear in roman text. Abbreviations are listed in Table 1. Text styles correspond to the resolved mitochondrial haplotypes (mitotypes)—bold for mitotype D, bold italics for mitotype E, and bold underline for F. Support values are bayesian posterior probabilities, parsimony bootstrap and neighbor-joining bootstrap, respectively. An "*" represents 100% support, whereas "-" means <50 % support for an analysis. The matrix shows the absolute number of nucleotide differences within and between the various mitotypes.

mentioned above. However, some cases were more extreme. Clones of Amar031501 varied by two substitutions and two deletions, while Aesc9803 clones differed by nine substitutions and four deletions. The most striking example of this phenomenon was from clones of Anana1749. Cloning resulted in five distinct sequences from seven clones, with two very distinct signatures. Once autapomorphies (likely PCR errors) were removed, one clone (D) grouped with an *Alaria marginata* isolate (Amar9794), whereas the others were identical to Anana1750, collected from the same area as Anana1749 (Fig. 6). This result is unlikely to be contamination because clone D was not identical to any of our other isolates, even with autapomorphies removed.

4. Discussion

4.1. Morphology and genetic differentiation

The most morphologically distinct member of *Alaria* is *A. fistulosa* based on the air bladders within the midrib of the blade, which allows the blade to float to the surface of

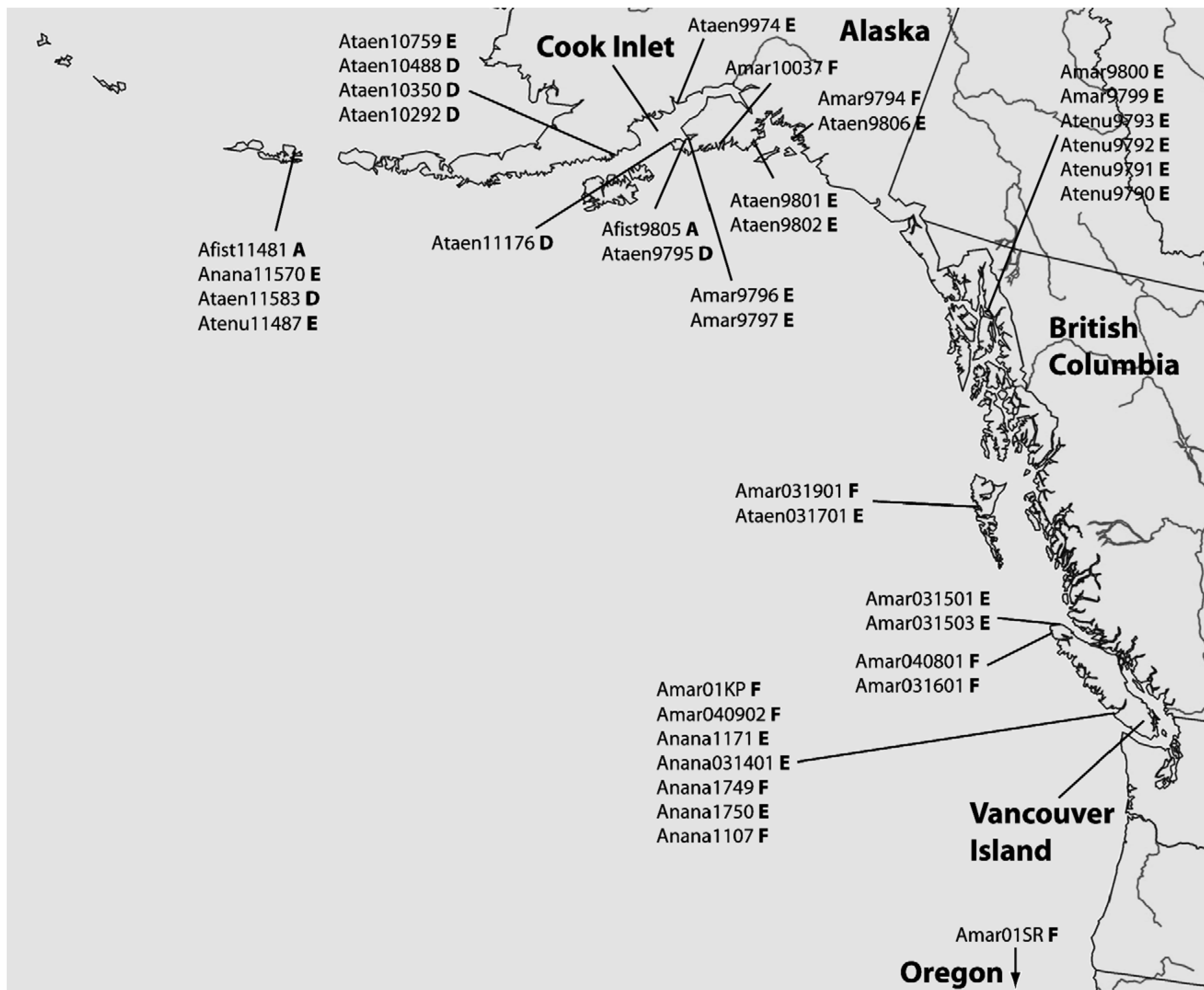


Fig. 4. Geographic distribution along the northeast Pacific coast of North America for isolates used in all analyses. Bold letters following the isolate designation correspond to the mitotype of each sample (Fig. 3).

