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RESEARCH ARTICLE

Pleistocene speciation of sister taxa in a North Pacific clade of brooding sea stars (*Leptasterias*)

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Abstract Although numerous coastal marine species show intra-specific lineage splitting and population divergence times that date to the period of glacial cycles during the Pleistocene epoch, reported instances of recent speciation in the coastal marine environment are relatively rare. Marine organisms with brood-protection and other reproductive modes with limited dispersal potential have been suggested to experience more frequent speciation and extinction events than related species with higher dispersal rates, but few studies have actually estimated divergence times of sister species in these organisms. Here, two mitochondrial gene regions (cytochrome oxidase subunit I, putative control region and upstream tRNAs) and a nuclear gene region (Elongation factor 1α subunit intron 4) provide evidence of recent (0.5–1.2 Mya) cladogenetic events in four pairs of putative sister taxa in a predominantly North Pacific brooding subgenus of sea stars (*Leptasterias* subgenus *Hexasterias*). Calibration is obtained from a trans-arctic migration in a related clade of sea stars (*Leptasterias* subgenera *Hexasterias* and *Nesasterias*) that is timed to the opening of the Bering Strait at 3.5 $\pm$ 0.25 Mya, and uncertainty in the calibration point is accommodated with a normally-distributed Bayesian prior probability. Similar estimates of population splitting times for two of the pairs of putative sister taxa were obtained by a multilocus coalescent analysis. Estimates of mitochondrial mutation rates (0.01/My) were approximately 50% of the values calibrated for sister species pairs in tropical sea stars and sea urchins.

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Introduction

Cycles of glacial advance and retreat from the late Pliocene to the Pleistocene epoch (which began 1.8 Mya) were associated with major changes in the coastal marine environment, including variation in sea surface and air temperatures and changes in sea level and coastal hydrography. Along the California coastline, periods of glacial advance were also associated with altered patterns of nearshore upwelling, as well as wetter climatic conditions near the coastline that led to increased runoff through exposed coastal canyons (see discussion and references in Dawson 2001; Jacobs et al. 2004; Hickerson and Cunningham 2005). These glacial cycles had important effects on the genetic structure of terrestrial and freshwater animal and plant species (e.g., Avise et al. 1998; Bernatchez and Wilson 1998; Hewitt 2004), probably due to cycles of range contraction and expansion coupled in some instances with range fragmentation into disjunct refugia. In agreement with temporal patterns commonly seen in non-marine vertebrate species, several marine species along the Pacific

coast of North America show Pleistocene-dated divergence of conspecific populations (e.g., Marko 2004; Wares and Cunningham 2005; Hickerson and Cunningham 2005). However, there are few examples for coastal marine animals in North America of divergence times of putative sister taxa that have been dated to the Pleistocene epoch, in contrast to terrestrial animals, for which numerous examples of Pleistocene-dated speciation have been reported (e.g., Knowles 2000; Lovette 2005). For example, only one of 14 molecular phylogenetic studies of marine animals along the Pacific coast of North America that were reviewed by Jacobs et al. (2004) reported interspecific divergence times younger than 1.8 million years (My). Such differences between terrestrial and marine systems could reflect differences in the geographic extent and connectivity of populations in the two environments (Dawson and Hamner 2008), or could reflect the fact that taxonomic delineation of marine species lags behind that of terrestrial species, causing some recent speciation events in marine animals to be unrecognized (Marko et al. 2003). A third possibility is that prior surveys have under-sampled marine species with brooded larvae and other non-dispersive modes of reproduction. The survey by Jacobs et al. (2004) did include several North Pacific molluscan genera that lack planktonic larvae, including one genus (*Nucella*) for which molecular and morphological evidence for Pleistocene speciation was recently reported by Marko et al. (2003), suggesting that a broader survey of divergence dates in marine invertebrates with brood protection and other non-dispersive modes of reproduction might yield additional evidence for recent speciation events. Examples of Pleistocene-dated speciation are known for some tropical and subtropical echinoderms that reproduce by pelagic planktrotrophic larvae, including the sea urchins *Diadema* (Lessios et al. 2001), *Echinometra* (McCartney et al. 2000) and *Eucidaris* (Lessios et al. 1999) and the sea cucumber *Holothuria* (Uthicke et al. 2005).

The present report concerns *Leptasterias*, a species-rich (45 described species) genus of sea stars in the family Asteriidae, order Forcipulatida that is distributed throughout the northern hemisphere in cold to temperate, predominantly shallow-water environments. Members of the genus *Leptasterias* are obligate lecithotrophic brooders (McEdward and Miner 2001), in which females retain fertilized eggs and larvae on the benthic surface, until the offspring metamorphose into a crawl-away juvenile form. Among *Leptasterias*, the documented evolutionary consequences of brooding (compared to related taxa with pelagic larvae) include a reduction in gene flow among local populations, an increase in the importance of genetic drift (measured as F_{ST}), a reduction in the amount of nucleotide diversity and an increase in the rate of non-synonymous substitution in protein-coding genes (Foltz et al. 1996a, 2004). An earlier report (Hrincevich et al. 2000) of recent lineage splitting events (0.04–2.3 Mya) in the genus *Leptasterias* involved both intra- and inter-specific divergences, due to limited taxonomic information. This earlier study also had other potential limitations, including a rate calibration from tropical sea stars and no nuclear sequence data. Here, nuclear and mitochondrial sequence data, calibrated from a trans-arctic comparison of species in *Leptasterias* subgenera *Leptasterias* and *Nesasterias*, are used to produce Bayesian estimates of divergence times for four putative sister species pairs in *Leptasterias* subgenus *Hexasterias*, all of which are documented as having a brood-protecting mode of reproduction. The estimated times of speciation (range 0.50–1.22 Mya) fall within the Pleistocene epoch, as does the bulk of the 95% highest probability density interval.

