Deep genetic subdivision within a continuously distributed and highly vagile marine mammal, the Steller's sea lion (*Eumetopias jubatus*)

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Abstract

The Steller's sea lion *Eumetopias jubatus* is an endangered marine mammal that has experienced dramatic population declines over much of its range during the past five decades. Studies using mitochondrial DNA (mtDNA) have shown that an apparently continuous population includes a strong division, yielding two discrete stocks, western and eastern. Based on a weaker split within the western stock, a third Asian stock has also been defined. While these findings indicate strong female philopatry, a recent study using nuclear microsatellite markers found little evidence of any genetic structure, implying extensive paternal gene flow. However, this result was at odds with mark–recapture data, and both sample sizes and genetic resolution were limited. To address these concerns, we increased analytical power by genotyping over 700 individuals from across the species' range at 13 highly polymorphic microsatellite loci. We found a clear phylogenetic break between populations of the eastern stock and those of the western and Asian stocks. However, our data provide little support for the classification of a separate Asian stock. Our findings show that mtDNA structuring is not due simply to female philopatry, but instead reflects a genuine discontinuity within the range, with implications for both the phylogeography and conservation of this important marine mammal.

Keywords: conservation genetics, genetic structure, gene flow, isolation by distance, microsatellite, phylogeography, pinniped, stocks

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Introduction

In common with many marine mammals, pinnipeds are capable of dispersing many hundreds or even thousands of kilometres. However, despite this potential mobility, females of most species show sufficiently strong philopatry to create a significant matrilineal substructure, as reflected by the distribution of mitochondrial DNA (mtDNA) lineages (Stanley *et al*. 1996; Andersen *et al*. 1998; Slade *et al*. 1998; Burg *et al*. 1999; Goldsworthy *et al*. 2000; Hoelzel *et al*. 2000; Wynen *et al*. 2000). Fewer studies of pinnipeds have used nuclear markers to examine patterns of male-mediated gene flow. Where this has been done, although

significant structuring is found, overall the patterns are weaker (Allen *et al*. 1995; Andersen *et al*. 1998; Goodman 1998; Slade *et al*. 1998; Burg *et al*. 1999; Goldsworthy *et al*. 2000) indicating that females are usually more philopatric than males.

The Steller's sea lion is the largest extant otariid, with bulls sometimes exceeding 1000 kg in mass (Loughlin *et al*. 1987), and is distributed across the North Pacific Rim and throughout the Bering and Okhotsk Seas (Fig. 1). The worldwide population was once estimated to number about a quarter of a million animals (Kenyon & Rice 1961) but by 1989 the count had fallen to a little over 100 000 (Loughlin *et al*. 1992). This dramatic decline attracted worldwide attention and led to the Steller's sea lion being listed as threatened under the United States Endangered Species Act. The underlying causes of the decline remain

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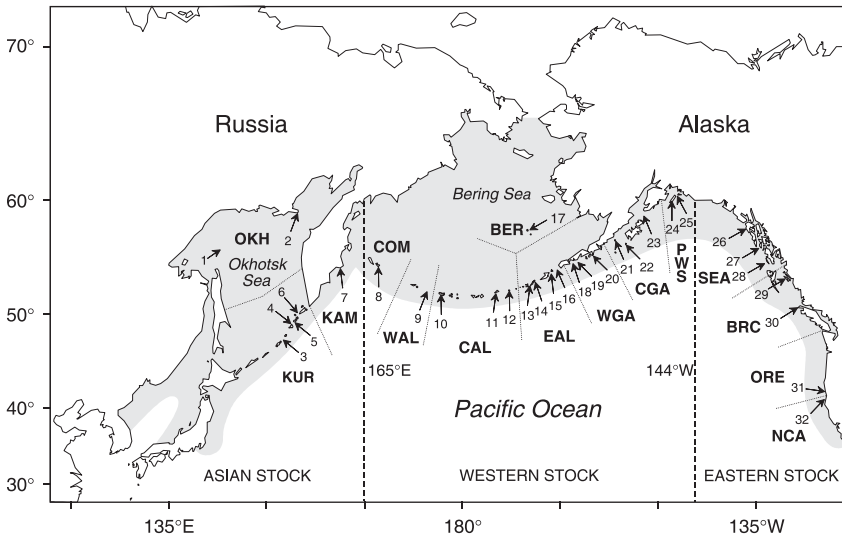
Fig. 1 Map indicating the locations of 32 Steller's sea lion rookeries sampled in this study. The grey area indicates the current distribution of the species. Stocks and regions are as defined by Baker *et al.* (2005). For details of regions and rookeries, including the numbers of individuals successfully genotyped at 10 or more microsatellite loci, see Table 1.

poorly understood and are almost certainly multifactorial (Loughlin *et al.* 1992; Loughlin 1998).

Early studies of Steller's sea lions using mtDNA polymorphisms found significant genetic divergence between populations lying to the west and east of 144°W and proposed that the overall population be divided into two management units: the western and eastern stocks respectively (Bickham *et al.* 1996; Bickham *et al.* 1998). Much of the western stock has experienced dramatic population declines during the past five decades. For example, the Alaskan western stock population, currently numbering 26 000 adults (Sease & Gudmundson 2002) is falling by about 5% per year (Sease *et al.* 2001) and according to Loughlin & York (2000) the population west of Cape Suckling, Alaska, to the westernmost tip of the Aleutian Islands, will number fewer than 1000 animals by the year 2020. In contrast, eastern stock numbers have increased from 13 000–15 000 during the period of 1965–1985, to 19 000 in 1995. To reflect this difference, the western stock was recently reclassified as endangered and the eastern stock as threatened (Calkins *et al.* 1999).

Two recent studies have further refined our picture of stock structure in Steller's sea lions. While early mtDNA studies (Bickham *et al.* 1996, 1998) indicated the presence of two discrete stocks, sample sizes were limited and a recent, more extensive analysis by Baker *et al.* (2005) concluded that the Asian rookeries should be classified as a separate stock. Elsewhere, nested-clade analysis based upon combined mitochondrial control-region and cytochrome *b* sequences (Harlin-Cognato *et al.* 2005) presented evidence that a series of population isolations, expansions and contractions were caused by the disappearance and subsequent reappearance of suitable rookery habitat during Pleistocene glacial and interglacial episodes. Isolation of the eastern stock was the oldest split and was repeated

multiple times, while the western and Asian stocks may have begun to differentiate more recently.

