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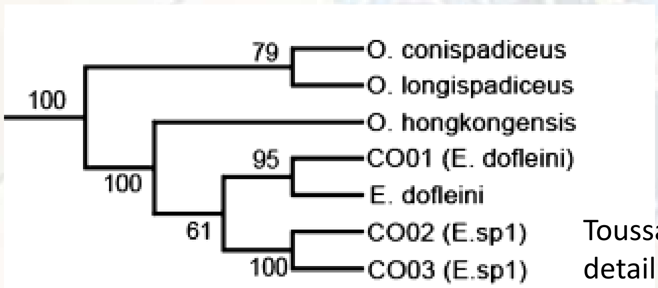
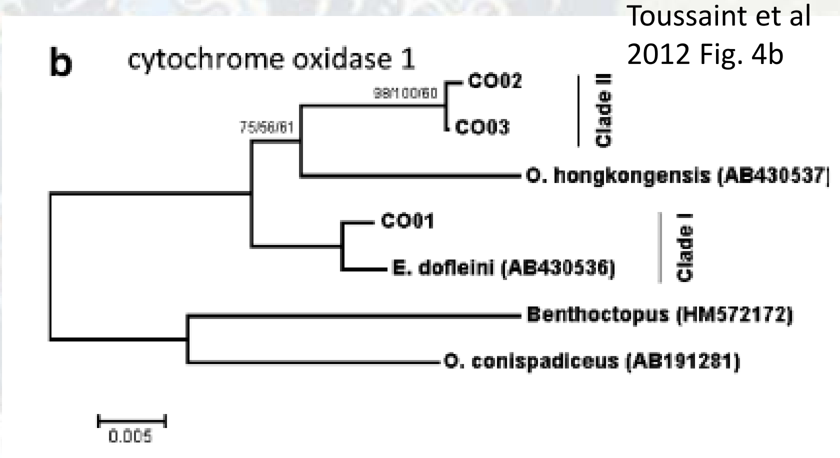
North American GPO Species



Toussaint et al 2012 detail from Fig. 5

Toussaint et al 2012 Fig. 4b



The so-called "*O. hongkongensis*" here is actually a misidentified GPO from northern Hokkaido, Japan

AB430536 is a GPO from deep water near Tokyo

Gleadall 2014 (conference talk with abstract) notes that O. hongkongensis AB430537 shown in Toussaint et al 2012 Fig 4b and Fig 5 is actually a mis-identified dofleini.

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RESEARCH ARTICLE

Nuclear and mitochondrial markers reveal evidence for genetically segregated cryptic speciation in giant Pacific octopuses from Prince William Sound, Alaska

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Abstract Multiple species of large octopus are known from the north Pacific waters around Japan, however only one large species is known in the Gulf of Alaska (the giant Pacific octopus, *Enteroctopus dofleini*). Current taxonomy of *E. dofleini* is based on geographic and morphological characteristics, although with advances in genetic technology that is changing. Here, we used two mitochondrial genes (cytochrome *b* and cytochrome oxidase I), three nuclear genes (rhodopsin, octopine dehydrogenase, and paired-box 6), and 18 microsatellite loci for phylogeographic and phylogenetic analyses of octopuses collected from across southcentral and the eastern Aleutian Islands (Dutch Harbor), Alaska. Our results suggest the presence of a cryptic *Enteroctopus* species that is allied to, but distinguished from *E. dofleini* in Prince William Sound, Alaska. Existence of an undescribed and previously unrecognized taxon raises important questions about the taxonomy of octopus in southcentral Alaska waters.

Keywords *Enteroctopus dofleini* · Phylogeographic · Phylogenetic · Microsatellite · Mitochondrial

Introduction

There are over 80 octopus species known from the shallow waters of the western Pacific Ocean off Japan (Kaneko et al. 2011). In contrast, fewer than ten species, including deep sea forms, are named from Alaskan waters in the eastern Pacific (Jorgensen 2009). Both regions support the widespread giant Pacific octopus (*Enteroctopus dofleini*, Wülker 1910, previously *Octopus dofleini*), which occupies the entire north Pacific Rim from California north to Alaska and west to Japan. The giant Pacific octopus is the most commonly encountered species in the eastern Pacific, is found from the intertidal zone (Scheel 2002) to depths of 1,000 m (Hochberg 1998), and makes up a significant component of by-catch in Alaskan commercial pot fisheries (Reuter et al. 2010). Although distinctive in the eastern Pacific due its large size (to >50 kg, Hartwick 1983), in the western Pacific, *E. dofleini* belongs to a larger group of common octopuses that may be morphologically difficult to distinguish (Gleadall 1993). With the advent of molecular techniques, the taxonomy of north Pacific octopuses is currently being reexamined (Hochberg 1998; Jorgensen et al. 2010; Kaneko et al. 2011).

Hochberg (1998) has reviewed the taxonomy of *E. dofleini*, yet the phylogenetic relationships and population structure of this species across its range remain unclear. As a result of changes in standards used for cephalopod identification and systematics (Nixon 1998; Allcock et al. 2008), as well as this species' variable morphological characteristics (Pickford 1964; Hochberg 1998), some researchers have identified subspecies based on geographic ranges and a few key morphological characteristics (such as ligula length, Berry 1912; Pickford 1964) while others have suggested an unresolved species complex (e.g. Hochberg 1998). The subspecies designation *E. d. dofleini* has been proposed for octopuses from the eastern Yellow Sea, Sea of Japan, and southern Sea of Okhotsk and nearby coastal waters. The subspecies *E. d. apollyon* has been proposed for octopuses from waters near Japan and Okhotsk to the Bering Sea and the Gulf of Alaska, and *E. d. martini* for octopuses from

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British Columbia to California (Pickford 1964; Nesis 1982). Hochberg (1998) found subspecies designations unsupported and suggested that *E. dofleini* currently comprises an unresolved species complex.

Life history and ecological characteristics of *E. dofleini* have been reviewed and described throughout the species' range (Hartwick 1983; Hartwick and Barriga 1997; Scheel 2002). Following a planktonic paralarvae stage that can last up to 90 days (Kubodera 1991), *E. dofleini* live 3–5 years (Hartwick 1983), with minimal overlap of generations. In near-shore environments off Hokkaido, Japan, *E. dofleini* makes a biannual onshore-offshore migration (Kanamaru and Yamashita 1969; Mottet 1975) in response to temperature fluctuations (Rigby 2004); however, similar migrations are unknown in Alaskan and other eastern Pacific waters where movements have been studied (Mather 1985; Hartwick et al. 1984; Scheel and Bisson 2012).

The limited genetic research on population structure or phylogeographic relationships of *E. dofleini* has been primarily based on nucleotide sequence data from the mitochondrial genes cytochrome c oxidase (CO) subunits I or III (Sosa et al. 1995; Söller et al. 2000; Takumiya et al. 2005; Jorgensen et al. 2010). Alone, neither gene is considered sufficiently polymorphic within species to answer questions regarding population genetics or phylogeographic relationships (Strugnell and Lindgren 2007). However, COI in particular has been useful for distinguishing among species of some cephalopods as well as many other taxa, and has been identified as a useful "barcoding" locus (Strugnell and Lindgren 2007; Kaneko et al. 2011). Recently, Barry (2010) found unexpectedly high sequence divergence of COI and COIII among individual *E. dofleini* from Alaska, consistent with the presence of a cryptic species. Similar studies of *Octopus vulgaris*, a widely distributed octopus species, uncovered genetic signatures of both regional isolation by distance and of cryptic species diversity using COI and COIII (Söller et al. 2000; Teske et al. 2007; Cabranes et al. 2008).