Materials and methods

DNA extraction, PCR and sequencing

DNA was extracted from fresh, frozen or alcohol-preserved sea star tube feet as in Foltz (2007) and from formalin-preserved tissues as in Foltz et al. (2007a). PCR reactions for mitochondrial sequences were set up using a scaled down version of the protocol in Hrincevich and Foltz (1996); details of primers and reaction conditions for nuclear sequences have been published elsewhere (Foltz et al. 2007b) or are in Electronic Supplementary Table S2. Cycle sequencing was performed as in Foltz et al. (2007a, b). Intra-individual nucleotide site heterozygosity and length variation heterozygosity for nuclear sequences were resolved as in Foltz (2007).

Taxon and gene selection

For phylogenetic reconstruction, data were obtained from 35 specimens of *Leptasterias* representing 13 nominal species or cryptic species (Bickford et al. 2006). The taxonomic status of the morphologically identified and/or genetically identified cryptic species of *Leptasterias* included in the analysis is discussed below. Four other forcipulate species (*Evasterias retifera*, *Urasterias lincki*, *Stephanasterias albula* and a composite of *Pisaster ochraceus* and *P. brevispinus*) were included as outgroups in the phylogenetic reconstruction (Electronic Supplementary Table S4). *Evasterias* was included in the analysis because prior studies (Knott and Wray 2000; Foltz et al. 2007a) suggested that this genus is the sister group to *Leptasterias*. The remaining outgroups were chosen more opportunistically, based on the ability of PCR primers to amplify the target regions and on the general rooting strategy of Smith (1994). Although the *Evasterias* + *Leptasterias* clade

is well supported (e.g., 100% bootstrap support from Foltz et al. 2007a), the sister group to this clade is currently unknown. The phylogenetic reconstruction was based on two nuclear gene regions and two mitochondrial regions. The nuclear regions were (1) intron 4 of the Elongation factor-1α subunit gene and (2) intron 5 and 7 combined for the ATP synthase β subunit gene. The mitochondrial regions were (1) a partial sequence for the cytochrome oxidase subunit I gene and (2) an approximately 335-bp control region with two upstream tRNA genes (Glu and Thr) and small portions of the flanking 12S and 16S rRNA genes. Further details are in Electronic supplementary Table S2.

Phylogenetic and population genetic methods

The best fit model from MrModelTest v. 2.2 (http://darwin.uvigo.es/software/modeltest.html), obtained for each region separately using the Akaike Information Criterion, is shown in Electronic supplementary Table S2. The combined data were then analyzed in MrBayes v. 3.1 (http://mrbayes.csit.fsu.edu), using the overall Bayesian-bootstrap approach of Foltz et al. (2007a) for the four regions treated separately. Nucleotide diversities (π) with bootstrapped standard errors were estimated using Tamura–Nei distances in MEGA v. 3.1 (http://www.megasoftware.net). The values were tested for heterogeneity among genes and taxa using type III sums of squares in PROC GLM in SAS v.9.1.3.