the sea. Moreover, this species has a large, complex conical holdfast (the attachment of the thallus to the substrate), a wide, flattened rachis (area where specialized reproductive structures, called sporophylls, are produced), which in section has distinctly raised margins (as does the midrib), characters that separate *A. fistulosa* from other species of *Alaria* (Widdowson, 1971a). This species also attains a significantly larger size than its congeners, with lamina reaching 25 m in length and 90 cm in breadth (Setchell and Gardner, 1925). Its fistulose midrib is unique among all of the genera of the Alariaceae and makes its ecology and ability to disperse different from other members of *Alaria*. Other species of *Alaria* are found in the intertidal or shallow subtidal waters, often in a distinct band just above the *Laminaria*/*Saccharina* band. *A. fistulosa* is the only species in the genus that is a true denizen of the subtidal environment, where it forms extensive offshore floating kelp beds. Although the gross morphology of *A. fistulosa* makes it unquestionably a member of the Alariaceae, its morphology and ecology clearly distinguish it from other species of *Alaria* and other genera of the family.

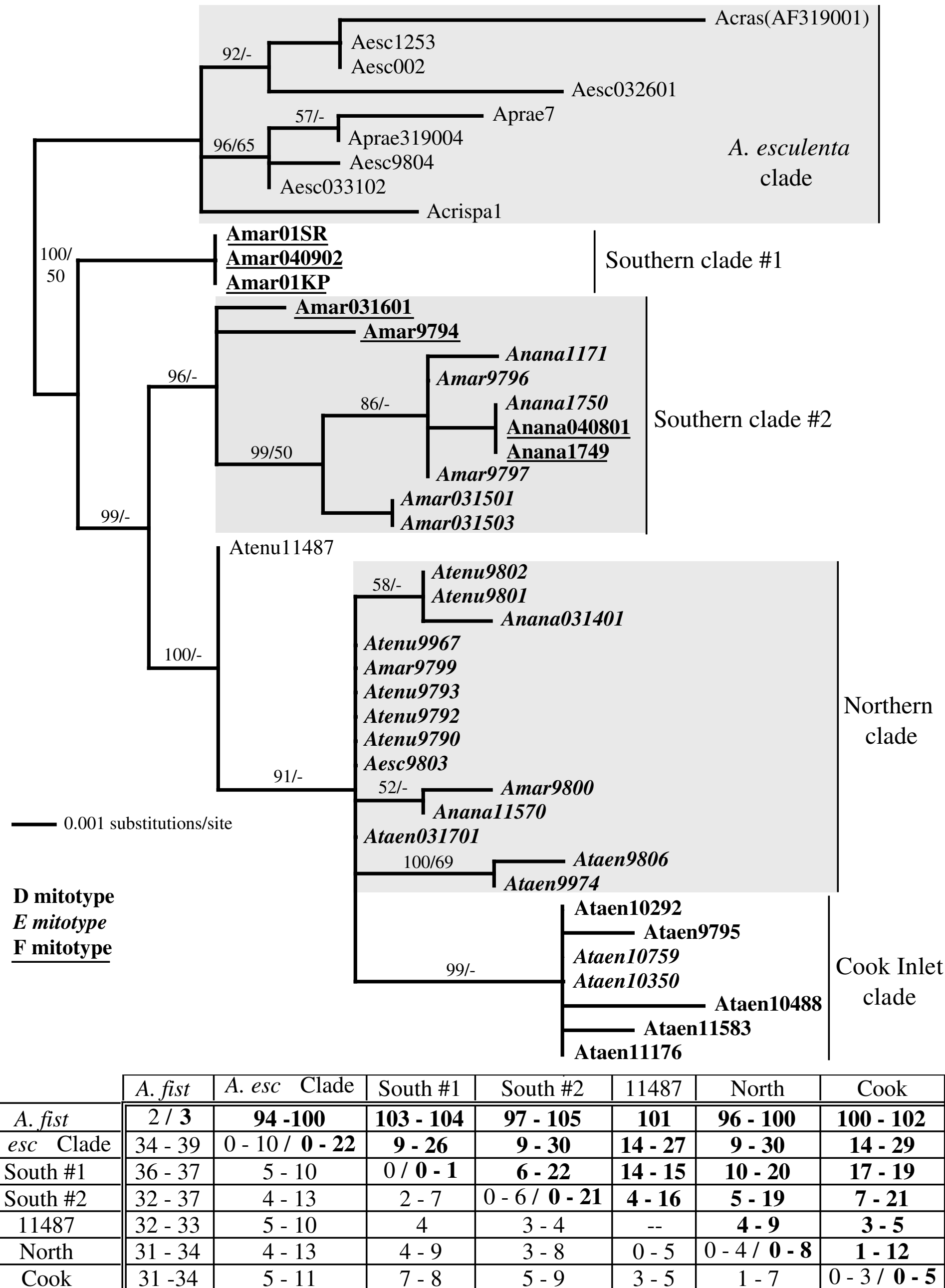
The data presented here (Figs. 1–3), indicate that *A. fistulosa* is genetically divergent from all other *Alaria* species and from other members of the Alariaceae. Analyses of the combined ITS and *cox*I-5′ data position *A. fistulosa* sister to other members of the Alariaceae, separate from the other *Alaria* spp. (Fig. 1). Thus, based on morphological, ecological and molecular data, we recognize *A. fistulosa* as distinct from other species of *Alaria* and propose a new genus to accommodate it.