In this study, we used sequence data from two mitochondrial (mtDNA) genes [cytochrome *b* (CYTB) and cytochrome oxidase subunit I (COI)], and three nuclear genes (rhodopsin, octopine dehydrogenase and paired box-6) to examine the genetic relationships among *E. dofleini* from Prince William Sound (PWS) to Dutch Harbor (DH), Alaska. We complemented these analyses with fragment data from 18 microsatellite loci.

Methods and materials

Sample collection

We collected tissue samples only (no whole specimens), putatively from *E. dofleini* from three locations in southcentral Alaska including PWS, Montague Strait (MS), and Kachemak Bay (KB) and from the eastern Aleutian Islands near Unalaska Bay at DH. Sample locals and sample size are listed in Fig. 1. Tissue samples were collected by biopsy of the arm tip from octopuses from the intertidal zone (PWS, KB) and from subtidal waters (PWS, KB, MS, and DH). Tissue samples were stored in 100 % ethanol at ambient temperatures prior to DNA extraction. Tissue sample vouchers will be held at the U.S. Geological Survey's Molecular Ecology Laboratory in Anchorage, Alaska and at the Santa Barbara Museum of Natural History (voucher numbers pending).

DNA extraction and sequencing

A chloroform-isoamyl CTAB-PVP extraction technique, modified after Stewart and Via (1993), was used to extract whole genomic DNA. Extracted DNA was quantified using fluorometry and diluted to 50 ng/μL working solutions. Primers were synthesized for the mtDNA and single-copy nuclear genes (Table 1) with a universal modified, unlabeled tail (M13F and M13R) added the primers to the 5′- and 3′ end, respectively (Oetting et al. 1995), and universal primers were added to the forward primer for each microsatellite locus (Toussaint et al. 2011). Functional coding regions of each nuclear gene were targeted. See Toussaint et al. (2011) for microsatellite isolation, development, and primer sequences.

Polymerase chain reaction (PCR) amplifications were conducted to target specific regions of each mitochondrial and single-copy nuclear gene for subsequent sequencing. PCR amplifications were conducted in 50 μL reaction volumes containing 3 mM $MgCl_2$, 100 mM KCl, 20 mM Tris/HCl, and 0.2 % gelatin, 10 mM dNTP's, 0.001 mg BSA, 1.5 units *Taq* polymerase, 2 pmol of the forward and reverse primers, and 1 μL of 50 ng/μL of template DNA. The thermal cycler profile consisted of a 2 min denaturation at 94 °C followed by 40 cycles of 45 s at 94 °C, 45 s at 50 °C, and 90 s at 72 °C. A final extension of 30 min at 72 °C was performed. PCR products were visualized using agarose gel electrophoresis and purified with ExoSapIt (Affymetrix Santa Clara, CA). Simultaneous bidirectional, universal, labeled-tail sequencing (Oetting et al. 1995) was conducted using SequilTherm EXCEL™ DNA sequencing kits-LC. Sequenced products were electrophoresed on 5.5 % 41 cm polyacrylamide gels on a LI-COR LongReadIR™ automated sequencing system. Sequences were analyzed using the LI-COR eSeq™ software and aligned in AlignIR 2.0™ software (Lincoln, Nebraska). Sequences for each octopus were assigned to a haplotype using FaBox (1.40) DNA Collapser (Villesen 2007). GenBank Accession Numbers are listed in Tables 2, 3, 4 and 5.

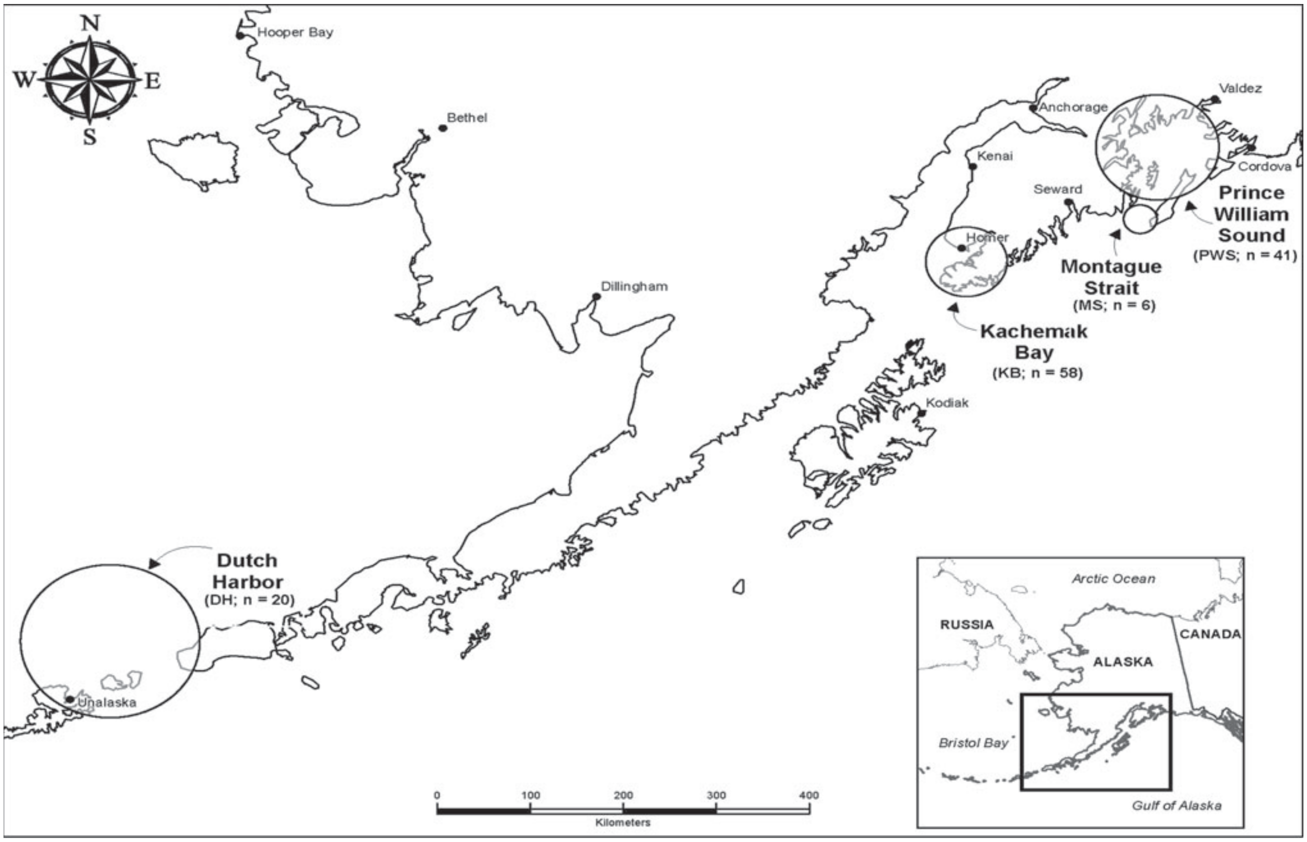

Fig. 1 Study location areas (*circles*) indicating where tissue samples from giant Pacific octopuses were collected. PWS and KB include samples collected from the intertidal and state waters (<3 miles from shore); MS from state waters, and near DH in the eastern Aleutians, samples were collected from state and federal waters (>3 miles from shore) of the Bering Sea (created in GIS)