Findings using mtDNA are consistent with high levels of female philopatry in Steller's sea lions. In contrast, a recent study by Trujillo *et al.* (2004) using nuclear microsatellite markers found little evidence of any genetic structure and concluded that this was likely to be the result of paternal gene flow. While this is a plausible explanation, it goes somewhat against the observation that the western and eastern stocks show opposite growth trajectories. In addition, studies of marked individuals have failed to reveal any movement of animals between the western and eastern stocks (Raum-Suryan *et al.* 2002). Given these concerns, it seems reasonable to ask whether the relatively small sample size ($n = 208$) and few loci ($n = 6$) used for the nuclear study provide sufficient genetic resolution to detect significant structure.

To address this question, we genotyped over 700 Steller's sea lions at 13 highly polymorphic microsatellite loci. Our aims were (i) to determine whether population structure is evident when both sample sizes and genetic resolution are increased; (ii) to ask whether our data support or refute the three-stock model of population structure proposed by Baker *et al.* (2005); and (iii) to test the hypothesis presented by Trujillo *et al.* (2004) that male Steller's sea lions exhibit higher rates of gene flow than females, resulting in a less clearly defined population subdivision in the nuclear genome compared to mtDNA.

Materials and methods

Tissue sample collection, DNA extraction and genotyping

A total of 709 tissue samples were collected from pups at their natal rookeries ranging from Iony Island in the

Table 1 Numbers of Steller's sea lion samples amplifying successfully at 10 or more microsatellite loci. Stocks and regions are as defined by Baker *et al.* (2005)

Stock	Region	Rookery	Coordinates	Number of samples amplifying at ≥ 10 loci
Asian stock (*n* = 115)	Sea of Okhotsk–OKH (*n* = 50)	1. Iony Island	56°24.3′N, 143°23.0′E	25
		2. Yamsky Island	59°15.0′N, 155°25.0′E	25
	Kuril Islands–KUR (*n* = 40)	3. Srednego Island	47°34.8′N, 152°54.5′E	7
		4. Raykoke Island	48°17.6′N, 153°14.0′E	9
		5. Lovushki Island	48°32.6′N, 153°41.7′E	15
		6. Antsiferova Island	50°11.1′N, 154°58.1′E	9
	Kamchatka Peninsula–KAM (*n* = 25)	7. Kozlova Cape	54°29.3′N, 161°42.4′E	25
Western stock (*n* = 440)	Commander Islands–COM (*n* = 25)	8. Medny Island	54°41.0′N, 167°40.0′E	25
	Western Aleutian Islands–WAL (*n* = 12)	9. Buldir Island	52°21.6′N, 175°58.4′E	12
	Central Aleutians–CAL (*n* = 70)	10. Kiska Island	51°52.5′N, 177°20.0′E	24
		11. Seguam Island	52°21.1′N, 172°34.4′W	24
		12. Yunaska Island	52°41.4′N, 170°36.4′W	22
	Eastern Aleutian Islands–EAL (*n* = 172)	13. Adugak Island	52°54.7′N, 169°10.5′W	9
		14. Ogchul Island	52°59.7′N, 168°24.2′W	7
		15. Akutan Island	54°03.5′N, 166°00.0′W	56
		16. Ugamak Island	54°13.5′N, 164°47.5′W	100
	Bering Sea–BER (*n* = 13)	17. Walrus Island	57°11.0′N, 169°54.2′W	13
	Western Gulf of Alaska–WGA (*n* = 30)	18. Clubbing Rocks	54°43.0′N, 162°26.5′W	19
		19. Pinnacle Rocks	54°46.1′N, 161°45.9′W	8
		20. Atkins Island	55°03.2′N, 159°17.4′W	3
	Central Gulf of Alaska–CGA (*n* = 43)	21. Chowiet Island	56°00.5′N, 156°41.4′W	25
		22. Chirikof Island	55°46.5′N, 155°39.5′W	9
		23. Sugarloaf Island	58°53.3′N, 152°02.4′W	9
	Prince William Sound–PWS (*n* = 75)	24. Fish Island	59°52.9′N, 147°20.6′W	25
		25. Seal Rocks	60°09.8′N, 146°50.3′W	50
Eastern stock (*n* = 113)	South Eastern Alaska–SEA (*n* = 45)	26. White Sisters Island	57°38.1′N, 136°15.4′W	9
		27. Hazy Island	55°52.0′N, 134°34.0′W	26
		28. Forrester Island	55°50.0′N, 133°31.0′W	10
	British Columbia–BRC (*n* = 18)	29. North Danger Rocks	55°15.3′N, 130°20.5′W	10
		30. Triangle Island	50°52.3′N, 129°04.6′W	8
	Oregon–ORE (*n* = 25)	31. Rogue Reef	42°26.4′N, 124°28.1′W	25
	Northern California–NCA (*n* = 25)	32. St. George Reef	41°44.7′N, 124°15.0′W	25
Entire range				668

Okhotsk Sea to St George Reef in northern California (Fig. 1). Samples were obtained from rear flipper punches and stored individually in the preservative buffer 20% dimethyl sulphoxide (DMSO) saturated with salt (Amos & Hoelzel 1991). Total genomic DNA was extracted using either a standard phenol-chloroform protocol (Sambrook *et al.* 1989) or an adapted Chelex 100 protocol (Walsh *et al.* 1991). All samples were then genotyped using a panel of 13 dinucleotide-repeat microsatellite loci previously characterized from a variety of pinniped species (Table 2). These loci were chosen because they amplified consistently and exhibited clear banding patterns. The amplification and screening of microsatellite loci was performed as described by Hoffman & Amos (2005). To maximize data quality, any reactions that failed or yielded uncertain genotypes (e.g. with faint or unclear bands) were repeated up to two times. Samples amplifying at fewer than 10 loci

(*n* = 41) were then excluded from the data set, leaving a total of 668 individuals (Table 1). Finally, since microsatellites are prone to genotyping error, we estimated the underlying rate of error for our data set following Hoffman & Amos (2005) by independently re-genotyping 29 individuals, each selected from a different rookery.

Microsatellite data were already available for a subset of 208 individuals genotyped at six microsatellite loci (Hg6.3, Hg8.10, Pv11, Pvc29, M11a and M2b) by Trujillo *et al.* (2004). However, calibrating data sets produced in different laboratories poses many potential problems, particularly (as here) when genotyping methods differ. For example, microsatellite stutter patterns and estimated allele sizes were found to vary across a network of European laboratories (Jones *et al.* 1997) and, in extensive comparisons between two centres specializing in high-throughput microsatellite genotyping, Weeks *et al.* (2002)

were unable to match allele sizes consistently for almost 20% of > 300 loci. Consequently, we examined inter-laboratory repeatability by re-genotyping 100 animals from Trujillo *et al.* (2004) at the original six microsatellite loci. After compensating for variation in estimated allele sizes due to different size standards being used (Jones *et al.* 1997), over 20% of reactions were found to yield incongruent genotypes. While there are a number of approaches that could be used to reduce the size of this discrepancy, overall we felt the problem was too great to risk incorporating the remaining 108 genotypes published by Trujillo *et al.* (2004).