Estimation of divergence times

The various approaches to dating events in a molecular phylogeny have been reviewed by Rutschmann (2006) and Welch and Bromham (2005). Unlike some other computer programs that require a fixed topology and/or a point estimate of one or more dated nodes, BEAST: Bayesian evolutionary analysis sampling trees ver. 1.4.5 (http://beast.bio.ed.ac.uk) allows for phylogenetic uncertainty as well as uncertainty in the calibration data. The former uncertainty is accommodated by estimating the phylogeny along with the divergence time in a Bayesian MCMC framework, and the latter uncertainty is accommodated by putting a probabilistic prior on the date of the calibration node (Drummond et al. 2006). Divergence times of nodes separating four pairs of putative sister species in the subgenus *Hexasterias* were estimated with BEAST, using the strict molecular clock model, the Yule speciation model and the same nucleotide substitution models that were used in the phylogenetic reconstruction (see Electronic supplementary Table S2 for details). *Leptasterias* generally live in high-energy rocky coastal environments at depths of less than 30 m (Fisher 1930), so fossil material is unavailable. In the absence of fossil-derived dates, calibration of divergence times was achieved by putting a prior probability distribution on the divergence time of a North Atlantic (*Leptasterias muelleri*) and two North Pacific (*L. fisheri* and *L. stolacantha*) representatives of *Leptasterias*. This analysis assumes that *L. fisheri* and *L. stolacantha* are more closely related to each other than either is to *L. muelleri*. This assumption is supported by the phylogenetic reconstruction in Fig. 1, even though *L. stolacantha* nominally belongs to a different subgenus (*Nesasterias*) than either *L. fisheri* or *L. muelleri*. *L. muelleri* (and other North Atlantic representatives of the subgenus *Leptasterias* that were not sampled in the present study) presumably represent trans-Arctic migrants of ancestral Pacific lineages after the opening of the Bering Strait. We modeled this uncertainty by using a normally-distributed prior on the divergence time of *L. muelleri* versus *L. fisheri* + *L. stolacantha* with a mean of 3.5 Mya and standard deviation of 0.25 Mya. A normally-distributed prior is appropriate for biogeographic calibration dates, but generally inappropriate for fossil calibration dates (Ho 2007). It is also generally assumed that the subsequent onset of Northern Hemisphere cooling has prevented more recent gene flow across the Arctic (Wares and Cunningham 2001, but see Addison and Hart (2005) for a counter-example). The amount of uncorrected sequence divergence between these two groups suggested that a normally-distributed prior on the mean rate of substitution of approximately 0.010 ± 0.004 substitutions/site/My for the two mitochondrial genes and 0.004 ± 0.002 substitutions/site/My for the EF-1 α gene was appropriate. In each case, the standard deviation was chosen to ensure that a range of substitution rates including those close to (but not below) 0 were proposed in the MCMC sampling. Because the divergence times from BEAST are TMRCAs (time to most recent common ancestor) rather than actual speciation times, we also calculated, where possible, divergence dates for pairs of putative sister species using the coalescent-based program IMa (http://lifesci.rutgers.edu/~heylab/HeylabSoftware.htm#IM).

Results

The alignment used for phylogenetic reconstruction consisted of 3,226 bp of concatenated mitochondrial and nuclear sequence. Individual specimens were represented in the analysis by 44.5–96.6% of this alignment; the average coverage was 73% (see Electronic supplementary Table S2). The analysis (Fig. 1) recovered nine clades corresponding to traditional taxonomic categories with bootstrap support >86%: genus *Leptasterias*, subgenus *Hexasterias*, section *Camtschatica* of subgenus *Hexasterias*, *L. alaskensis*, *L. fisheri*, *L. hexactis*, *L. leptodoma*, *L. polaris* and *L. stolacantha*. Two clades with 98–100% bootstrap support conflicted with traditional taxonomy:

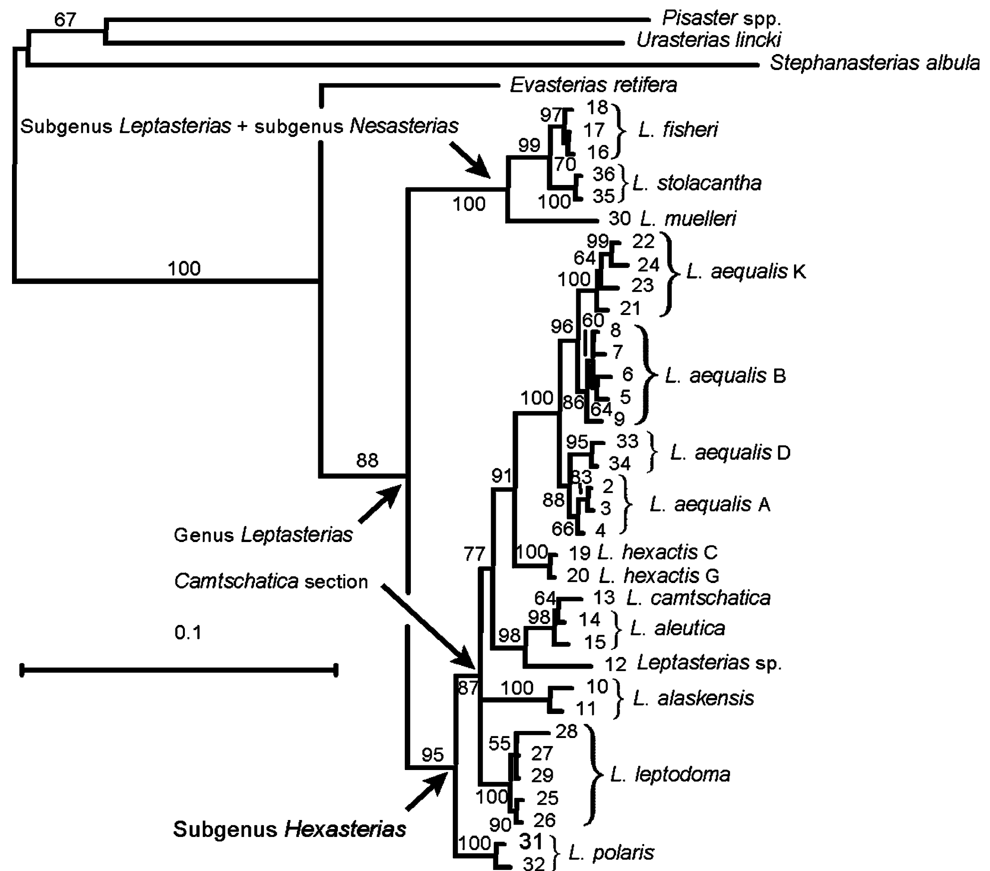

Fig. 1 Bootstrap consensus tree (100 replicates) from a gene-partitioned analysis in MrBayes v. 3.1.1 of two mtDNA genes and three introns of two nuclear genes. Bootstrap support values as percentages are shown for all nodes. The *scale bar* shows the expected number of substitutions per nucleotide site. Letters *A*, *B*, *C*, *D*, *G* and *K* in species names refer to putative sister species listed in Table 2