Table 1 Primers and sequence results for two mtDNA and three nuclear genes for octopuses from southcentral and the eastern Aleutian Islands (DH), Alaska

Gene	Primer sequence (5′–3′)	Target fragment length (bp)	Fragment length (bp)	Variable sites	Parsimony informative (PI)	Variable amino acids (AA)
Cytochrome *b*	F: GGAACGAAGAATAGCATAAGC					
	R: ATATGCTTAGTTCATTACG	815	597	28	24	2
Cytochrome oxidase I	F: GGTCAACAAATCATAAAGAYATYGG					
	R: TAAACTTCAGGGTGACCAAARAAYCA	708	594	17	0	0
Rhodopsin	F: GCTTTCCTCATGATTATC					
	R: TTCTCCATCATTGCCATC	653	552	4	0	0
Octopine dehydrogenase	F: AAATCCCGACCAAACATG					
	R: GTTAAGTTTGTACCAGTC	618	528	4	0	2
Paired box-6	F: TTGTMAAYGGACGGCCGCTACC					
	R: GTCTCTGATYTCCCAGGCA	232	253	0	0	0

Note All forward sequences are preceded by the M13F universal tail: CACGACGTTCTAAAACGAC; all reverse sequences are preceded by the M13R universal tail: GGATAACAATTTCACACAGG

Phylogenetic analyses and genetic diversity indices

A portion of the CYTB gene was sequenced for all octopus samples. Subsets of $n = 14$–16 (see "Results" section) octopuses were selected for additional sequencing of the COI mitochondrial gene and the three nuclear genes. From these sequences, variable and parsimony informative (PI) sites were determined for each gene using MEGA 3.1

Table 2 Distribution of mtDNA CYTB haplotypes among 125 octopuses of southcentral (PWS, KB, MS) and the eastern Aleutian Islands, (DH) Alaska

Haplotype	Variable nucleotide site position																												Number of octopuses per population			
CYTB (GenBank Accession)	066	087	117	126	138	174	180	183	195	199	216	223	226	234	243	250	288	318	396	429	471	477	516	534	540	546	561	570	PWS	KB	MS	DH
CB01 (Pending)	G	C	C	A	C	C	C	G	G	C	C	A	T	A	A	T	C	A	A	C	A	A	C	G	G	A	A	G	11	21	4	8
CB02 (Pending)	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	2	0	0
CB03 (Pending)	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	14	23	1	8
CB04 (Pending)	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	7	0	1
CB05 (Pending)	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	0
CB06 (Pending)	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0	1
CB07 (Pending)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	0	0	0	2
CB08 (Pending)	A	T	T	.	T	T	T	A	A	T	T	.	C	G	T	C	T	.	.	T	G	G	T	A	A	G	G	A	1	0	0	0
CB09 (Pending)	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	0
CB10 (Pending)	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	2	1	0
CB11 (Pending)	A	T	T	.	T	T	T	A	A	T	T	.	C	G	T	C	T	.	G	T	G	G	T	A	A	G	G	A	8	0	0	0
CB12 (Pending)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	0	1	0	0

CYTB cytochrome *b*, *PWS* Prince William Sound, *KB* Kachemak Bay, *MS* Montague Strait, *DH* Dutch Harbor

Table 3 Distribution of mtDNA COI among 16 octopuses of southcentral (PWS, KB, MS) and the eastern Aleutian Islands, (DH) Alaska

Haplotype	Variable nucleotide site position																	Number of octopuses per population			
COI (GenBank Accession)	006	084	117	141	144	165	210	249	282	316	349	363	426	441	463	511	588	PWS	KB	MS	DH
CO01 (Pending)	T	C	C	T	A	C	C	C	A	T	C	T	T	G	C	C	A	2	4	1	1
CO02 (Pending)	C	T	T	C	G	T	T	T	G	A	T	C	C	A	T	T	G	6	0	0	0
CO03 (Pending)	.	T	T	C	G	T	T	T	G	A	T	C	C	A	T	T	C	2	0	0	0

COI cytochrome oxidase subunit I, *PWS* Prince William Sound, *KB* Kachemak Bay, *MS* Montague Strait, *DH* Dutch Harbor

Table 4 Distribution of nuclear RHO alleles among 15 octopuses of southcentral (PWS, KB, MS) and the eastern Aleutian Islands, (DH) Alaska

Allele	Variable nucleotide site position				Number of alleles per population			
RHO (GenBank Accession)	240	315	351	459	PWS	KB	MS	DH
RHO01 (Pending)	T	C	T	C	4	4	2	2
RHO02 (Pending)	.	.	C	.	0	3	0	0
RHO03 (Pending)	.	.	.	T	0	1	0	0
RHO04 (Pending)	C	T	.	.	10	0	0	0

Four octopuses with RH04 at one allele did not reveal a clean sequence for the homologous allele, giving $n = 26$ rather than 30 nuclear alleles

RHO rhodopsin, *PWS* Prince William Sound, *KB* Kachemak Bay, *MS* Montague Strait, *DH* Dutch Harbor

Table 5 Distribution of nuclear OCDE alleles among 15 octopuses of southcentral (PWS, KB, MS) and the eastern Aleutian Islands, (DH) Alaska

Allele	Variable nucleotide site position				Number of alleles per population			
OCDE (GenBank Accession)	384	443	468	510	PWS	KB	MS	DH
OCDE01 (Pending)	C	G	G	T	4	8	2	2
OCDE02 (Pending)	T	A	A	G	14	0	0	0

OCDE octopine dehydrogenase, *PWS* Prince William Sound, *KB* Kachemak Bay, *MS* Montague Strait, *DH* Dutch Harbor

(Tamura et al. 2011). Unbranched median joining networks were created in Network 4.6.0 (Fluxus Technology Ltd. 2004) to visualize the divergence between haplotypes/alleles for each gene. Standard diversity indices for mtDNA included haplotype (h) and nucleotide diversity (π) and neutrality (Tajima 1989; Fu 1997) and were obtained using ARLEQUIN (Schneider et al. 2000).

Prompted by results of the sequence data and following methods in Toussaint et al. (2011), we generated individual genotypes for PWS samples at 18 microsatellite loci, We tested for significant deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium using GENEPOP 3.2 (Raymond and Rousset 1995). We used microsatellite toolkit (Park 2001) to determine number of alleles (A) and estimated observed (H_O) and expected (H_E) heterozygosity. We used Genetix 4.05 (Belkhir et al. 2004) to perform a factorial correspondence analysis, and a Bayesian analysis (BAPS version 5.1; Corander et al. 2004) to test for subdivision across microsatellite loci within the PWS samples. We used Population 1.2.30 (Langella 1999) and Tree View (Page 2001) to construct neighbor-joining trees showing the proportion of shared alleles among the polymorphic microsatellite loci.