Druehlia C.E. Lane & G.W. Saunders, gen. nov

Type. *Druehlia fistulosa* (Postels & Ruprecht) C.E. Lane & G.W. Saunders, *comb. nov.*

Basionym. *Alaria fistulosa* Postels and Ruprecht (1840), Illustrationes algarum p. 11.

Diagnosis. Membrum familiae Alariaceae (Laminariales) ab generibus aliis costo fistuloso differens. Member of the Alariaceae (Laminariales) differing from other genera by its fistulose midrib.



	A. fist	*A. esc* Clade	South #1	South #2	11487	North	Cook
A. fist	2 / **3**	**94 -100**	**103 - 104**	**97 - 105**	**101**	**96 - 100**	**100 - 102**
A. esc Clade	34 - 39	0 - 10 / **0 - 22**	**9 - 26**	**9 - 30**	**14 - 27**	**9 - 30**	**14 - 29**
South #1	36 - 37	5 - 10	0 / **0 - 1**	**6 - 22**	**14 - 15**	**10 - 20**	**17 - 19**
South #2	32 - 37	4 - 13	2 - 7	0 - 6 / **0 - 21**	**4 - 16**	**5 - 19**	**7 - 21**
11487	32 - 33	5 - 10	4	3 - 4	--	**4 - 9**	**3 - 5**
North	31 - 34	4 - 13	4 - 9	3 - 8	0 - 5	0 - 4 / **0 - 8**	**1 - 12**
Cook	31 -34	5 - 11	7 - 8	5 - 9	3 - 5	1 - 7	0 - 3 / **0 - 5**

Fig. 5. Bayesian tree for ITS sequences from *Alaria* isolates. Geographic groupings among the northeast Pacific samples are indicated, but some isolates are not from the same area as the majority of isolates in their clade (eg., Ataen031701 groups with members of the Northern clade even though it was collected in the south of the range discussed here, whereas the inverse is true for Amar9794). The *Alaria esculenta* clade was used as an outgroup to reduce artifacts (see text). The matrix shows the absolute differences between the clades, both with gaps included (bold numbers) and excluded (roman type). Abbreviations are as in Table 1 and the text styles represent the mitotype of each isolate (Fig. 3). Support values are posterior probabilities and neighbor-joining bootstrap values, respectively.

Etymology. Named in honor of Prof. Louis Druehl for his life-long dedication to the study of kelp throughout the Northern Pacific.

A monotypic genus containing only the type species.

Lectotype collection. Stockholm A2533, Postels & Ruprecht, Unalaska, Aleutian Islands, AK, USA (Stockholm A2534 is annotated by S. Lindstrom as either being part of A2533 or an isotype). Lectotypified by Widdowson (1971b). Additional isotypes in Komarov Botanical Institute, St. Petersburg (LE).

Among species of *Alaria* reported from the northwest Pacific, *A. crassifolia*, *A. crispa* and *A. praelonga* were included in our study based on the following material. The *rbc*Sp sequences of *A. crassifolia* deposited in GenBank (Kraan and Guiry, 2000; Yoon et al., 2001) indicated that this species is distinct from the other species included