Data analysis

We calculated expected heterozygosity (H_E) for each locus and tested for evidence of deviation from Hardy–Weinberg equilibrium and linkage equilibrium using the program GENEPOP 3.3 (Raymond & Rousset 1995). For each test, we set the dememorization number to 30 000, the number of batches to 1000 and the number of iterations per batch to 300 000. To compensate for multiple statistical tests, levels of significance were adjusted using the sequential Bonferroni method (Rice 1989) with $\alpha = 0.05$.

Genetic differentiation among regions and rookeries was estimated using Wright's *F*-statistics (Wright 1951) calculated in ARLEQUIN 2.0 (Schneider *et al.* 2000). Significance testing of F_{ST} values was conducted using 30 000 permutations of the data set. To enable direct comparison with previous studies of Steller's sea lions (e.g. Trujillo *et al.* 2004), a small transformation (Slatkin 1995) was applied when calculating F_{ST} to linearize population divergence time and distance. Neighbour-joining trees at the regional and rookery levels were then constructed from the resulting genetic distance matrices using the program POPULATIONS 1.2.28 (Langella 2002). Finally, we conducted Mantel tests (Mantel 1967) using GENALEX 6 (Peakall & Smouse 2006) to test for associations between Slatkin's linearized F_{ST} and straight-line geographic distance among rookeries.

Alternative measures of genetic distance include R_{ST}, an analogue of F_{ST} designed for microsatellite data that incorporates a stepwise mutation model (see Slatkin 1995). However, we did not calculate R_{ST} because this tends to perform worse than F_{ST} when sample sizes are relatively small and fewer than twenty loci are used (Estoup *et al.* 1995; Ruzzante 1998; Gaggiotti *et al.* 1999). To test whether our analyses were sensitive to the specific measure of genetic distance chosen, we calculated instead a second genetic distance matrix based on Cavalli-Sforza and Edward's chord distance D_c (Cavalli-Sforza & Edwards 1967). When this was used to generate further phylogeographic trees, the resulting topographies were very similar to those using Slatkin's linearized F_{ST} (data not shown) indicating that our analyses are robust to the choice of measure.

Finally, cluster analysis of microsatellite genotypes was conducted using the program STRUCTURE 2.0 (Pritchard *et al.* 2000). STRUCTURE uses an iterative approach to cluster microsatellite genotypes into *K* populations without using the geographic locations of individuals. The approach essentially subdivides genotypes in a way that maximizes the Hardy–Weinberg equilibrium and the linkage equilibrium within the resulting clusters. The membership of each individual in a population is then estimated as *q*, which varies between 0 and 1, with the latter indicating full population membership. We ran two independent runs for $K = 1$–5 using 3×10^5 MCMC iterations after a burn-in of 10^5, the correlated allele frequencies model and assuming admixture.

Table 2 Summary of the 13 microsatellite loci used in this study including polymorphism characteristics for 668 Steller's sea lions. GenBank accession numbers were not available for all of the loci

Locus	Isolated from species	Reference	GenBank accession number	Number of alleles	Allelic size range (bp)
Hg1.3	Grey seal (*Halichoerus grypus*)	Gemmell *et al.* 1997	AF055864	6	250–260
Hg6.3	Grey seal (*Halichoerus grypus*)	Allen *et al.* 1995	G02092	7	225–237
Hg8.10	Grey seal (*Halichoerus grypus*)	Allen *et al.* 1995	G02093	4	172–178
Hl4	Leopard seal (*Hydrurga leptonyx*)	Davis *et al.* 2002	AF417693	9	139–155
Lc28	Crabeater seal (*Lobodon carcinophagus*)	Davis *et al.* 2002	AF140584	9	146–174
Lw10	Weddell seal (*Leptonychotes weddellii*)	Davis *et al.* 2002	AF140592	10	107–133
M2b	Southern elephant seal (*Mirounga leonina*)	Hoelzel *et al.* 1999	—	11	92–118
M11a	Southern elephant seal (*Mirounga leonina*)	Hoelzel *et al.* 1999	—	9	152–170
OrrFCB1	Atlantic walrus (*Odobenus rosmarus rosmarus*)	Buchanan *et al.* 1998	G34933	10	194–212
OrrFCB8	Atlantic walrus (*Odobenus rosmarus rosmarus*)	Buchanan *et al.* 1998	G34929	11	190–210
SGPV11	Harbour seal (*Phoca vitulina*)	Goodman 1997	U65444	4	170–186
Pvc29	Harbour seal (*Phoca vitulina*)	Coltman *et al.* 1996	L40987	8	128–144
Ssl15	Steller's sea lion (*Eumetopias jubatus*)	J. Bickham, unpublished data	—	5	178–186

Results

The microsatellite data set

We used 13 polymorphic microsatellite loci to genotype 709 Steller's sea lion pups sampled from 32 natal rookeries representing 15 regions and three putative stocks (Fig. 1). After excluding individuals that failed to amplify at 10 or more loci, 668 microsatellite genotypes were available for analysis (Table 1). The rate of genotyping error, estimated by independently re-genotyping 29 individuals at all 13 loci, was low at 0.008 per reaction or 0.004 per allele.

Moderate levels of genetic variability were found, with each locus yielding between four and 11 alleles (Table 2) and expected heterozygosity ranging from 0.237 to 0.843 (Table 3). Small, weakly significant deviations from Hardy–Weinberg equilibrium were detected at a number of loci (Table 3). However, these were not consistent across stocks and none of these P values remained significant following table-wide sequential Bonferroni correction for multiple statistical tests. Similarly, tests for linkage disequilibrium among pairs of loci yielded a small number of significant P values (Table 4), but these did not remain significant following sequential Bonferroni correction.