L. aleutica + *L. camtschatica* and *L. fisheri* + *L. stolacantha*. There was also support (66–100%) for morphologically-identified cryptic species within *L. aequalis*. The taxonomic implications of the phylogeny are discussed below. The topology in Fig. 1 was unchanged if the analysis was run after excluding the 140-bp repeat sequence present in introns 5 and 7 of the ATP synthase β subunit gene (see Electronic supplementary Table S2 for details). The nucleotide diversity values (Table 1) excluded the four outgroup taxa in Electronic supplementary Table S4, but included additional sequence data from 39 individuals (Electronic supplementary Table S5) that lacked sequence information for two or three of the gene regions included in the phylogenetic reconstruction. Nucleotide diversity values (π) varied widely but not significantly ($P > 5\%$) among genes and taxa.

Divergence times were estimated in BEAST for four pairs of putative sister taxa in the subgenus *Hexasterias* (Table 2) and for the trans-arctic comparison of *L. muelleri* (Atlantic) with *L. fisheri* and *L. stolacantha* (Pacific). Three *Hexasterias* taxa that each lacked an identified sister taxon along the Pacific coast of North America (*L. alaskensis*, *L. leptodoma* and *L. polaris*) and one unidentified species (*Leptasterias* sp. in Fig. 1) were excluded from the analysis. Also excluded from this analysis were the ATP synthase β subunit intron sequences, because of the unusual evolutionary behavior of the 140 bp repeat sequence (see Electronic supplementary Table S2). To validate the use of the strict molecular clock model in BEAST, log-likelihood values for phylogenies estimated with and without a molecular clock assumption were compared in PAUP* (Huelsenbeck and Crandall 1997), using best-fit models of substitution. For each of the three included gene regions, the log-likelihood test statistic was not significant ($P > 0.05$, see Electronic supplementary Table S2 for details). The estimated divergence times of sister taxa for the control region and flanking sequences ranged from 0.5 to 1.2 Mya (Fig. 2), and the highest posterior density (HPD) upper bounds were 1.9 Mya or less. For this region, the *L. aequalis* clade was dated at 1.9 Mya. The divergence times of sister taxa that were estimated from the COI gene fragment ranged from 0.5 to 1.0 Mya (Fig. 3). The 95% HPD intervals for these nodes were all less than 1.8 Mya. For COI, *L. aequalis* A and *L. aequalis* D were not jointly monophyletic. Although BEAST can calculate the divergence time of a non-monophyletic assemblage, the resulting value would not have the same meaning as for monophyletic assemblages. The *L. aequalis* clade was dated at 1.7 Mya. For the COI data, the only node that was older than 1.8 Mya was the divergence between *L. aequalis* and *L. hexactis*. Finally, for EF-1 α, missing data for *L. camtschatica* meant that the divergence time of this

Table 1 Number of individuals examined (N) and nucleotide diversities (π, as percentage, with standard error) for three gene regions in sea stars of the genus *Leptasterias*

Taxa	Control region + flanking sequences		Cytochrome oxidase I		Elongation factor-1α subunit, intron 4	
	N	$\pi \pm$ SE	N	$\pi \pm$ SE	N	$\pi \pm$ SE
L. aequalis A	9	0.17 ± 0.08	3	0.12 ± 0.11	4	0 ± 0
L. aequalis B	8	0.54 ± 0.17	8	0.44 ± 0.16	6	0.18 ± 0.13
L. aequalis D	4	0.46 ± 0.20	2	0.53 ± 0.30	1	–
L. aequalis K	6	1.37 ± 0.33	5	0.57 ± 0.21	2	1.42 ± 0.54
L. hexactis G	4	0.09 ± 0.08	3	0 ± 0	4	0 ± 0
L. hexactis C	5	0 ± 0	2	0 ± 0	3	0.80 ± 0.39
L. aleutica	3	0.62 ± 0.26	3	0.35 ± 0.20	0	–
L. camtschatica	4	1.15 ± 0.33	4	0.47 ± 0.20	2	0.39 ± 0.28
L. leptodoma	5	0.23 ± 0.13	3	0.94 ± 0.33	5	0.83 ± 0.26
L. alaskensis	2	0 ± 0	3	0.65 ± 0.30	2	2.64 ± 0.73
L. stolacantha	2	0 ± 0	2	0.19 ± 0.18	2	0.20 ± 0.19
L. fisheri	3	0.37 ± 0.21	3	0.90 ± 0.59	3	0 ± 0
L. polaris	2	0.98 ± 0.42	1	–	3	0.65 ± 0.28
L. muelleri	2	0 ± 0	2	0 ± 0	1	–

Table 2 Summary of genetic and morphological differences between four pairs of putative sister species of *Leptasterias* whose divergence times are estimated in Figs. 2, 3, and 4