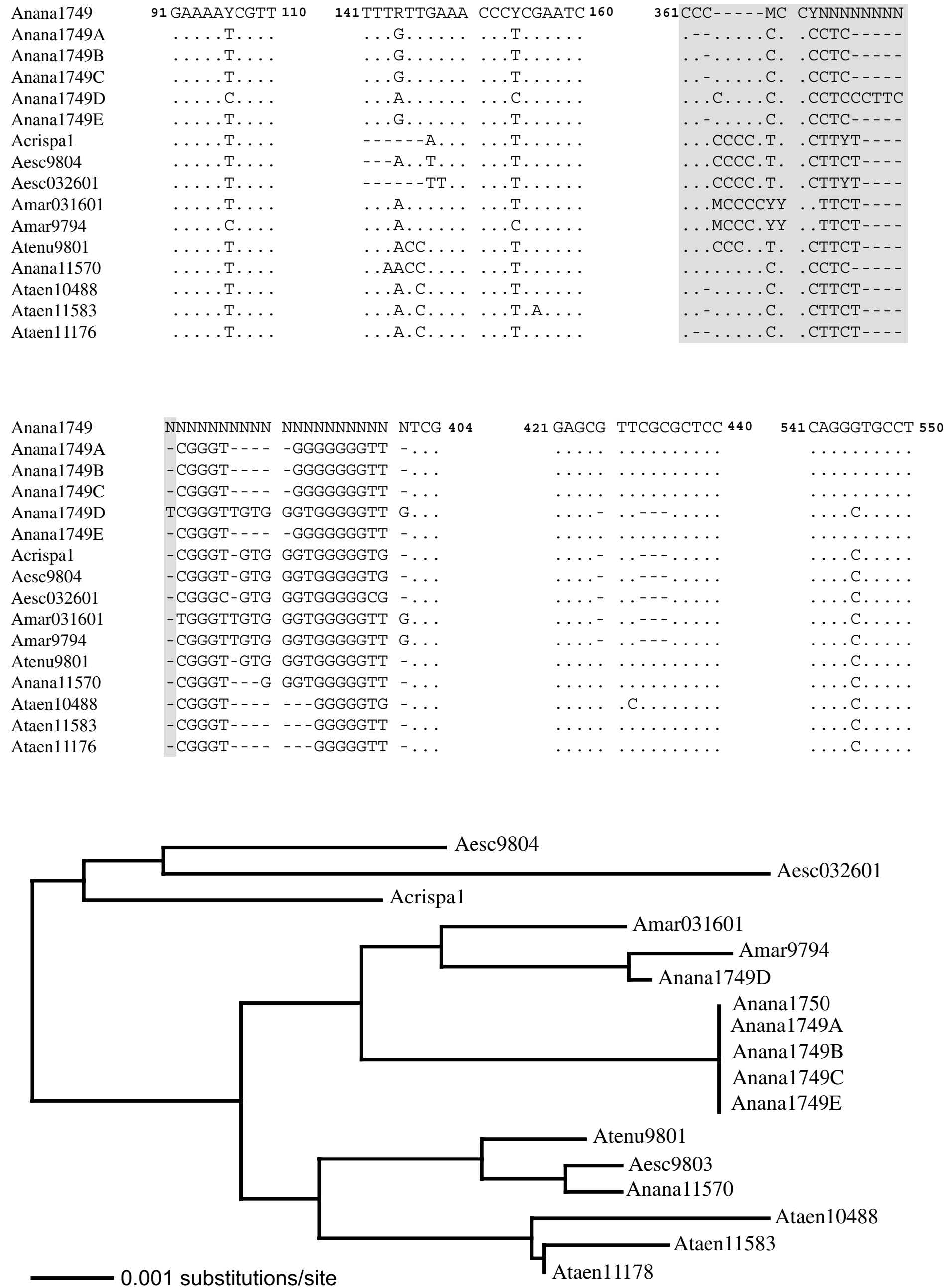


Fig. 6. Sequence comparison and resulting NJ tree from ITS sequences of five clones (A–E) from Anana1749 and representative samples from other *Alaria* clades. Clone 1749D was substantially different from the other clones from this isolate (even after cloning artifacts were removed), indicating more than one distinct copy of this region is present in the nuclear genome. Positions with the same nucleotide as the reference sequence are represented with ".", whereas indels are indicated with a "-". Alignment positions for the displayed nucleotides are given next to the sequence and positions that were excluded from all analyses are shaded. Abbreviations are as in Table 1.

in our study (Fig. 2). Our collection of *A. crispa* from the type locality of St. Lawrence Island revealed it to be distinct from other northeast Pacific *Alaria* in all three of our data sets, and both *rbc*Sp (Fig. 2) and *cox*I-5′ (Fig. 3) data support a close relationship between *A. crispa* and *A. praelonga*. *Alaria praelonga* (type locality the Commander Islands) was represented in the present study by Aprae7, AF318960, AF109801 and Aprae319004 (Kraan and Guiry, 2000; Yoon and Boo, 1999; Yoon et al., 2001). Although reported along the Pacific coast of Alaska (across the Aleutian Islands to the Commander Islands) to northern Japan (Widdowson, 1971a) none of our northeast Pacific isolates originally field identified as *A. praelonga* (Amar9796, Amar9799 and Ataen9806) grouped with Japanese *A. praelonga*, indicating that they are genetically different and suggesting that *A. praelonga* is confined to the western Pacific. Examples of North American *A. praelonga* were re-examined and determined to be misidentifications of *A. marginata* or *A. taeniata*. Examination of *A. praelonga* material from its type locality is warranted,

however, since Selivanova and Zhigadlova (1997) included Commander Islands records of this species under *A. marginata*.