Phylogeographic patterns

First, we explored patterns of genetic variation at the regional level. To enable direct comparison with the results of Trujillo *et al.* (2004) and Baker *et al.* (2005) we used Slatkin's linearized F_{ST}. However, the specific measure seems unimportant because we also calculated Cavalli-Sforza and Edward's chord distance D_c and this yielded similar results. Slatkin's linearized F_{ST} values calculated for each possible pairwise regional comparison ranged from 0 to 0.082 (Appendix I). Significant levels of subdivision ($P < 0.05$) were detected for 76 out of 105 comparisons (54 following sequential Bonferroni correction). However, the proportion of significant F_{ST} values was lower within stocks than among stocks (32.4% vs. 94.1%, respectively, $\chi^2 = 45.6$, 1 d.f., $P < 0.001$) indicating that stock substructure was relatively weak. The neighbour-joining tree (Fig. 2) reflects this pattern, revealing two distinct clades corresponding to the eastern stock and the combined Asian and western stocks, but with relatively short branch lengths within these clades indicating low levels of differentiation within stocks. As expected, populations in close geographic proximity to one another clustered together, with the exception of the Commander Islands, which grouped together with the eastern Aleutian populations as found also by Baker *et al.* (2005). Notably, the Asian regions cluster together within the western/Asian clade but do not form a sister group to the western regions as was reported for mtDNA by Baker *et al.* (2005).

Table 3 Microsatellite heterozygosity and Hardy–Weinberg equilibrium (HWE) P values calculated for the total population and for each stock separately. Significant values ($P < 0.05$) are highlighted in bold. None of these P values remained significant following table-wide sequential Bonferroni correction for multiple statistical tests

Locus	Total population			Asian stock			Western stock			Eastern stock		
	Expected heterozygosity (H_E)	Observed heterozygosity (H_O)	HWE P value	Expected heterozygosity (H_E)	Observed heterozygosity (H_O)	HWE P value	Expected heterozygosity (H_E)	Observed heterozygosity (H_O)	HWE P value	Expected heterozygosity (H_E)	Observed heterozygosity (H_O)	HWE P value
Hg1.3	0.546	0.525	**0.020**	0.639	0.661	0.618	0.548	0.529	**0.020**	0.379	0.372	0.147
Hg6.3	0.237	0.239	0.886	0.261	0.281	0.865	0.256	0.260	0.764	0.135	0.115	0.098
Hg8.10	0.577	0.542	0.242	0.567	0.548	0.339	0.576	0.533	0.169	0.592	0.571	0.910
Hl4	0.726	0.729	0.159	0.745	0.768	0.866	0.739	0.736	0.233	0.602	0.664	0.432
Lc28	0.708	0.680	**0.007**	0.722	0.681	0.385	0.726	0.725	**0.026**	0.493	0.504	0.459
Lw10	0.655	0.665	0.606	0.539	0.574	0.622	0.662	0.680	0.631	0.710	0.699	0.721
M2b	0.843	0.810	0.284	0.829	0.765	0.168	0.845	0.823	0.517	0.836	0.805	0.749
M11a	0.833	0.804	0.720	0.807	0.826	0.528	0.827	0.806	0.698	0.804	0.770	0.947
Orr1	0.814	0.797	**0.037**	0.805	0.765	0.658	0.798	0.813	0.768	0.818	0.770	**0.024**
Orr8	0.819	0.806	0.168	0.814	0.847	0.918	0.824	0.822	0.480	0.752	0.700	0.151
Pv11	0.593	0.578	0.567	0.630	0.649	0.198	0.581	0.540	**0.033**	0.594	0.655	0.173
Pvc29	0.731	0.699	0.064	0.633	0.609	0.629	0.750	0.729	0.105	0.715	0.673	0.257
Ssl15	0.481	0.464	0.452	0.513	0.535	0.665	0.438	0.441	0.478	0.550	0.482	**0.034**

Table 4 Results of tests for linkage disequilibrium among 13 microsatellite loci. *P* values were calculated for the total population (below diagonal) and also controlling for stock substructure (above diagonal). Significant comparisons (*P* < 0.05) are highlighted in bold. None of these *P* values remained significant following table-wide sequential Bonferroni correction for multiple statistical tests

	Hg6.3	Hg8.10	Pv11	PvcE	M11A	M2B	Hg1.3	Lw10	SSL15	Orr1	Lc28	Orr8	Hl4
Hg6.3	*	0.265	0.531	0.090	0.167	0.335	0.845	0.535	0.754	**0.012**	0.275	0.672	**0.016**
Hg8.10	0.381	*	**0.027**	0.852	0.454	0.325	0.741	0.746	0.081	0.274	0.282	0.857	0.126
Pv11	0.392	0.124	*	0.682	0.714	0.739	**0.018**	0.406	0.389	0.725	0.703	0.265	0.556
PvcE	0.366	0.268	0.921	*	**0.023**	0.158	0.896	0.059	0.411	0.130	0.766	0.231	0.061
M11A	0.863	0.282	0.946	0.069	*	**0.003**	0.327	0.160	0.858	0.547	0.242	0.090	0.547
M2B	0.909	0.127	0.650	0.644	**0.022**	*	0.829	0.266	0.320	0.231	0.893	0.810	**0.008**
Hg1.3	0.789	0.570	**0.002**	0.598	0.789	0.741	*	0.599	0.918	0.409	0.980	0.880	0.792
Lw10	0.823	0.993	0.865	0.089	0.055	0.102	0.863	*	0.830	0.134	0.656	**0.040**	0.435
SSL15	0.253	**0.030**	0.138	0.541	0.961	**0.013**	0.692	0.607	*	0.512	**0.048**	0.472	0.892
Orr1	0.211	0.164	0.918	0.448	0.297	0.818	0.091	0.293	0.868	*	0.218	0.110	0.397
Lc28	0.301	0.066	0.824	0.308	**0.012**	0.665	0.927	0.428	0.139	0.240	*	0.137	0.340
Orr8	0.840	0.776	0.218	0.482	0.104	0.344	0.981	0.138	0.244	**0.049**	0.135	*	0.269
Hl4	0.068	0.441	0.251	0.051	0.970	**0.030**	0.450	0.439	0.911	0.264	0.156	0.124	*
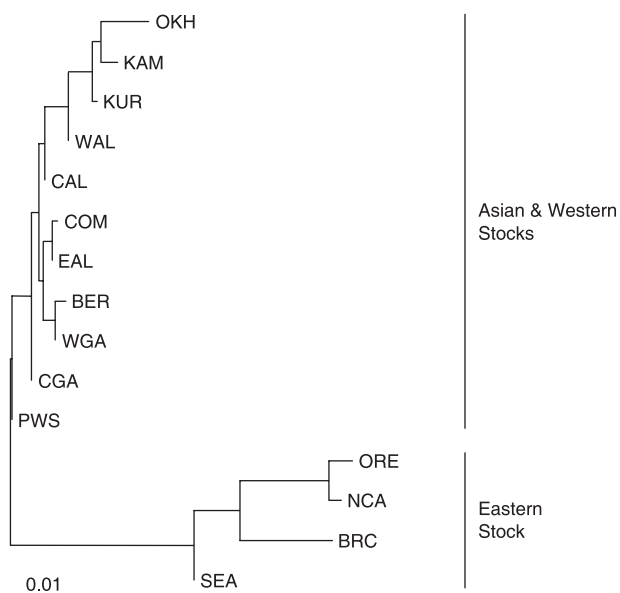
Fig. 2 Neighbour-joining tree showing genetic relationships among 668 Steller's sea lions based on Slatkin's linearized F_{ST} at the regional level (for regional designations see Table 1).