We compared our sequence data with homologous sequence data from up to 42 octopus species across seven genera in the family Octopodidae, archived in GenBank. Together, the percent of similarity, obtained through GenBank's Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990), and weighted pairwise distances, computed in MEGA 3.1 (Tamura et al. 2011), allowed us to

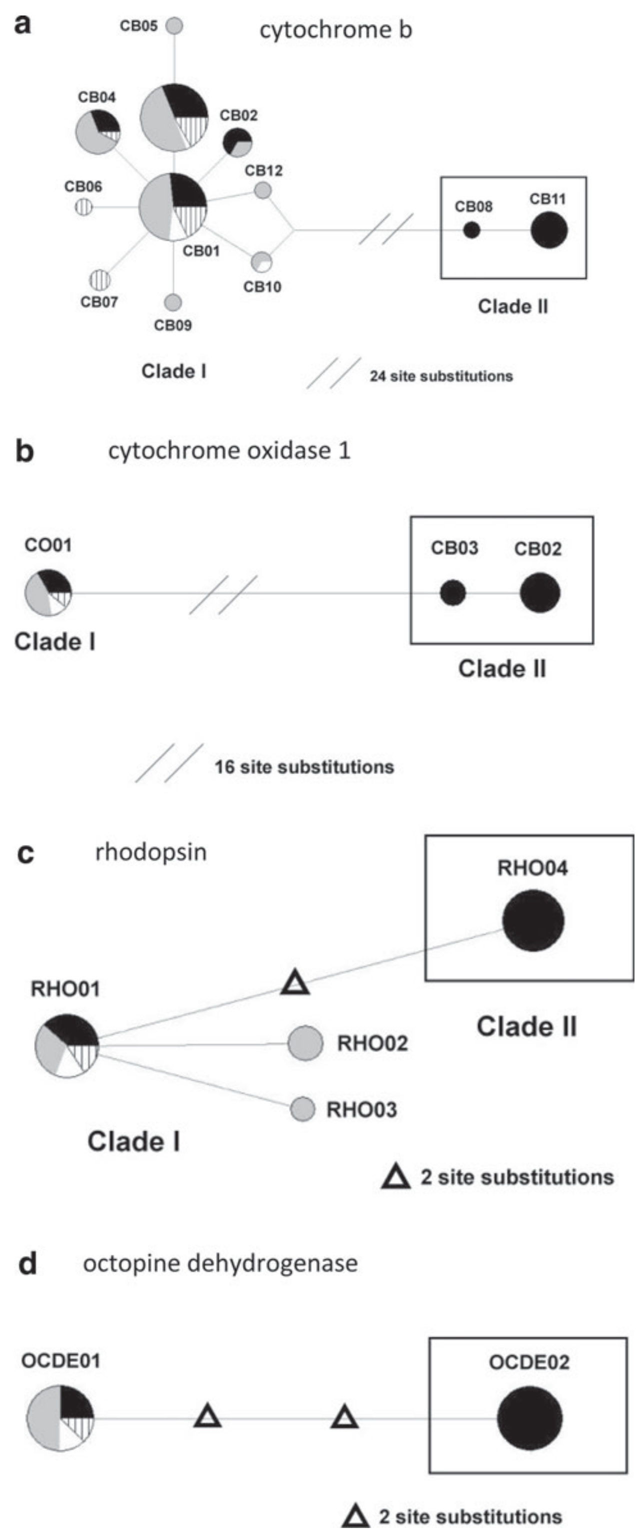

Fig. 2 Median joining networks of the mitochondrial genes cytochrome *b* (**a**) and cytochrome oxidase I (**b**) and nuclear genes rhodopsin (**c**) and octopine dehydrogenase (**d**) of giant Pacific octopuses. *Circle size* is proportional to the number of individuals with each haplotype (**a**–**b**) or with each allele (**c**–**d**); the smallest = 1 octopus (**a**, **c**), and the largest = 46 (**a**). Pie charts represent the proportion of individuals from each geographic location with each unique haplotype or allele; PWS (*black*), KB (*grey*), MS (*white*), and eastern Aleutians (DH; *cross-hatch*). All haplotypes were separated by one site substitution unless otherwise indicated. *Box outlines* indicate Clade II octopuses from PWS with the rare haplotypes or alleles. (Created in Network)

compare our sequence data to data collected from elsewhere in the species' range and select outgroups for phylogenetic analyses of sequence data (see "Results" section, Fig. 2 for Accession Numbers). Phylogenetic analyses of sequence data were conducted using PAUP* 4.0 beta 10 (Swofford 2003), using maximum-likelihood (ML), minimum evolution (ME), and maximum parsimony (MP) distance methods. We selected evolutionary models, using MODELTEST Version 3.06 (Posada and Crandall 1998) under Akaike's information criterion in ML and ME distance tree reconstructions. Phylogenetic trees were constructed in PAUP* 4.0 beta 10 (Swofford 2003) employing MP, ML, and ME analyses. Heuristic tree searches were conducted for each analysis, with 100 random additions of taxa for ML and parsimony analyses, each followed by tree bisection-reconnection topological rearrangements. Robustness of nodes was assessed using tree reconstructions of bootstrap-resampled data sets for 1,000 replicates under distance and parsimony criteria, and 50 replicates for ML criteria.

Results

Cytochrome *b*

We recovered a 597 bp fragment of CYTB from 125 individuals yielding 12 haplotypes that formed two distinct clades and differed at a total of 28 sites (24 PI sites including 2 non-synonymous changes; Table 1) The two haplotypes CB01 and CB03 were the most common ($n = 90$) and were found at all sample locations (Table 2; Fig. 2a); these formed one clade. Haplotypes CB08 and CB11 differed by 24 and 25 site substitutions, respectively, from CB01 (Table 2; Fig. 2a), and comprised a second clade. CB08 and CB11 occurred only in nine octopuses from PWS (22 % of $n = 41$) and at no other sample locations. A subset of eight octopuses (two from PWS, four from KB, and one each from MS and DH) representing the first CYTB clade, and eight octopuses of the second CYTB clade (CB11 only) were sequenced for mtDNA COI and three nuclear genes.

Cytochrome oxidase I

We recovered a 594 bp fragment of COI from these 16 octopuses yielding three haplotypes that similarly comprised two clades that differed at 16 and 17 sites and that corresponded to the two CYTB clades (Table 3; Fig. 2a, b).

Table 6 Estimates of genetic diversity from octopuses in PWS, Alaska

Taxa	Number of individuals (n)	Number of haplotypes or alleles	Haplotype diversity (h)	Nucleotide diversity (π)	Tajima's D	Fu's F
mtDNA CYTB						
Clade I	32	10	0.688	0.002	0.510	0.231
Clade II	9	2	0.222	0.000	−1.089	0.263
mtDNA COI						
Clade I	2	1	0.000	0.000	0.000	–
Clade II	8	2	0.429	0.001	0.333	0.536
nDNA RHO						
Clade I	2	3	0.000	0.000	0.000	–
Clade II	7	1	0.000	0.000	0.000	–
nDNA OCDE						
Clade I	2	1	0.000	0.000	0.000	–
Clade II	7	1	0.000	0.000	0.000	–

There were no PI sites or non-synonymous changes between COI haplotypes (Table 1).

Rhodopsin

We recovered a 653 bp fragment of rhodopsin (RHO) from 15 of the 16 octopuses that yielded four different alleles that formed two clades, corresponding to the two CYTB clades (Fig. 2c). At least two site substitutions separated the two RHO clades; no PI sites or non-synonymous changes observed (Fig. 2c).

Octopine dehydrogenase

We recovered a 618 bp fragment of octopine dehydrogenase (OCDE) from the 15 octopuses which among them, yielded two alleles, one found only in octopuses comprising the first CYTB clade, and the other found only in octopuses comprising the second CYTB clade (Fig. 2d). Four site substitutions were observed (Table 5).

Paired box-6

We recovered a 232 bp fragment (GenBank Accession Number pending) of paired box-6 from 14 octopuses; all possessed the identical allele (Table 1).