Putative sister species[a]	Genetic differences[b]	Morphological differences	Ranges[c]
L. aleutica versus *L. camtschatica*	Flowers and Foltz (2001), AMOVA $P < 0.001$	Morphologically distinct when sympatric at Kanaga Is., AK (Fig. 3 in Flowers and Foltz 2001)	*aleutica*: Kanaga Is. to Unalaska Is., AK *camtschatica*: Attu Is. to Unalaska Is., AK + Pribilofs Is.
L. hexactis G versus *L. hexactis* C	Allozymic differences per Foltz et al. (1996a)	No. of actinal spines per ray (Flowers and Foltz 2001)	*hexactis* G: Cordova, AK to Balance Rock, BC *hexactis* C: Port Renfrew, BC to Sekiu, WA
L. aequalis B versus *L. aequalis* K	No data	No. of actinal spines per ray (Flowers and Foltz 2001)	*aequalis* B: Balance Rock, BC to Puget Sound, WA *aequalis* K: Patricks Pt., CA to San Simeon, CA
L. aequalis A versus *L. aequalis* D	No data	Morphologically distinct when sympatric at Sekiu, WA (Fig. 4 in Foltz et al. 1996b)[d]	*aequalis* A: Port Renfrew, BC to Doran Rocks, CA *aequalis* D: Sekiu, WA to San Simeon, CA

[a] Note on taxonomy: *L. aequalis* K consists of PCR-RFLP haplotypes K/L/V; *L. aequalis* B consists of PCR-RFLP haplotypes B/X; *L. aequalis* D consists of PCR-RFLP haplotype D; *L. aleutica* and *L. camtschatica* are from Flowers and Foltz (2001); *L. aequalis* A and *L. hexactis* C are from Foltz and Flowers (2007); and *L. hexactis* G is from Foltz et al. (1996a)

[b] Genetic differences other than those depicted in Figs. 1, 2, 3 and 4

[c] See Fig. S1 in the Electronic supplement for sources of distributional data

[d] *L. aequalis* D also has relatively fewer major and minor adambulacral pedicellariae than does *L. aequalis* A (Foltz et al. 1996b)

species and *L. aleutica* could not be estimated. Also, *L. aequalis* B and *L. aequalis* K were not jointly monophyletic for this gene, nor were *L. aequalis* A and *L. aequalis* D. As a result, for these four taxa only the divergence time of the *L. aequalis* complex could be dated, at 2.3 Mya. The only pair of putative sister taxa for which a divergence time could be estimated from the EF-1 α gene fragment was *L. hexactis* C and *L. hexactis* G, at 1.3 Mya. Other estimated dates for this gene (Fig. 4) were also uniformly larger than the corresponding dates for the two mitochondrial genes.

An alternative method of obtaining species divergence times that were corrected for ancestral polymorphism was provided by the program IMa (see Electronic supplementary Table S3 for details). This program provides a multilocus coalescent-based Bayesian analysis of isolation and migration between two species that are thought to have diverged

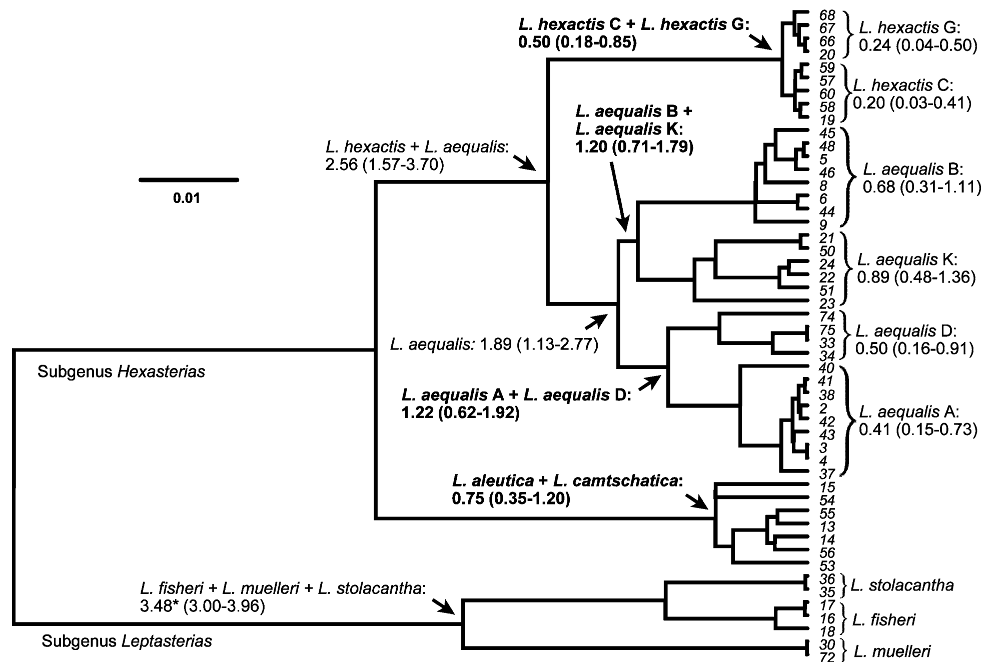

Fig. 2 Consensus tree from BEAST for the putative mitochondrial control region and flanking regions for four pairs of sister taxa (shown in *bold* font) in the subgenus *Hexasterias*, with members of the subgenus *Leptasterias* included for the purpose of calibration. For the calibration node (marked with an *asterisk*), the posterior distribution is necessarily the same as the prior distribution. Sequences are numbered as in Electronic supplementary Tables S4 and S5. Estimates of divergence times in million years are shown for 13 clades [with 95% highest posterior density (HPD) intervals in *parentheses*]. *L. aleutica* and *L. camtschatica* do not form reciprocally-monophyletic clades in this analysis. The *scale bar* shows the expected number of substitutions per nucleotide site. Alignment lengths are shown in Electronic supplementary Table S2; other details as in Fig. 1