Fig. 3 Neighbour-joining tree showing genetic relationships among 668 Steller's sea lions based on Slatkin's linearized F_{ST} at the rookery level.

Next, we analysed genetic differentiation at the level of individual rookeries. Slatkin's linearized F_{ST} values calculated for each possible pairwise comparison ranged from 0 to 0.121 (Appendix II). Again, the majority of F_{ST} values were significant at *P* < 0.05 (257/496, 51.8%, 114 following Bonferroni correction). As found previously, the proportion of significant F_{ST} values was lower within stocks than among stocks (22.6% vs. 70.8%, respectively, χ^2 = 110.1, 1 d.f., *P* < 0.001). Also as before, the neighbour-joining tree (Fig. 3) shows a clear separation of eastern stock rookeries from those of the Asian and western stocks. In addition, all

but one of the Asian stock rookeries (Antsiferova Island) group together, indicating that they are broadly differentiated from those of the western stock.

Isolation by distance

To further explore patterns of genetic divergence, we analysed the relationship between genetic differentiation and straight-line geographic distance among rookeries (Fig. 4). When all rookeries were analysed together, a strong isolation-by-distance pattern was obtained (Mantel test, *r* = 0.60, *n* = 32, *P* < 0.001). However, no relationship between genetic and geographic distance was found
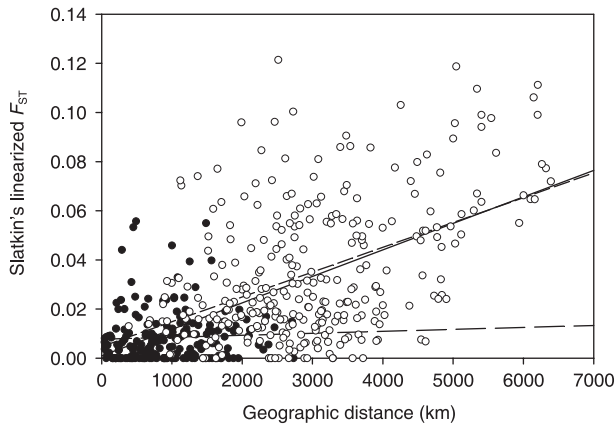
Fig. 4 The relationship between geographic and genetic distance (Slatkin's linearized F_{ST}) among 32 Steller's sea lion rookeries. Black points indicate within-stock comparisons and white points indicate among-stock comparisons. Linear regression lines are shown to indicate the underlying trends (solid line = total population, $r^2 = 0.361$; long-dashed line = within-stock comparisons, $r^2 = 0.002$; short-dashed line = among-stock comparisons, $r^2 = 0.207$).

Table 5 Results of two independent runs of the program STRUCTURE (Pritchard *et al*. 2000) for each hypothesized number of populations (*K*) using 668 Steller's sea lion multilocus genotypes. Consistent estimates of ln $P(x | K)$ were obtained for the two runs. The estimated posterior probabilities of *K* for the data set, given by $P(K | x)$, are indicative of which models are consistent with the data

Run	Number of clusters (*K*)	ln $P(x \mid K)$	Average ln $P(x \mid K)$	$P(K \mid x)$
1	1	−23801	−23801	~0
2		−23801		
1	2	−23712	−23716	~1
2		−23719		
1	3	−23858	−23857	~0
2		−23855		
1	4	−24245	−24262	~0
2		−24279		
1	5	−25108	−25529	~0
2		−25950		

within each of the three stocks (Asian stock, Mantel's $r = 0.36$, $n = 7$, P = NS; western stock, Mantel's $r = 0.05$, $n = 18$, P = NS; eastern stock, Mantel's $r = 0.03$, $n = 7$, P = NS) suggesting that the overall pattern is driven largely by among-stock comparisons.

Bayesian cluster analysis

Arguably the most versatile tests of population structure need not rely on knowledge of where individuals were sampled. Consequently, we implemented a Bayesian cluster analysis using the program STRUCTURE (Pritchard *et al*. 2000) to test whether genetic structure could be detected in the absence of a priori geographic data. STRUCTURE uses an approach that subdivides the data set in such a way that any departures from the Hardy–Weinberg equilibrium and the linkage equilibrium are minimized. In support of the previous analyses, the most likely genetic structure for the data set was found to consist of two clusters (*K* = 2, Table 5, Fig. 5), which broadly correspond

to the eastern and western/Asian stocks. When animals were classified according to the stocks they were sampled from, cluster membership coefficients varied significantly among the three stocks (mean = 0.40, 0.45 and 0.74 for the Asian, western and eastern stocks, respectively; ANOVA, $F_{2,665} = 264.7$, $P < 0.0001$). Assigning individuals to the clusters in which their membership coefficients were greatest, all but three of the eastern stock animals (110/113, 97.3%) were placed in one cluster. However, this cluster also included appreciable numbers of the Asian (25/115, 21.7%) and western (159/440, 36.1%) animals, indicating imperfect assignment.

Discussion

We used a panel of 13 nuclear microsatellite loci to examine population genetic structure across the geographic range of the Steller's sea lion. In contrast to a previous microsatellite study of this species (Trujillo *et al*. 2004), we detected significant genetic structure. Our findings have important implications for both the phylogeography and conservation of Steller's sea lions.
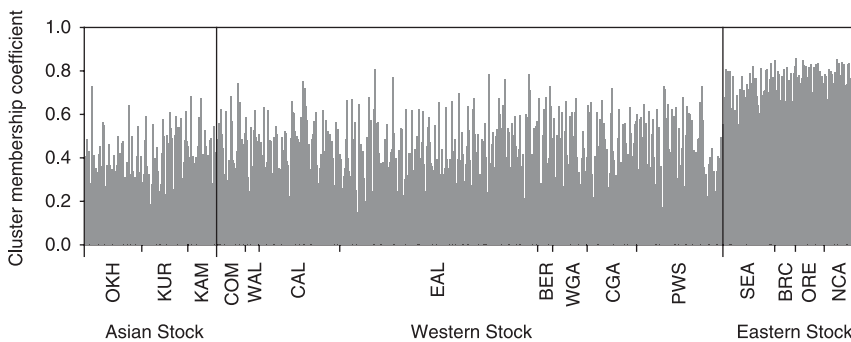
Fig. 5 Best clustering result (*K* = 2 clusters) for 668 Steller's sea lions using the program STRUCTURE (Pritchard *et al*. 2000). Each individual is represented by a vertical line partitioned into grey and white segments, the lengths of which indicate the probability of membership in each cluster.