The rbcSp was uninformative for resolving relationships among the northeast Pacific samples (Fig. 2), supporting close relationships within this group. The same region was used unsuccessfully by Kraan and Guiry (2000) to resolve phylogenetic structure among *A. esculenta* isolates from the North Atlantic. Our sequences produced a similar level of divergence within northeast Pacific collections as they observed among North Atlantic *A. esculenta* isolates, and the rbcSp only served to separate the northeast Pacific isolates from other geographic areas, albeit without strong support (Fig. 2). The rbcSp data did indicate a distinction between *A. esculenta* and the northwest Pacific taxa included in the dataset, which was not apparent in the ITS trees. The relationship among these taxa needs further study.

Our ITS data positioned the three West Pacific species (*A. crassifolia*, *A. crispa* and *A. praelonga*) among various isolates of *A. esculenta* (Fig. 5), but the low levels of taxon sampling and the irregularities of the ITS in *Alaria* (discussed below) are likely responsible for the discrepancies between the organellar data (Figs. 2 and 3) and the ITS tree (Fig. 5). A wider sampling of these taxa, using nuclear and organellar markers, may shed light on the detailed relationships among these entities. *A. esculenta* isolates were collected from a wide range of geographic locations (Table 1) and, with the exception of Aesc9803 (presumably misidentified as *A. esculenta* based on collection location), are genetically distinct from all of our northeast Pacific samples in all analyses (Figs. 2, 3 and 5).

Morphological identification of our northeast Pacific *Alaria* isolates (except *D. fistulosa*) based on Widdowson's (1971a) key was not reflected in any of the trees recovered from the separate data sets. In fact, taxa with distinct morphologies (Fig. 7), clearly representative of different morphological species, have nearly identical sequences from the three regions examined here (Figs. 2, 3 and 5). However, some loose patterns of morphology were apparent from the trees such that, with the exception of the Northern clade, each clade includes a subset of morphologies, but with no morphology exclusive to any clade. The Southern clades (Fig. 5) are composed of samples identified as *A. marginata* and *A. nana* H.F. Schrader, two 'species' suspected of regular hybridization because individuals can be found with morphologies that form a continuous grade (Widdowson, 1971b). The Northern clade consists mainly of taxa identified in the field as *A. tenuifolia*, but all of the northeast Pacific morphologies are represented in the clade. Some isolates from lower Cook Inlet and western Alaska, with identical sequence in all of our data sets, share a similar morphology, with long, lanceolate sporophylls and a large blade that flares out almost immediately above the transition zone (Fig. 8). The only exception to this morphology among the samples from the Cook Inlet clade is Ataen10759, which has numerous small sporophylls and a blade that does not flare out above the transition zone. As noted above and again below, Ataen10759 is also the only sample from the ITS Cook Inlet clade that has an E mitotype, indicating that this sample may be a hybrid. The Cook Inlet morphology resembles most closely the figures of *A. taeniata* in Widdowson's monograph (Widdowson, 1971a, Figs. 21a and b).

4.2. Discrepancies in the molecular data

Previous barcoding studies have found an average divergence of 11.3% between closely related species of the animal kingdom, whereas values of less than 1% are normally recovered between isolates within a species (Hebert et al., 2003b). As another comparison to our data, Saunders (2005) uncovered less than 0.5% *cox*I-5′ divergence within red algal species and 4.5 to 13.6% between species within a genus. However, Saunders also reported divergence values of only 0.8–1.2% between two closely related species pairs. Sequence variation presented here between mitotypes B, C and the D/E/F complex (Fig. 2) ranged from 2.2–4.7%, whereas values between genera of the Alariaceae ranged from 6.9–12%. Divergence within our individual northeast *Alaria* mitotypes was low, less than 1% in all cases, but divergence values between mitotypes D, E and F were only slightly greater (1.1–2.3%)—equivalent to or slightly greater than values between closely related species pairs of red algae (Saunders, 2005). If the barcoding results were taken in isolation, we would probably recognize three closely related species of *Alaria* in the northeast Pacific, corresponding to mitotypes D, E and F. However, all of the data together suggest a more complicated story.

Divergence values for the ITS sequences within our northeast Pacific clades are surprisingly large and overlap with the values between clades (Fig. 5). Comparisons to other genera in the Alariaceae to see if within-genus ITS variation is high in this family are not possible because the Alariaceae is composed of mainly monospecific genera. However, in a red algal ITS study, Ross et al. (2003) found fewer than four ITS nucleotide differences within isolates of two closely related *Mazzaella* species, and nine to twelve differences between them. In a green algal ITS study, Lindstrom and Hanic (2005) observed up to 12 nucleotide differences within three closely related *Urospora* species, and up to 30 nucleotide differences among the species. As a more appropriate comparison within the Laminariales, isolates of *Saccharina latissima* (L.) C.E. Lane, Mayes, Druehl *et* G.W. Saunders from each coast of North America have only zero to two nucleotide changes in the ITS (Lane, unpubl.) and closely related species of *Saccharina* differed by as few as five to seven substitutions (Lane et al., 2006). Thus, the ITS divergence values observed among *Alaria* isolates in the northeast Pacific exceed species level differences observed in other taxa in the Laminariales. However, rather than resolving distinct species-level groups (0–4 changes), the data revealed essentially a continuum of ITS divergence across isolates, at odds with the mitochondrial