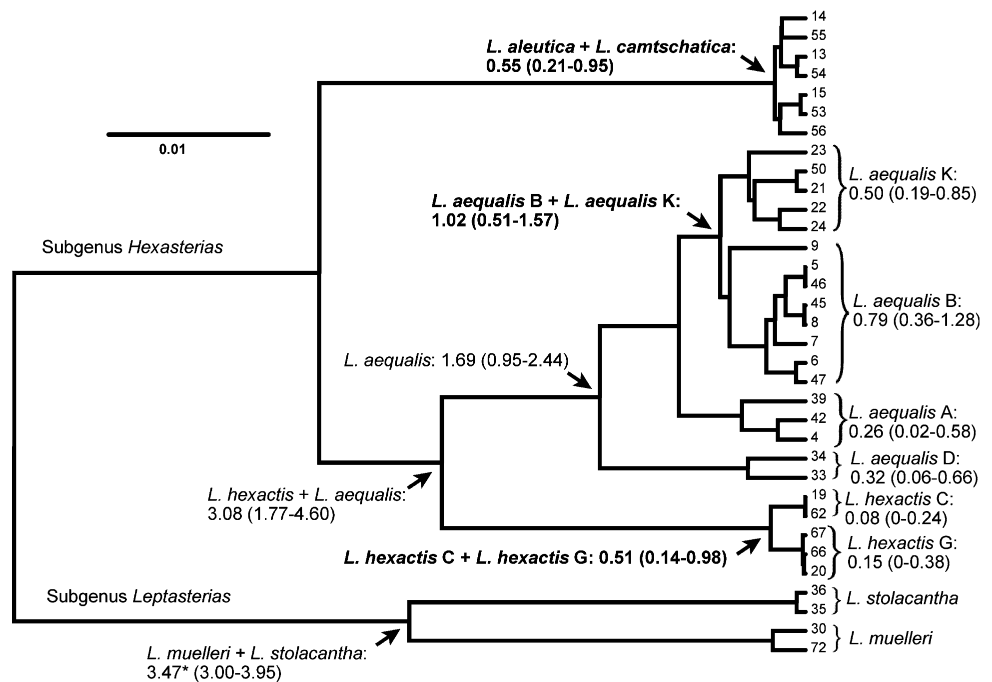

Fig. 3 Consensus tree from BEAST for the cytochrome oxidase subunit I gene, with divergence times shown for 12 clades. Other details as in Fig. 2

from a common ancestral species in the recent past. Because of missing data, the analysis could not be performed on the COI sequence data or for the *L. aleutica*/*L. camtschatica* comparison. Also, the posterior density curve of the divergence time estimate (t) was flat for the *L. hexactis* C/L. *hexactis* G comparison, possibly because the low within-species nucleotide diversity values (see Table 1) prevented the accurate estimation of effective population