Our key finding is that of a clear phylogenetic break between populations of the eastern stock and those of the Asian and western stocks. This pattern is found regardless of the analytical approach used (from phylogenetic reconstruction using F_{ST} values through to Bayesian cluster analysis). It also concurs with previous mtDNA studies of this species (Bickham *et al.* 1996, 1998; Trujillo *et al.* 2004; Baker *et al.* 2005; Harlin-Cognato *et al.* 2005) and is consistent with a recent study of over 8500 branded Steller's sea lions that found no interchange of animals between the western and eastern stocks over a 24-year period (Raum-Suryan *et al.* 2002). Two other species of marine mammals, the harbour seal (Westlake & O'Corry-Crowe 2002) and the sea otter (Cronin *et al.* 1996) each show a major break between populations east and west of 144°W. Such shared phylogenetic patterns among unrelated species with similar distributions are indicative of a common historic influence on intraspecific genetic architecture. In this case, Steller's sea lion stock structure most probably reflects historic isolation in Pleistocene glacial refugia (Harlin-Cognato *et al.* 2005).

While early mtDNA studies of Steller's sea lions indicated the presence of two discrete stocks (Bickham *et al.* 1996, 1998; Trujillo *et al.* 2004), sample sizes were limited and a recent more extensive analysis by Baker *et al.* (2005) concluded that the Asian rookeries should be classified as a separate stock. Our microsatellite data indicate that the Asian stock rookeries do group together, but that they form part of a lineage comprising both the Asian and western rookeries. Our data alone provide little support for the classification of a separate Asian stock. Nonetheless, the nuclear microsatellite loci confirm the mtDNA pattern that the three Asian regions are closely related and form a branch separate from all other populations.

At the regional level, the neighbour-joining tree revealed strong concordance between genetic divergence and the geographic distribution of rookeries. The exception was Medney Island, the only rookery of the Commander Islands, which clustered together with the eastern Aleutian rookeries rather than with those of the geographically closer western Aleutian or Kamchatka Peninsula populations. Baker *et al.* (2005) also placed Medney Island together with the Aleutian populations based on their analysis of control region sequences. These authors postulate that this recently founded rookery was probably colonized by immigrants from the Aleutian Islands.

While the genetic patterns that we found at the regional level were broadly reflected in the tree of individual rookeries, structure within stocks was less pronounced. This is consistent with previous studies (e.g. Baker *et al.* 2005) and probably reflects a combination of weak stock substructure and smaller sample sizes. In line with this finding, the isolation-by-distance analysis revealed a significant relationship when all rookeries were analysed together but no pattern within each of the individual stocks. Again, this parallels previous work based on mtDNA (Baker *et al.* 2005), although the F_{ST} values for nuclear markers are somewhat lower in our study. Such a pattern is consistent with a social structure in which females exhibit stronger philopatry than do males.

Bayesian analysis implemented in STRUCTURE was used to test the hypothesis that different stocks exist even when the analysis is not guided by a priori information about sampling sites. In agreement with other analyses of our microsatellite data, STRUCTURE identified two groups, corresponding roughly to the eastern and western/Asian stocks. Interestingly, an asymmetry was noted in the individual assignment coefficients of animals, with almost all individuals from the eastern stock being placed together in one cluster while animals from the other stock were split approximately 70:30. Such a pattern implies some level of demographic asymmetry, possibly arising through asymmetric gene flow or large differences in population size. In order to find the probable cause of this pattern, further simulation studies will be required.

Our results support the pattern identified by mitochondrial DNA alone, but they appear at odds with the prior study of Trujillo *et al.* (2004). However, Trujillo *et al.* (2004) cautioned that their microsatellite analysis was limited in terms of both sample size and the number of available loci. Our study addresses these shortcomings by more than doubling the number of microsatellite loci screened (from 6 to 13) and trebling the number of samples genotyped (from 208 to 709). The resulting increase in statistical power may well explain the difference in outcome, though other factors may also need to be considered. For example, Kalinowski (2004) showed that more individuals need to be sampled when F_{ST} is small than when it is large, and suggested that 20 individuals per population may be adequate when F_{ST} is > 0.05 but nearer 100 are needed when F_{ST} is ~0.001. In our study, Slatkin's linearized F_{ST} values reached a maximum of 0.082 for regional comparisons and 0.121 among rookeries, suggesting that sample sizes in the previous study may have been inadequate, at least for many of the comparisons. Another possible contributory factor could be genotyping errors. Discrepancies between laboratories are to be expected given the numerous pitfalls in cross-calibrating data sets (Jones *et al.* 1997; Weeks *et al.* 2002), but the incongruence rate of ~20% that we found shows how these problems can become extreme when manual and automated approaches (including allele calling and binning) are compared.

Conclusion

Using increased sample sizes and more loci, biparentally inherited microsatellite markers reveal significant genetic structure in Steller's sea lions. All of our analyses support

the recognition of two primary stocks comprising the eastern rookeries and all others. Within the latter, phylogenetic analysis reveals two subclusters corresponding to the western and Asian rookeries. The fact that stronger support for the differentiation between the western and Asian stocks was found for mtDNA than for microsatellites is consistent with dispersal rates being higher for males than females. Our results reinforce previous findings using mtDNA that an apparently continuous population includes a strong division (Bickham *et al.* 1996, 1998; Baker *et al.* 2005). Furthermore, these findings have potential implications for conservation since not only do the stocks have different demographies, but also the declining stock may not be replenished from the expanding stock despite its close geographic proximity.