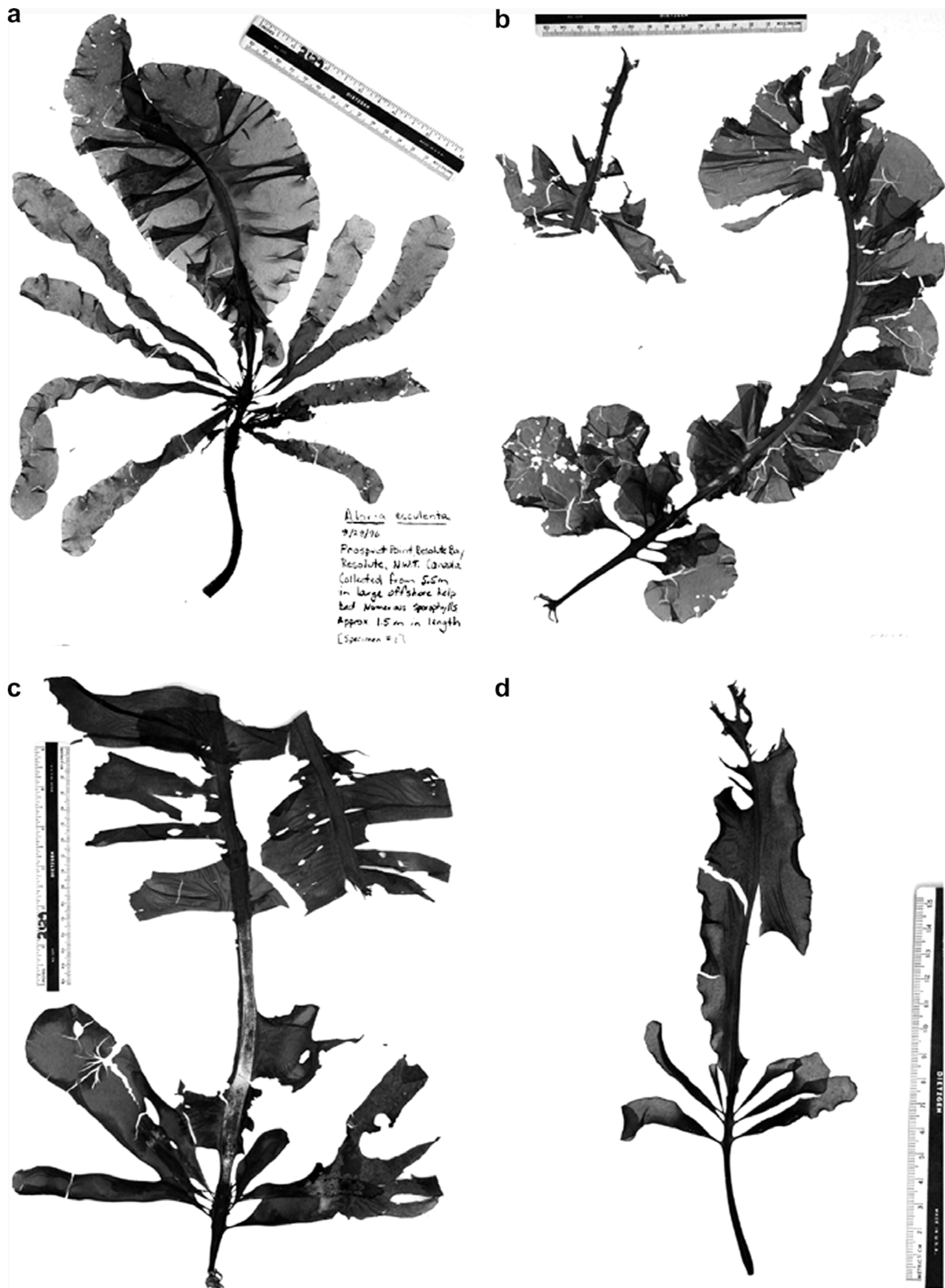


Fig. 7. Examples of the morphological variation of northeast Pacific isolates. Pictured are: (a) Aesc9803, (b) Atenu9793, (c) Amar9799, and (d) Anana031401. Isolates a, b and c were all genetically identical in ITS and barcode analyses, whereas sample d contained minor sequence differences in these genes. Despite the close relationship, these isolates display a wide range of morphological variation. Centimeter rule.

haplotypes, and trees with low resolution. This situation is in contrast to what would be expected among species of a genus.