To summarize the sequencing results of the four variable genes, there was concordance between mitochondrial and nuclear signals for the two separate groups: (1) a common lineage(s) that occurred at all sample locations, and (2) a rare lineage(s) found only in PWS (Fig. 2). Given this concordance, hereafter, these will be referred to as Clade I and Clade II, respectively. Haplotype diversity values for each gene by clade are reported in Table 6. No significantly positive or negative values of Tajima's D or Fu's F were observed (Table 6), confirming selective neutrality of the sequence data.

Microsatellite loci

Of the 18 microsatellites analyzed (Toussaint et al. 2011), nine (Table 7) were shared among, and polymorphic within both Clade I and Clade II octopuses. The number of alleles at each locus varied from 2 to 28, (mean = 9 per locus); H_O ranged from 0.30 to 1.00 (mean = 0.68), and H_E ranged from 0.34 to 0.97 (mean = 0.76; Table 7). One locus (Edoμ6) deviated from HWE expectations ($P < 0.05$) within Clade I due to heterozygote deficiency (Table 7). We found no evidence of linkage disequilibrium based on 72 pairwise comparisons ($P < 0.05$, Bonferroni correction applied).

Neighbor-joining trees based on Cavalli-Sforza Edwards (CSE) chord distances revealed two groupings (with 41 % bootstrap support) from microsatellite data (Fig. 3a) corresponding to Clades I and II identified through analyses of the mitochondrial and three variable nuclear genes as described above. The factorial correspondence analysis of microsatellite loci clearly delineated the same two distinct groups among the PWS samples (Fig. 3b). Bayesian analyses of the microsatellite data also detected two clusters that corresponded to the two clades ($K = 2$, LnL = −358.3; Fig. 3c).

Table 7 Genetic diversity of nine polymorphic microsatellite loci developed for *E. dofleini* collected from PWS, Alaska

Locus	Number of alleles (A)	Size range (bp)	Observed heterozygosity (HO)	Expected heterozygosity (HE)	Polymorphism information content (PIC)
Edoμ6					
Clade I	12	180–220	**0.458**	**0.793**	0.758
Clade II	4	180–192	0.333	0.712	0.599
Edoμ8					
Clade I	13	179–219	0.862	0.843	0.814
Clade II	3	181–233	0.571	0.473	0.386
Edoμ11					
Clade I	15	187–219	0.931	0.911	0.886
Clade II	7	137–163	1.000	0.864	0.765
Edoμ105					
Clade I	5	187–205	0.345	0.336	0.312
Clade II	5	191–211	0.500	0.667	0.586
Edoμ110					
Clade I	15	124–170	0.793	0.903	0.878
Clade II	4	124–132	0.667	0.608	0.533
Edoμ118					
Clade I	18	225–269	0.897	0.926	0.903
Clade II	6	225–249	0.500	0.850	0.766
Edoμ122					
Clade I	25	178–236	0.815	0.934	0.912
Clade II	7	181–228	0.833	0.894	0.796
Edoμ125					
Clade I	28	168–268	0.750	0.968	0.948
Clade II	7	160–208	0.833	0.879	0.782
Edoμ129					
Clade I	9	139–159	0.793	0.755	0.712
Clade II	2	127–133	0.286	0.440	0.325

Notes Values in bold signify deviations from HWE ($P < 0.05$)

Clade I $n = 29$ of 32 PWS octopuses, Table 2. The other three did not yield clean sequences

Clade II $n = 9$ PWS octopuses (Table 2; Fig. 2)

Phylogenetic results

BLAST comparison of our Clade I CO01 haplotype sequence to homologous sequences archived in GenBank demonstrated that Clade I sequences were 99 % similar to published *E. dofleini* sequences from individuals sampled from the eastern and western Pacific regions (Strugnell et al. 2004; Kaneko et al. 2011; Accession Numbers: AY545190 and AB430536 respectively). In contrast, Clade II haplotype CO02/CO03 were only 97 % similar to the same *E. dofleini* sequences. Similar BLAST comparisons revealed 97 % similarity of both Clade I and Clade II haplotypes to both *Octopus hongkongensis* and *O. conispadiceus*. Weighted pairwise distances for COI ranged from 0.0 to 0.08 (Table 8). Clade I/*E. dofleini* (Accession Number: AB430536), Clade II, and *O. hongkongensis* (Accession Number: AB430537), appear to be equidistant. All are approximately 0.07–0.08 distant (Table 8) from *O. conispadiceus* (Accession Number: AB191281).

Phylogenetic trees of each polymorphic gene depict separation into clades previously identified Clade I and Clade II, and the distinctiveness was supported by high bootstrap values for MP, ML, and ME analyses (Fig. 4a–d). Comparison of the COI haplotype sequences recovered from our samples to homologous data from 35 species presented by Kaneko et al. (2011) separated the Clade I haplotype (CO01) from Clade II haplotypes (CO02 and CO03) with 61 % bootstrap support (Fig. 5).