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Appendix I

Genetic differentiation among regions, estimated using Slatkin's linearized F_{ST} (above diagonal). P values, calculated using 30 000 permutations of the data set, are given below the diagonal. Significant P values ($P < 0.05$) are highlighted in bold, and those that remained significant following table-wide sequential Bonferroni correction for multiple statistical tests are underlined. Regional abbreviations correspond to those given in Table 1

		Asian stock			Western stock								Eastern Stock			
		OKH	KUR	KAM	COM	WAL	CAL	EAL	BER	WGA	CGA	PWS	SEA	BRC	ORE	NCA
Asian stock	OKH	*	0.009	0.009	0.017	0.005	0.012	0.016	0.026	0.014	0.011	0.014	0.047	0.077	0.082	0.072
	KUR	**0.004**	*	0.005	0.015	0.000	0.006	0.009	0.016	0.007	0.004	0.006	0.029	0.065	0.071	0.072
	KAM	**0.016**	0.100	*	0.013	0.002	0.009	0.014	0.020	0.011	0.011	0.007	0.035	0.068	0.067	0.064
Western stock	COM	**< 0.001**	**< 0.001**	**0.014**	*	0.000	0.002	0.000	0.004	0.008	0.002	0.003	0.027	0.061	0.054	0.047
	WAL	0.206	0.449	0.345	0.743	*	0.000	0.000	0.008	0.000	0.000	0.000	0.012	0.047	0.050	0.048
	CAL	**< 0.001**	**0.008**	**0.008**	0.253	0.814	*	0.002	0.005	0.002	0.000	0.004	0.024	0.052	0.054	0.052
	EAL	**< 0.001**	**< 0.001**	**< 0.001**	0.469	0.868	0.076	*	0.003	0.000	0.000	0.005	0.026	0.053	0.055	0.056
	BER	**< 0.001**	**0.008**	**0.009**	0.294	0.182	0.166	0.248	*	0.001	0.000	0.012	0.021	0.044	0.047	0.050
	WGA	**< 0.001**	**0.031**	**0.017**	**0.047**	0.706	0.223	0.869	0.396	*	0.000	0.006	0.024	0.044	0.061	0.066
	CGA	**< 0.001**	0.059	**0.008**	0.296	0.829	0.462	0.534	0.437	0.567	*	0.000	0.018	0.042	0.048	0.048
	PWS	**< 0.001**	**0.015**	**0.020**	0.139	0.920	**0.026**	**< 0.001**	**0.026**	**0.030**	0.560	*	0.016	0.047	0.045	0.045
Eastern Stock	SEA	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.024**	**< 0.001**	**< 0.001**	**0.002**	**< 0.001**	**< 0.001**	**< 0.001**	*	0.012	0.008	0.013
	BRC	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.010**	*	0.036	0.022
	ORE	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.027**	**< 0.001**	*	0.005
	NCA	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.004**	**0.004**	0.204	*

Appendix II

Genetic differentiation among rookeries, estimated using Slatkin's linearized F_{ST} (above diagonal). *P*-values, calculated using 30 000 permutations of the data set, are given below the diagonal. Significant *P*-values ($P < 0.05$) are highlighted in bold, and those that remained significant following table-wide sequential Bonferroni correction for multiple statistical tests are underlined. Rookery numbers correspond to those given in Table 1