This previous paradox was accompanied by another anomalous aspect of northeast Pacific *Alaria* ITS sequences—they are characterized by an unusual number

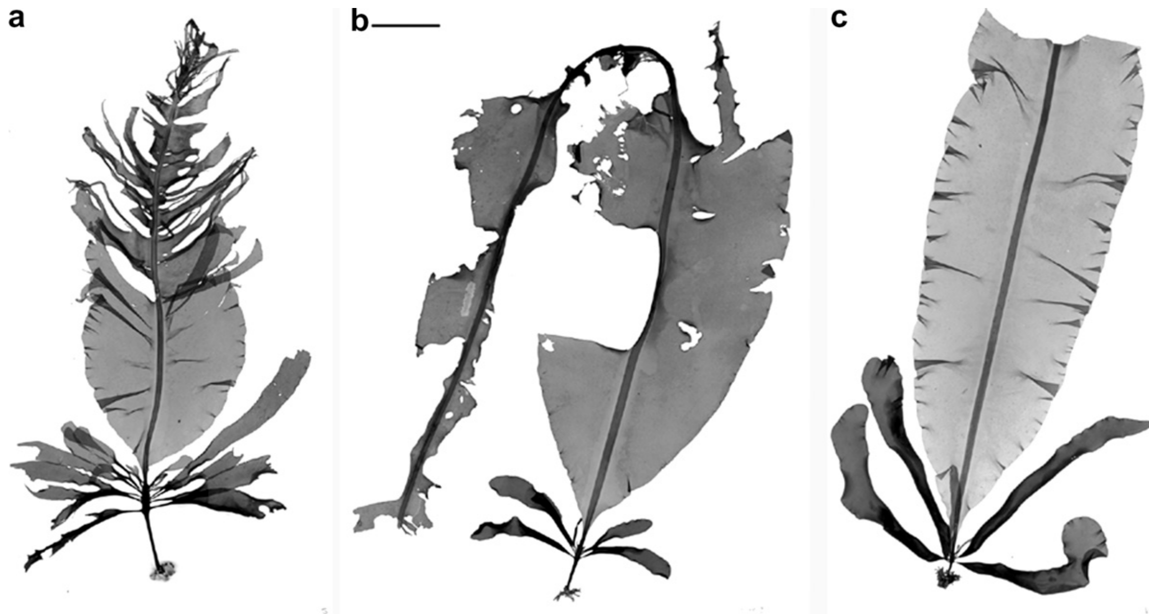


Fig. 8. Three examples of the morphology of *Alaria* isolates from the mouth of Cook Inlet. The samples pictured are: (a) Ataen10488; (b) Ataen10350; and (c) Ataen10292, respectively. *Alaria* isolates from this area had the most stable morphology of any of the northeast Pacific collection locations, but the sample size included here is small. Scale equals 5 cm.

of ambiguous nucleotide positions. Sixteen of forty-six sequences contained at least one ambiguous nucleotide (a nucleotide that is not assigned to a single state by the sequencing software due to multiple peaks in the same position; most contained at least three ambiguities), including all but four of the isolates in the Southern clade, three of which had an E mitotype. In contrast, an ITS data set from a previous kelp study (Lane et al., 2006) had no sequence ambiguities among 43 diverse taxa of the Laminariales. Identification of distinct ITS clones, with different copies of the ribosomal cistron, from some individuals of northeast Pacific *Alaria* explains these ambiguities in our directly sequenced data. Based on the large number of ambiguous nucleotides in our ITS sequence data, the occurrence of multiple ITS sequences within individuals of *Alaria* from this region is likely widespread. We are confident that the discrepancies among clones from each sample was due to ITS variants rather than contamination from other kelp taxa (e.g., Lane and Saunders, 2005) because all clones grouped within the northeast Pacific clade in phylogenetic analysis, even in the most extreme cases (Fig. 6).

The combination among northeast Pacific *Alaria* isolates of distinct mitotypes and larger than expected ITS variation within and between clades, which nonetheless failed to form distinct species assemblages, is consistent with a hypothesis of formerly isolated species recently reverting to a continuous breeding population, rather than with an incipient species beginning to diverge. The distinct mitotypes resolved with the DNA barcode data are an indication of a period of former genetic isolation, which was long enough for the mitochondrial DNA (as well as ITS) to diverge. However, the resolving power expected for ITS data at the species level is being degraded by recombination events between the multiple variants being exchanged within the current interbreeding population, which includes remnants of the variants of the former species, explaining the high levels of ITS divergence we observed. Since the mitochondrial genome does not undergo recombination, the three mitotypes are retained within the interbreeding northeast Pacific species complex. Eventually, if species boundaries continue to collapse, one mitotype, as well as a stable nuclear ribotype, will become fixed through stochastic processes. Further evidence of interbreeding among the northeast Pacific isolates is the prevalence of samples that have identical ITS sequences but different mitotypes (Fig. 5).