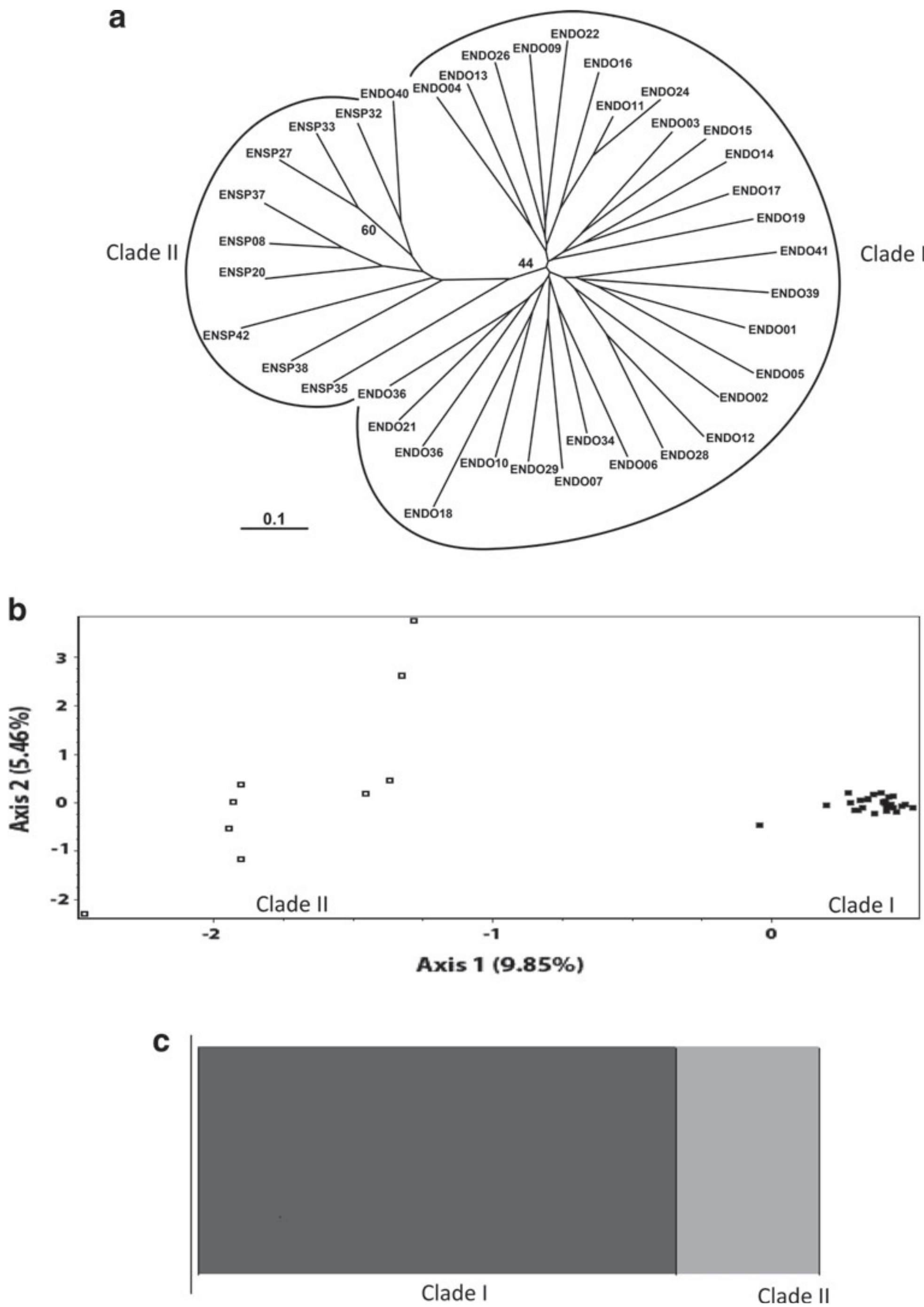

Fig. 3 Shared alleles among giant Pacific octopuses from PWS, Alaska across nine microsatellite loci. A neighbor-joining network (**a**) revealed two distinct clades (*left* and *right* sides emphasized by the *external lines*). The two groups consist of individual octopuses possessing either the common, Clade I haplotypes, *dofleini* or the rare, Clade II haplotypes. *Bootstrap* values are marked at the supported nodes. A factorial correspondence analysis (**b**) separates the samples into the same two distinct clades. The *solid black squares* represent the common, Clade I haplotype, and the *open boxes* represent the rare, Clade II haplotype. A Bayesian clustering analysis (**c**) indicates two distinct groups ($K = 2$) within this sample set (created in Tree View, Genetix, BAPS)

Discussion

Our results revealed that two distinct octopus groups (Clade I and Clade II), separated by 3–4 % sequence divergence within COI, occupy PWS. Within-clade divergence was less than 1 %. Clade I octopuses were found from four sampling locals across southcentral and eastern Aleutians, Alaska, whereas Clade II octopuses were only found in PWS. Separation between the two clades was consistent across four polymorphic genes (two mtDNA and two nuclear, Figs. 2, 5) and ten microsatellite loci (Fig. 3).

No Clade II haplotypes were observed in samples collected west of PWS. Barry (2010) similarly found high sequence divergence (COI) among giant Pacific octopus forming two groups, the first comprised of octopuses from PWS and southeast, Alaska, and the second of octopuses from southcentral (KB, PWS), and Glacier Bay (southeast AK). Barry (2010) suggested the possibility of cryptic speciation. However, inference of species relationships

Table 8 Cavalli-Sforza chord pairwise distances of our three cytochrome oxidase I haplotypes, (CO01, CO02, and CO03) compared to archived sequence data of closely related species *O. hongkongensis* (*O. hong*; AB430537), *O. conispadiceus* (*O. cons*; AB430533), and *E. dofleini* (*E. dofl*; AB430536, Kaneko et al. 2011)

	CO01	*E. dofl*	CO02	CO03	*O. hong*	*O. cons*
CO01	–					
E. dofl	0.01	–				
CO02	*0.03*	*0.03*	–			
CO03	*0.03*	*0.03*	0.00	–		
O. hong	*0.04*	*0.04*	*0.03*	*0.03*	–	
O. cons	0.07	0.07	0.08	0.07	0.07	–

Our haplotypes were obtained from giant Pacific octopuses collected throughout southcentral and the eastern Aleutian Islands (Dutch Harbor) Alaska. italics values indicates equidistant clades (see "Results" section)

based on data from mitochondrial DNA benefits from corresponding analyses of independent genes (Edwards and Beerli 2000); we have added in this case evidence from two nuclear, functional genes and corroborative evidence from neutral microsatellite loci. Therefore, our nuclear data confirm unexpected levels of diversity in southcentral (and presumably southeast) Alaska and indicate a previously unknown lineage of *Enteroctopus* occupies Alaskan waters.

Under the assumption that genetic data sets reflect lineage divergence and therefore can be used to delimit species, a number of researchers have attempted to define divergence thresholds that indicate speciation (Hebert et al. 2004; Papadopoulou et al. 2008). It is reasonable to assume that as lineages diverge, genetic differences accrue, and therefore the identification of a threshold of divergence that delineates species is attractive. However, the rate of divergence may vary across lineages (Hoffman et al. 2009; Sala-Bozano et al. 2009), precluding a strict application of divergence thresholds to species determination, and the rates of molecular and morphological evolution are not tightly correlated (Bromham et al. 2002; Davies and Salvolainen 2006), even at the species level. Relationships between phenotypic and genetic divergences within species are less well studied (Humphries and Winker 2010), and divergence at the mtDNA, considered by many researchers to be a 'leading' indicator of divergence (Zink and Barrowclough 2008), can overlap substantially at both the intra- and the interspecific level in marine species (Keever et al. 2009; McGovern et al. 2010), including octopus species.

Kaneko et al. (2011) found that intraspecific sequence divergences within Octopodidae ranged from 0.0 to 7.0 % (mean = 2.0 %). This range overlapped with interspecies divergences, which ranged from 4.0 % (sibling species) to more than 50 %. BLAST and distance analyses suggest that octopuses with Clade I haplotypes are <1 % divergent from *E. dofleini* from waters off British Columbia (Strugnell et al. 2004) and Japan (Kaneko et al. 2011; Takumiya et al. 2005) suggesting they are the same species. In contrast, the Clade II haplotypes are 3–4 % divergent from the Clade I/*E. dofleini* sequences. The level of divergence observed between Clade I and Clade II is therefore at upper level of the range found within other *Octopus* species, and within the lower range of the magnitude observed between sister species within Octopodidae (Kaneko et al. 2011).

Nevertheless, the two clades we identified appear to be reciprocally monophyletic at both the mtDNA and two nuclear genes. This is notable, since nuclear genes have larger effective population size than mtDNA, and thus nuclear lineages take longer to sort out into distinct lineages following divergence (Zink and Barrowclough 2008). Again, since it is reasonable to assume that lineages that have diverged accrue genetic differences over time, and even if those differences vary across lineages, the observation of reciprocal monophyly in both mtDNA and nuclear lineages lends strong evidence that the two clades in PWS have been diverged for sufficient evolutionary time to attain reproductive isolation, and maintain it while in sympatry. Given the influence of Pleistocene glacial cycles and dispersal barriers on the distribution of genetic variation in populations and species occupying marine waters in the Pacific Northwest and Alaska (Keever et al. 2009; McGovern et al. 2010), we suggest a critical next step is to increase sampling of *Enteroctopus* both eastward and westward to delineate the geographic extent of both clades.