		Asian stock							Western stock																		Eastern stock						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Asian stock	1	*	0.014	0.025	0.014	0.017	0.025	0.011	0.016	0.012	0.018	0.010	0.021	0.031	0.013	0.016	0.018	0.030	0.015	0.017	0.023	0.029	0.010	0.017	0.029	0.019	0.052	0.053	0.032	0.096	0.094	0.077	0.072
	2	**0.010**	*	0.002	0.001	0.020	0.005	0.014	0.025	0.005	0.017	0.019	0.019	0.028	0.019	0.021	0.022	0.029	0.016	0.027	0.030	0.017	0.006	0.032	0.019	0.014	0.055	0.053	0.036	0.103	0.083	0.098	0.084
	3	**0.027**	0.416	*	0.005	0.009	0.007	0.011	0.031	0.014	0.020	0.019	0.025	0.018	0.037	0.027	0.025	0.034	0.023	0.032	0.032	0.023	0.000	0.024	0.022	0.018	0.060	0.026	0.024	0.050	0.099	0.099	0.079
	4	0.072	0.429	0.331	*	0.008	0.003	0.000	0.029	0.004	0.015	0.027	0.010	0.013	0.014	0.016	0.021	0.040	0.014	0.020	0.000	0.015	0.008	0.036	0.007	0.014	0.052	0.049	0.045	0.119	0.110	0.106	0.111
	5	**0.011**	**0.005**	0.185	**0.189**	*	0.000	0.008	0.010	0.000	0.001	0.009	0.005	0.010	0.006	0.004	0.006	0.008	0.006	0.018	0.002	0.005	0.008	0.016	0.015	0.007	0.034	0.025	0.024	0.089	0.060	0.065	0.065
	6	**0.005**	0.254	0.183	0.289	0.345	*	0.004	0.001	0.000	0.007	0.007	0.000	0.017	0.000	0.000	0.003	0.000	0.000	0.000	0.004	0.007	0.000	0.018	0.007	0.000	0.021	0.008	0.007	0.075	0.059	0.055	0.066
	7	**0.025**	**0.009**	0.136	0.558	0.088	0.244	*	0.013	0.001	0.007	0.013	0.014	0.016	0.011	0.008	0.018	0.020	0.012	0.015	0.000	0.016	0.003	0.024	0.011	0.007	0.046	0.029	0.027	0.078	0.080	0.067	0.064
Western stock	8	<u>**0.002**</u>	<u>**<0.001**</u>	**0.009**	**0.002**	0.071	0.359	**0.013**	*	0.000	0.010	0.000	0.005	0.011	0.000	0.000	0.002	0.004	0.013	0.000	0.009	0.005	0.003	0.013	0.015	0.000	0.022	0.028	0.022	0.086	0.056	0.054	0.047
	9	0.058	0.232	0.141	0.297	0.476	0.731	0.341	0.745	*	0.002	0.001	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.013	0.009	0.011	0.068	0.048	0.050	0.048
	10	**0.001**	**0.001**	**0.048**	0.060	0.359	0.170	0.072	**0.035**	0.329	*	0.003	0.007	0.024	0.009	0.009	0.010	0.010	0.013	0.006	0.006	0.009	0.012	0.016	0.017	0.005	0.035	0.033	0.023	0.086	0.056	0.072	0.067
	11	**0.035**	**0.002**	0.067	**0.011**	0.065	0.171	**0.010**	0.665	0.372	0.229	*	0.008	0.020	0.006	0.001	0.004	0.006	0.007	0.000	0.014	0.008	0.000	0.005	0.019	0.004	0.023	0.019	0.016	0.058	0.056	0.050	0.038
	12	<0.001	**0.002**	**0.031**	0.145	0.212	0.803	**0.014**	0.181	0.974	0.097	**0.069**	*	0.009	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.004	0.000	0.010	0.006	0.001	0.012	0.029	0.022	0.081	0.061	0.055	0.065
	13	**0.006**	**0.011**	0.124	0.171	0.136	0.058	0.058	0.133	0.641	**0.019**	**0.038**	0.192	*	0.005	0.005	0.020	0.025	0.018	0.017	0.009	0.023	0.000	0.039	0.013	0.011	0.045	0.035	0.048	0.121	0.100	0.091	0.086
	14	0.078	**0.031**	**0.017**	0.155	0.213	0.744	0.096	0.855	0.699	0.134	0.211	0.886	0.271	*	0.000	0.000	0.000	0.000	0.000	0.001	0.004	0.000	0.012	0.010	0.000	0.006	0.031	0.024	0.096	0.041	0.057	0.070
	15	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.008**	**0.031**	0.161	0.629	**0.018**	0.562	0.930	**0.012**	0.326	0.575	0.247	0.904	*	0.001	0.001	0.000	0.000	0.003	0.010	0.000	0.012	0.014	0.001	0.023	0.028	0.026	0.085	0.058	0.058	0.059
	16	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.013**	**0.011**	0.109	0.360	<u>**<0.001**</u>	0.316	0.689	**0.004**	0.122	0.612	**0.030**	0.718	0.196	*	0.005	0.000	0.000	0.000	0.003	0.000	0.012	0.015	0.004	0.014	0.028	0.018	0.071	0.041	0.055	0.055
	17	<u>**<0.001**</u>	<0.001	**0.013**	<u>**<0.001**</u>	0.140	0.407	**0.009**	0.297	0.179	0.079	0.207	0.191	**0.025**	0.468	0.416	0.210	*	0.000	0.003	0.015	0.008	0.000	0.008	0.029	0.005	0.017	0.023	0.013	0.072	0.038	0.047	0.050
	18	**0.009**	**0.009**	**0.034**	0.091	0.151	0.755	**0.023**	**0.014**	0.698	**0.015**	0.114	0.597	0.055	0.738	0.805	0.628	0.529	*	0.004	0.012	0.010	0.000	0.007	0.019	0.004	0.016	0.023	0.022	0.066	0.036	0.063	0.066
	19	0.059	**0.014**	0.063	0.111	0.064	0.390	0.078	0.720	0.557	0.264	0.468	0.664	0.148	0.773	0.522	0.693	0.412	0.344	*	0.000	0.009	0.000	0.009	0.024	0.002	0.033	0.038	0.031	0.096	0.062	0.074	0.081
	20	0.137	0.093	0.174	0.678	0.358	0.229	0.652	0.306	0.504	0.322	0.236	0.764	0.232	0.383	0.385	0.560	0.243	0.258	0.728	*	0.000	0.003	0.021	0.000	0.000	0.000	0.007	0.008	0.054	0.029	0.044	0.063
	21	<u>**<0.001**</u>	**0.002**	**0.032**	0.062	0.164	0.175	**0.003**	0.131	0.571	**0.049**	0.057	0.188	**0.022**	0.281	**0.006**	0.188	0.137	**0.036**	0.188	0.456	*	0.009	0.031	0.005	0.002	0.019	0.025	0.022	0.077	0.039	0.054	0.054
	22	0.119	0.219	0.438	0.181	0.145	0.657	0.260	0.283	0.743	0.071	0.483	0.469	0.706	0.547	0.894	0.635	0.533	0.960	0.418	0.274	0.135	*	0.005	0.002	0.000	0.016	0.010	0.016	0.061	0.048	0.055	0.049
	23	**0.039**	**0.003**	0.070	**0.014**	0.061	0.058	**0.011**	0.087	0.147	0.057	0.266	0.177	0.007	0.197	0.059	0.071	0.265	0.237	0.313	0.186	**0.003**	0.284	*	0.044	0.012	0.031	0.033	0.017	0.074	0.064	0.066	0.067
	24	<u>**<0.001**</u>	<0.001	**0.036**	0.189	**0.015**	0.149	**0.021**	**0.004**	0.549	**0.002**	<0.001	0.131	0.090	0.121	<u>**<0.001**</u>	<u>**<0.001**</u>	<0.001	**0.002**	**0.017**	0.600	0.125	0.304	<u>**<0.001**</u>	*	0.006	0.015	0.022	0.015	0.070	0.050	0.054	0.052
	25	<u>**<0.001**</u>	<0.001	**0.046**	**0.048**	0.080	0.961	**0.032**	0.513	0.921	0.070	0.139	0.385	0.119	0.651	0.290	**0.033**	0.166	0.181	0.391	0.637	0.268	0.790	0.079	**0.042**	*	0.014	0.015	0.011	0.072	0.044	0.044	0.046
Eastern stock	26	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.004**	<u>**<0.001**</u>	**0.002**	**0.038**	<u>**<0.001**</u>	**0.015**	0.093	<u>**<0.001**</u>	**0.010**	0.104	**0.002**	0.274	**0.004**	**0.050**	0.071	0.067	**0.041**	0.642	**0.031**	0.069	**0.022**	0.051	**0.044**	*	0.005	0.000	0.053	0.007	0.011	0.020
	27	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.021**	<u>**<0.001**</u>	<u>**<0.001**</u>	0.158	<u>**<0.001**</u>	<u>**<0.001**</u>	0.086	<u>**<0.001**</u>	**0.001**	<u>**<0.001**</u>	**0.004**	**0.003**	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.004**	<0.001	**0.001**	0.337	<u>**<0.001**</u>	0.113	**0.001**	<u>**<0.001**</u>	<u>**<0.001**</u>	0.239	*	0.000	0.024	0.024	0.011	0.015
	28	<0.001	<u>**<0.001**</u>	**0.026**	**0.001**	**0.007**	0.126	**0.003**	**0.009**	0.091	**0.006**	**0.036**	**0.009**	**0.001**	**0.029**	**0.001**	**0.014**	0.088	**0.008**	**0.038**	0.225	**0.013**	**0.038**	0.062	**0.038**	0.067	0.762	0.915	*	0.022	0.009	0.000	0.000
	29	<u>**<0.001**</u>	<u>**<0.001**</u>	<0.001	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<0.001	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.048**	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.002**	**0.008**	**0.040**	*	0.056	0.055	0.040
	30	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.001**	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.001**	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.015**	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.006**	**0.002**	**0.004**	0.155	**0.001**	**0.002**	**0.001**	<u>**<0.001**</u>	<u>**<0.001**</u>	0.321	**0.023**	0.186	**0.002**	*	0.046	0.033
	31	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.046**	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	0.129	**0.025**	0.629	<u>**<0.001**</u>	<u>**<0.001**</u>	*	0.005
	32	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<0.001	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	**0.015**	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	<u>**<0.001**</u>	0.053	**0.006**	0.474	<u>**<0.001**</u>	**0.009**	0.199	*