A similar pattern of mitochondrial divergence, which is not concordant with nuclear DNA variation, has been demonstrated for European plankton (Peijnenburg et al., 2006). In this study, nuclear microsatellite markers failed to recover genetic structure within the study population of *Sagitta*, whereas significant mitochondrial sequence divergence was uncovered (up to 26%). The authors hypothesize that the more variant haplotypes may be the remnants of an ancestral mitochondrial lineage that has not been lost by lineage sorting. The observed mitochondrial variation in *Alaria* is considerably lower and likely occurred recently, during the Pleistocene glaciation. At that time, the northeast Pacific coast was heavily affected by ice cover, salinity and sea temperature changes (Hetherington et al., 2003) that may have created ideal conditions for allopatric speciation. Without a physical separation, mitochondrial variation would have had to occur sympatrically, which is hypothesized to be rare among high-dispersal marine organisms (Palumbi, 1992) and would be unlikely to reverse into the observed interbreeding population. With

the retreat of the glacial ice sheet and subsequent rise in sea level, incipient *Alaria* species may have regained contact before adequate barriers to hybridization had accumulated. This scenario would explain our multiple mitotypes that do not strictly correspond to the pattern shown by the nuclear data, which itself is marred by intra-individual variation that masks the phylogenetic structure.

4.3. Implications on using coxI-5′ as DNA barcode marker in brown algae

The *cox*I-5′ region has been mainly tested in animals, where it has been established as a tool for differentiating species (e.g., Gomez et al., 2007; Hebert et al., 2003a; Hebert et al., 2003b), as well as for phylogenetic inference (Remigio and Hebert, 2003). Here we employed the *cox*I-5′ in attempt to better understand species boundaries within the brown algal genus *Alaria*. Our *cox*I-5′ data were able to resolve reasonably well-supported groupings of mitochondrial haplotypes in *Alaria*, but the number of differences between groups (e.g., 7–11 between mitotypes D and E) was equivalent to those observed between only the most closely related of red algal sibling species pairs (Saunders, 2005). In the red algal cases genetic isolation of the species pairs was supported by clearly divergent (although very similar) nuclear ITS clusters (Ross et al., 2003). When our *Alaria cox*I-5′ data are compared to data from the commonly used nuclear ITS marker, each mitotype is not clearly associated with a nuclear genotype, consistent with our hypothesis of incipient speciation followed by hybridization and possibly a breakdown in species boundaries. Instances of hybridization and introgression are particular cases that have been recognized as requiring supplementary analyses to understand the complexities of species boundaries (Hebert et al., 2003a). In the example outlined here, low levels of *cox*I-5′ sequence divergence and nucleotide position ambiguity in our ITS sequences were diagnostic of a relationship between the northeast Pacific isolates that was more complicated than sibling species. This is not a problem limited to brown algae, and from these data, it would seem prudent to investigate any putatively closely related species, resolved with *cox*I-5′ sequences, using other parts of the genome. However, ongoing work (Saunders Laboratory, unpublished) indicates that the *cox*I-5′ region is a useful tool for reliably separating species in other genera of brown algae and this interesting exception should not be considered as a failure of DNA barcoding, but as an opportunity to recognize the dynamic nature of evolutionary processes. Thus, we suggest that the *cox*I-5′ shows promise for use among brown algae as tool to differentiate taxa at the level of species. Additionally, the *cox*I gene may be considered a suitable phylogenetic tool in brown algae based on the reasonable amount of signal in the 5′ end of the gene. However, for phylogenetic analysis, we recommend using a portion at least twice as large as the *cox*I-5′ to maximize the number of informative characters.

4.4. Taxonomy of the northeast Pacific Alaria

Based on the fact that all of the *Alaria* populations we recover in the northeast Pacific are interbreeding (causing a lack of phylogenetic signal within the ITS region) and that morphology in this region appears to be largely an environmental artifact, we recommend recognizing *Alaria* in the northeast Pacific as an *A. marginata* species complex. Accordingly, we recommend that *A. nana*, *A. taeniata*, and *A. tenuifolia* be regarded as synonyms of *A. marginata* and exclude *A. crispa* and *A. praelonga* from the flora of the northeast Pacific. Several subspecies, varieties, or ecads could be recognized within our definition of *A. marginata*, but genetic distinctions would be useless in the field, and morphological designations would have no basis in genetics. With the possible exception of the Cook Inlet clade (Fig. 8; except for Ataen10759, which has an E mitotype), individuals within the various clades of northeast Pacific *Alaria* encompass all of the distinct morphologies on which species in this area were previously distinguished (Widdowson, 1971a), including every imaginable intermediate form (Fig. 7). The biogeography of the isolates in this group may provide the best indication of the genetic affinities of an isolate, but even this would provide a weak probability of correct assignment.

Note added in Proof

DeCew's Guide to the Seaweeds of British Columbia, Washington, Oregon, and Northern California (http://ucjeps.berkeley.edu/guide/index.html) lists *A. tenuifolia* as a later homonym of *A. fragilis* Saunders. Although *A. fragilis* (Saunders, 1901) was published earlier than *A. tenuifolia* (Setchell and Gardner, 1903) there remains some debate as to whether these species encompass the same concept, as Setchell and Gardner included *A. fragilis* in their 1903 publication. However, *A. fragilis* undoubtedly falls under our expanded concept of *A. marginata*.

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