Understanding the significance of the divergence between the two clades relative to other octopus species benefits from comparison at a larger phylogenetic level. Re-creation of the maximum-parsimony tree presented by Kaneko et al. (2011) based on COI sequences also showed that Clade II haplotypes are distinct from Clade I/*E. dofleini* haplotypes. However, all three haplotypes cluster with *O. hongkongensis* and *O. conspadiceus*. The group of four *Octopus* species (including *O. hongkongensis* and

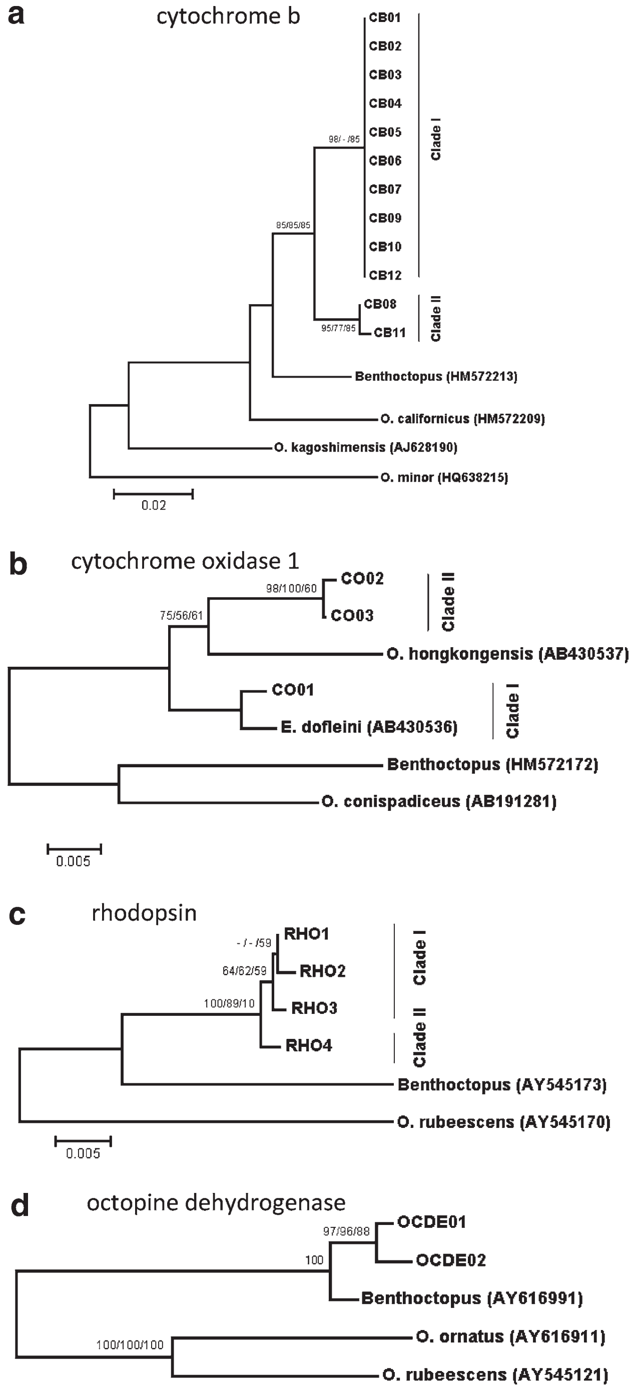

Fig. 4 Phylogenetic relationship among octopuses from southcentral and eastern Aleutians, Alaska based on haplotypes of mtDNA cytochrome *b* (**a**) and cytochrome oxidase I (**b**) nuclear genes rhodopsin (**c**) and octopine dehydrogenase (**d**). Accession Numbers are listed after each species used as an outgroup. *Bootstrap* values are listed at each supported node in order of maximum parsimony, maximum likelihood, and minimum evolution (created in MEGA)

O. conspadiceus that occupy waters off of Japan) shown by Kaneko et al. (2011) to group with *E. dofleini* occurred in a highly supported monophyly. As a result, Kaneko et al. (2011) suggest these four *Octopus* species should be reassigned and placed within the genus *Enteroctopus*. Unlike in Japanese waters where there are multiple large (>50 kg; Hartwick 1983) octopus species (Gleadall 1993; Kaneko et al. 2011), there are no other large species of benthic octopuses similar to *E. dofleini* described from the waters of southcentral Alaska (Jorgensen 2009). Therefore, although Clade I and Clade II octopuses are potentially different species based on their clear distinctiveness at both mitochondrial and nuclear genes. We are unable to assign species membership to individuals possessing the Clade II mitochondrial and nuclear sequences. Until morphological analyses can contribute to the determination of the species status of the taxon comprising Clade II, it is unclear where our Clade II octopuses will eventually be placed. However, the genetic data place Clade II within what is currently called *Enteroctopus*.

Although the finding of two clades of presumably *Enteroctopus* within PWS alone was unexpected, cryptic lineage divergence or speciation among marine species is very common and found in sponges, (Muicy et al. 1996; Erpenbeck et al. 2004) nematodes, (Derycke et al. 2005), fish (Terry et al. 2001; Griffiths et al. 2010), and other octopus (Söller et al. 2000). Based on our results, we suggest that further investigation and analysis of the nature and extent of differences between individuals representing Clade I and Clade II could include the determination of the extent of range and habitat overlap, including confirmation of range disjuctions and phylogeographic breaks (Keever et al. 2009) suggested by our study and research by Barry (2010), identification of functional differences in additional nuclear coding genes, and description of morphological characteristics.

All Clade II individuals ($n = 9$) were collected in PWS by pot-fishing at depths of 95.5–121 m on the western side of PWS. Eighteen Clade I individuals were also collected from same depth range and location (Fig. 6). No Clade II individuals occurred in samples collected from the intertidal ($n = 9$) to depths of 95 m ($n = 6$). This suggests there may be selection for different ecologies. Although six to eight other species of octopus occur in Alaskan waters (Jorgensen 2009), the only octopuses commonly collected by pot gear at these depths or locations, key to *E. dofleini* (Kozloff 1996; Jorgensen 2009). Again, samples collected from similar depths and with the same method in MS, KB, and DH did not contain any Clade II individuals. Additional sampling may determine if Clade II is restricted to depths >95 m. Additionally, all Clade II were collected within PWS; sampling further offshore may determine whether Clade II octopuses are restricted to bays or sounds.

There may also be functional differences in coding genes between individuals comprising the two clades. One of the nucleotide changes between CB01 and CB08/CB11