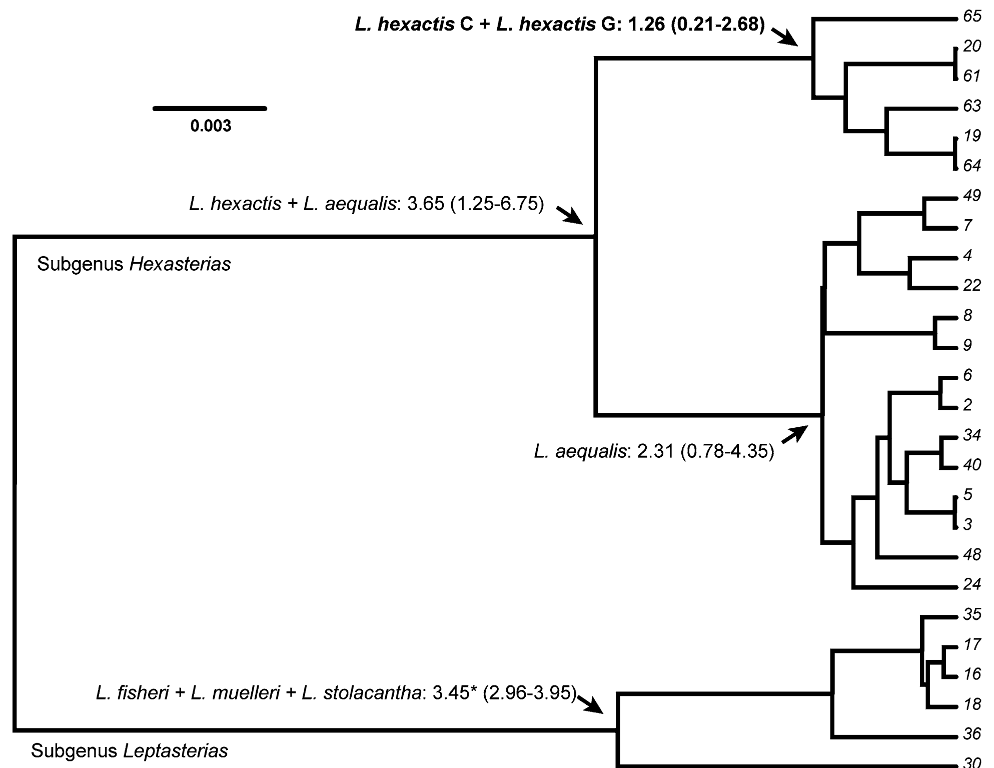

Fig. 4 Consensus tree from BEAST for the Elongation factor-1α subunit gene, with divergence times shown for four clades. Other details as in Fig. 2

sizes (Ne) for this pair of species. For the remaining two pairs of putative sister species, the estimated divergence time from IMa for *L. aequalis* A/*L. aequalis* D was 0.6 Mya (95% range 0.1–1.2 Mya) and the corresponding value for *L. aequalis* B/*L. aequalis* K was 0.7 Mya (95% range 0.2–1.6 Mya).

Discussion

We hypothesized, based on earlier studies with smaller numbers of taxa (Hrincevich et al. 2000; Foltz et al. 2007a), that traditional taxonomic categories within the genus *Leptasterias*, including the subgenera and sections depicted in Fig. 1, would be reciprocally monophyletic. This hypothesis was rejected for the subgenus *Leptasterias*, because the clustering of *L. stolacantha* with two species in the subgenus *Leptasterias* (*L. muelleri* and *L. fisheri*) suggested that the monotypic subgenus *Nesasterias* should be dissolved and *L. stolacantha* re-assigned to the subgenus *Leptasterias*. Other phylogenetic relationships in Fig. 1 agreed with past studies, such as (1) the position of *Evasterias* as the sister taxon to *Leptasterias*, previously noted by Knott and Wray (2000) and Foltz et al. (2007a), (2) the monophyly of the genus *Leptasterias* and of the subgenus *Hexasterias* (Foltz et al. 2007a) and (3) the monophyly of the *Camtschatica* section (Hrincevich et al. 2000). Concordant mitochondrial and nuclear gene trees (details not shown) for two nominal species, *L. leptodoma* and *L. alaskensis*, provided additional support for their status as species with no demonstrable taxonomic heterogeneity (Flowers and Foltz 2001).

The tangled taxonomic history of sea stars in the predominantly North Pacific subgenus *Hexasterias* (six-rayed forms) has been extensively discussed by Flowers and Foltz (2001) and is briefly reviewed in the Electronic Supplement and in Table 2. Two sympatrically distributed pairs of putative sister species (*L. aleutica*/*L. camtschatica* and *L. aequalis* A/*L. aequalis* D) show genetic and morphological differences beyond those depicted in Figs. 1, 2, 3 and 4 and likely correspond to conventional species. Two other parapatrically distributed pairs of sister species with less certain taxonomic status (*L. hexactis* C/*L. hexactis* G and *L. aequalis* B/*L. aequalis* K) might be phylogroups (in the sense of Avise 2000) rather than sister species. However, conclusions about the Pleistocene dating of lineage splits and speciation events in the subgenus *Hexasterias* are largely unaffected by this taxonomic uncertainty. In particular, estimated divergence times of lineages within the nominal species *L. aequalis*, which includes at least two biological species (*L. aequalis* A and *L. aequalis* B) are 1.9 Mya for the putative control region and flanking sequences, and 1.7 Mya for the COI gene (Figs. 2, 3).

As noted above, the divergence times estimated in Figs. 2, 3 and 4 are TMRCAs rather than estimates of actual species divergence dates. As a result, the time estimates inevitably include an unknown but potentially long period of time when the extant alleles were polymorphic in

the ancestral species. This ancestral polymorphism is typically accounted for by either calculating the "net" between-species divergence (e.g., Avise et al. 1998) or using the internal branch length separating sister taxa (e.g., Wares and Cunningham 2001). Both of these methods assume that present-day diversities within sister taxa provide good estimates of the amount of diversity and duration of the period of ancestral polymorphism in the immediate ancestor of the taxa concerned. In the present study, the approach of Avise (2000) is used, in which the TMRCA for a pair of sister taxa provides an upper (older) bound estimate of the divergence time and the within-species TMRCAs provide a lower (younger) bound estimate of divergence time. Subtracting the average within-species TMRCA from the between-species TMRCA is another way of correcting for ancestral polymorphism, but this method involves the same assumption as those described above. For some comparisons (e.g., *L. aequalis* B/*L. aequalis* K), the corrected divergence times are dramatically reduced (from 1.2 to 0.4 Mya for the control region and from 1.0 to 0.4 Mya for COI). These corrected estimates are similar to the IMa estimate of 0.7 Mya. Other comparisons (*L. hexactis* C/L. *hexactis* G) involve taxa with relatively little intraspecific divergence and are largely unaffected by the correction (from 0.5 to 0.3 Mya for the control region and from 0.5 to 0.4 Mya for COI). Corrected values could not be calculated for the remaining comparisons in Figs. 2, 3 and 4, except that the estimated divergence date for *L. aequalis* A/*L. aequalis* D for the control region changed from 1.2 to 0.8 Mya, which is similar to the IMa estimate of 0.6 Mya.

The mitochondrial substitution rates estimated by BEAST (0.011 per My for the control region and 0.009 per My for COI) are lower than values estimated for the COI gene in tropical geminate species pairs in sea stars (0.025 per My: Hart et al. 1997) and sea urchins (0.018 per My: Lessios et al. 2001). The assumptions involved in calibrating substitution rates from comparisons of geminate species pairs were discussed by Lessios (1998) and Lessios et al. (2001), who noted that two tropical geminate species pairs in the sea urchin genera *Diadema* and *Meoma* gave nominal rate calibrations that were only 30–40% as fast as for species pairs in six other sea urchin genera. Given the variation seen among tropical echinoderms in divergence values for geminate species pairs, meaningful comparison to data for polar and subpolar species will require more data. Although substitution rates have sometimes (e.g., Crame 1997) been suggested to be positively correlated with temperature, there is little direct evidence to support this hypothesis (Held 2001).

Opening of the Bering Strait has been conventionally dated to 3.5 Mya (e.g., Vermeij 1991) with new fossils extending the opening date to 5.3 Mya (Marincovich and Gladenkov 1999; Gladenkov et al. 2002; Marincovich et al. 2002). The 3.5 Mya date was chosen as a more conservative calibration point, based on the relatively unambiguous Pacific to Arctic–Atlantic exchange, as observed from fossils (e.g., Vermeij 1991). The older 5.3 Mya date supports an earlier opening of the Bering Strait, but suggests Arctic to Pacific faunal interchange, with relatively little evidence for the Pacific to Arctic exchange observed in *Leptasterias*. Using a 5.0 Mya calibration point with a standard deviation of 0.25 to estimate divergence times in BEAST produced means that were about 40% greater than those in Figs. 2, 3 and 4. The older date for the opening of the Bering Strait still gave mean divergence times for pairs of sister taxa that were less than 1.8 Mya, but the 95% HPD intervals included dates as old as 2.7 Mya (or 3.7 Mya for EF-1 α). This late Pliocene date coincides approximately with the onset of Northern Hemisphere glaciation (Ravelo et al. 2004), and supports the suggestion that hydrographic and climatic changes associated with glacial cycles over the past 2.7 My have had important effects on the genetic structure of *Leptasterias* populations.

The failure of any putative pair of sister taxa to show reciprocal monophyly for the EF-1 α intron 4 sequences (Fig. 4), as well as the larger divergence time estimates for this gene when compared to the mitochondrial sequences, are both consistent with the expected longer coalescence times for nuclear sequences versus mitochondrial sequences. However, the magnitude of the difference in speed of coalescence depends in part on population structure and possible fluctuations in population size, as well as on possible differences between the nuclear and mitochondrial genomes in mutation rates or natural selection (Hudson and Turelli 2003; Ray et al. 2003). For the present data, the 95% HPD intervals for the dates of particular nodes overlapped extensively among genes, reflecting the inherent stochasticity of the coalescent process. As a result, the observed among-gene heterogeneity in divergence time estimates is not statistically significant, and demonstrating a genomic effect (nucleus vs. mitochondrion) on divergence times of sister taxa would require a larger sample of genes from the nuclear genome.

The evidence for genetic signatures of glaciation-related diversification in *Leptasterias*, apart from recent speciation events, is somewhat equivocal. The π values in Table 1 for the cytochrome oxidase subunit I gene were significantly smaller ($P < 0.05$ by one-way ANOVA in PROC GLM in SAS after arcsine transformation) than the data for the mostly tropical and temperate echinoderm species with planktotrophic larvae that were reviewed by Foltz et al. (2004), and the mean π value from Table 1 (0.37%) was slightly smaller than that previously calculated for *Leptasterias* from a smaller number of sequences and species (0.41%). Three species (*L. aequalis* A, *L. hexactis* G and *L. stolacantha*) showed little or no polymorphism for any of the three surveyed regions, suggestive of severe bottlenecks.

L. hexactis G also has reduced allozyme heterozygosity compared to other *Leptasterias* species (Foltz et al. 1996a), which is consistent with the suggestion that some force has reduced the genetic diversity throughout the nuclear and mitochondrial genomes in this taxon.

Leptasterias is somewhat atypical of Arctic and sub-Arctic sea stars, being one of the few genera (along with *Henricia* and *Pteraster*) that have both a high latitudinal distribution and a documented obligate brood-protecting mode of reproduction (McEdward and Miner 2001). However, numerous major clades of Antarctic marine invertebrates are composed largely or exclusively of brooding lineages, many of which are species-rich and all of which are potentially impacted by southern hemispheric glacial cycles (Pearse and Lockhart 2004). Detailed molecular population genetic analysis of these lineages may well uncover many more examples of Pleistocene-dated speciation events.

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