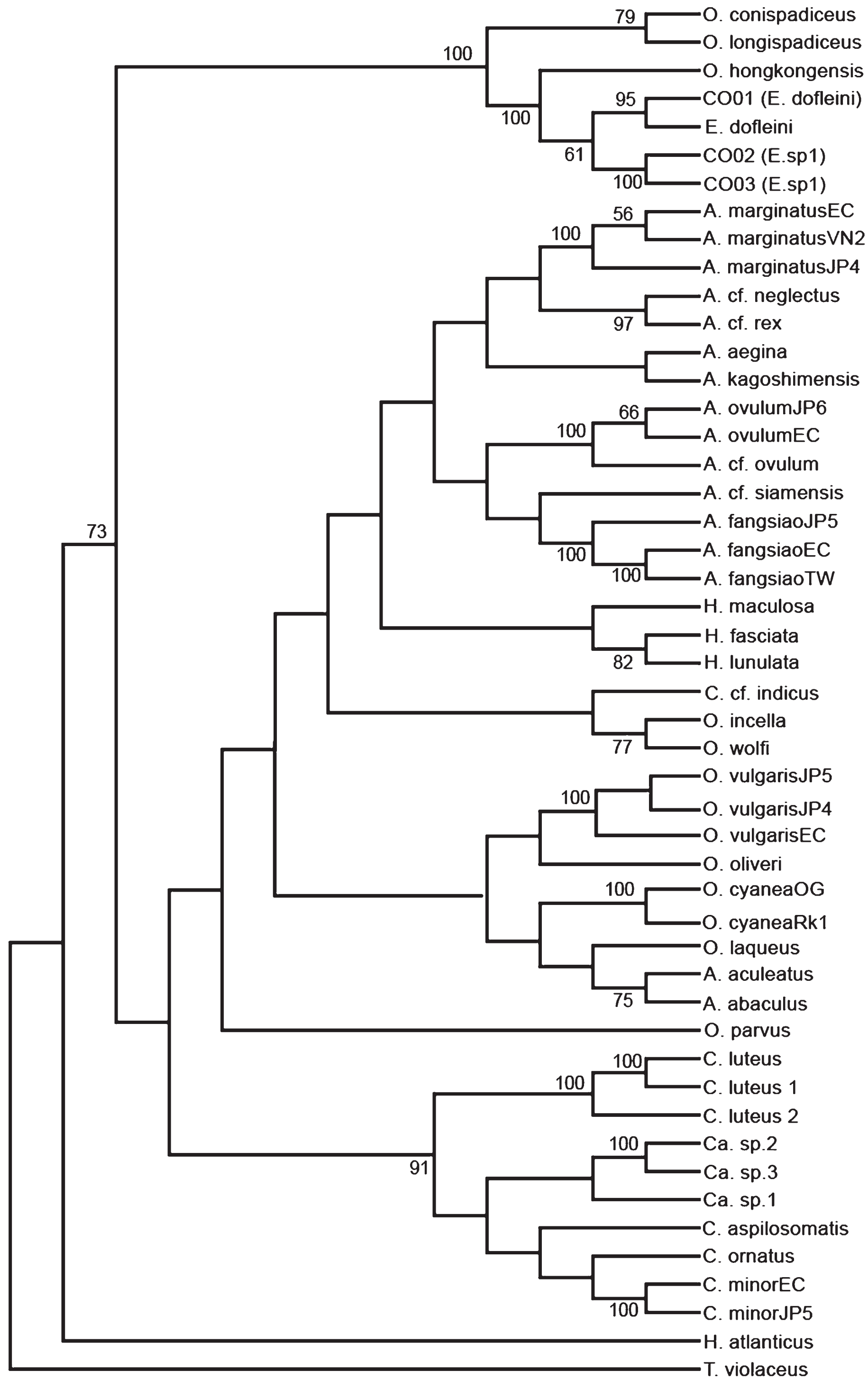

Fig. 5 Phylogenetic tree (maximum parsimony) of shallow-water benthic octopuses re-created from Kaneko et al. (2011) with the addition of our three cytochrome oxidase 1 haplotypes (CO1-3) from southcentral and the eastern Aleutian Islands (DH), Alaska. *Bootstrap* values over 50 are shown (created in MEGA)

was a first position codon non-synonymous transition, located at amino acid residue 113 ($Tyr^{113}His$), which occupies a part of the transmembrane cytochrome *b* protein. Two of the four site substitutions in OCDE are third position, non-synonymous amino acid changes ($Gly^{x}Asp$ and $Asp^{x}Glu$). Additional research into the three observed non-synonymous changes, as well as other genes would clarify whether these changes resulted in functional differences between the two clades.

We did not perform morphological analyses of individuals representing Clades I and II. Based on our genetic evidence of a cryptic taxon, we recommend further studies to delineate the frequency and distribution of the variant lineage, including detailed concomitant morphological and

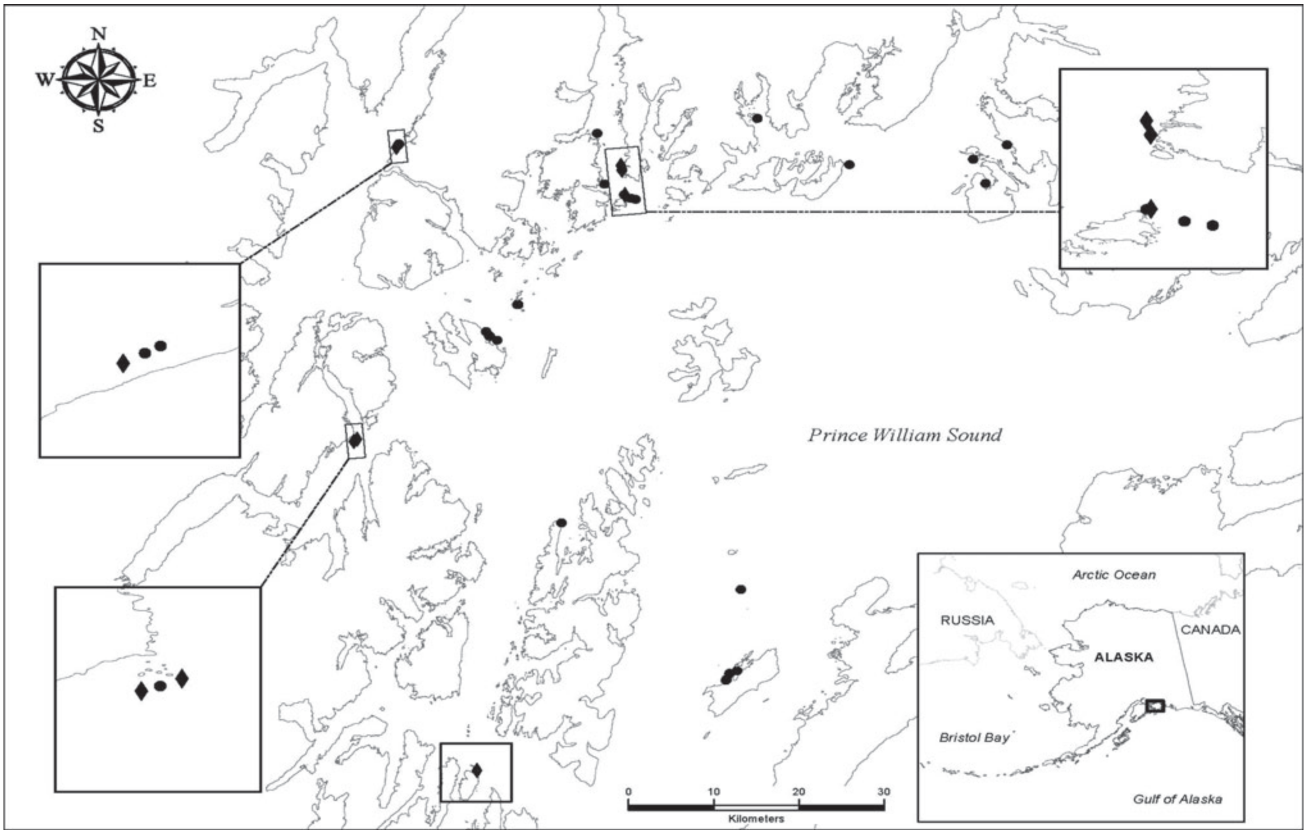

Fig. 6 Collection locations of giant Pacific octopuses from PWS, Alaska indicating individuals of common haplotypes found throughout the study region and which are 99 % similar to published mitochondrial DNA sequences of *E. dofleini* (*circles*); and individuals of a rare haplotype, *Enteroctopus* sp. 1 found only in western PWS (*black diamonds*). (Created in GIS)

phylogenetic assessments of *Enteroctopus* in eastern north Pacific waters off Alaska, Canada, and the continental United States. Extensive morphological analyses will be needed before fully resolving taxonomic status and verifying that the diversity is actually cryptic, and if so, for providing nomenclature for the cryptic diversity uncovered in this study, and to provide, if possible, field identification protocols to distinguish between individuals belonging to Clade I and Clade II.

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