

Lack of genetic evidence for the subspeciation of *Pisaster ochraceus* (Echinodermata: Asteroidea) in the north-eastern Pacific Ocean

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The existence of two Pisaster ochraceus subspecies has been proposed; one located northwards (P. ochraceus ochraceus) and the other southwards (Pisaster ochraceus segnis) from Point Conception. We used polymerase chain reaction–restriction fragment length polymorphism of the CO I and CO III mitochondrial genes to assess the degree of population structure from 126 samples collected along the western coast of North America, from Vancouver, Canada to Punta San Carlos, of Baja California, Mexico. The genetic structure was tested through molecular analysis of variance and by Monte Carlo simulations of the original data set. The phylogeographical pattern was deduced from a minimum spanning network analysis. No genetic structure was detected. Instead, a high degree of genetic homogeneity along the species distribution was evident from haplotype frequencies at each location. Two haplotypes, Po1 and Po5, were predominant along the distribution and were considered ancestral because of their central position in the minimum spanning network. Since Pisaster ochraceus depicts a planktotrophic larval stage with very long duration before settlement, it seems to be able to surpass the biogeographical boundary that limits other species around Point Conception, thereby maintaining homogeneity of its genetic pool. Results of this study recognize P. ochraceus as a single species.

Keywords: biogeographical provinces, invertebrate larval transport, metapopulation, mitochondrial DNA, *Pisaster ochraceus*, RFLP, starfish

Submitted 13 February 2007; accepted 7 December 2007

INTRODUCTION

Point Conception, in California, USA, located at 34.5°N, has been considered a boundary between the Oregonian province and the Californian biogeographical provinces (Briggs, 1974; Doyle, 1985; Gobalet, 2000). However, there are species whose distribution range includes both provinces (Newell, 1948; Briggs, 1974; Hayden & Dolan, 1976; Horn & Allen, 1978; Newman, 1979). Therefore, this boundary is referred to by other authors (Seappy & Littler, 1980) as a transitional zone. Burton (1998) considers that, in the marine environment, detailed research is necessary to clarify the hypothetical concordance of the biogeographical and phylogeographical boundaries originally proposed by Avise *et al.* (1987) and Avise (1992). The complex interaction of the oceanographic variables may favour dispersion and recruitment of some species with planktonic larval stages highly capable of remaining in the pelagic region, but their dispersion might be otherwise limited by seasonal and local oceanographic events.

The starfish *Pisaster ochraceus* is distributed along the north-western coast of North America, occupying both the Oregonian and the Californian provinces. Its northern limit is considered to be in the area of Prince William Sound (Alaska) and the southern at Cedros Island (Baja California, Mexico) (Lambert, 2000). It is commonly found in the intertidal and rocky subtidal zones, where it feeds mainly on mussels, crustaceans and algae. Ecologically, this species plays an important role as it impacts the structure and diversity of the intertidal community, particularly over the populations of the mussel *Mytilus californianus* that constitutes its preferred prey (Ricketts & Calvin, 1952; Paine, 1966; Mauzey *et al.*, 1968).

Pisaster ochraceus undergoes external fertilization and the fertilized eggs produce after approximately six days a planktotrophic larva called bipinnaria. These larvae can survive between 76 and 228 d (Strathman, 1978). Afterwards, they present the brachiolaria phase, which develops up to the juvenile star (Carefoot, 1977). In California, the reproductive cycle of the populations of *P. ochraceus* has been described by Pearse & Eemisee (1982). The spawning season lasts from March to May; the gonadic indices are low until October, then they begin to increase until the following spring when the next spawning takes place.

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The genus *Pisaster* (species, subspecies, forms and/or varieties) exhibits considerable morphological variation within and among its populations (Fisher, 1930). This author suggested eight taxa, including four of them on the *P. ochraceus* complex, by establishing synonymies between some of the 13 taxa previously recognized by Verrill (1914). Later, Clark (1996) recognized only three species in the genus and four subspecies, distinguishing two *P. ochraceus* subspecies according to their distribution: *P. ochraceus ochraceus* from Alaska to Point Conception, in California, and *P. ochraceus segnis* from the northern end to the middle portion of the Baja California Peninsula. However, Lambert (2000) recently recognized *P. ochraceus* as a single species whose distribution ranges from south-eastern Alaska to Cedros Island, in Baja California, Mexico.

The aforementioned reviews of *P. ochraceus* considered exclusively morphological characters. The research of Stickle *et al.* (1992) is the only one based on molecular genetic markers; they used allozymes and found genetic homogeneity among populations in their northern distribution range, supporting the taxonomic classification proposed by Lambert (2000).

In this paper, we used a different molecular marker and extended the area to include the southern populations of *P. ochraceus*. We tested for the Point Conception hypothetical breakdown in the connectivity between the populations from the Oregonian and Californian biogeographical provinces by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) of the mitochondrial partial genes CO I and CO III.

MATERIALS AND METHODS

Tube feet tissue of *Pisaster ochraceus* (approximately 200 mg) was cut from field or museum specimens representing 17 localities along the north-western coast of North America, between British Columbia, Canada, and Baja California,

Mexico. Additionally, we obtained tissue from some *Pisaster giganteus* specimens at three localities to be used as an external group in phylogenetic analyses (Table 1; Figure 1). The tissue was obtained by cutting the tip of an arm with sterilized scissors and storing it in Eppendorf tubes with 70% ethanol for preservation. Tissue from museum specimens was donated by the Natural History Museum of Los Angeles in California.

Prior to DNA extraction, ethanol was removed and samples were re-hydrated for 12 h in distilled, deionized and sterilized water. The salt extraction method described by Miller *et al.* (1988) was used with slight modifications. Briefly, 50–100 mg of tissue was homogenized in 400 µl of salt extraction buffer (200 mM Tris–HCl pH 7.4, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) and incubated at room temperature under agitation for 15–30 minutes. Cellular debris was eliminated by centrifugation and the supernatant was recovered in another tube. The supernatant was mixed with 250 µl of preheated CTAB buffer (100 mM Tris–HCl pH 8.0, 20 mM EDTA pH 8.0, 20% CTAB, 0.1% PVPP, 0.1% SDS, 0.2% mercaptoethanol). The mixture was incubated at 65°C for 10 minutes and centrifuged at 12000 × g for 5 minutes. Purification of DNA was done in one volume of chloroform and precipitated with one isopropanol volume. The pellet was washed with a volume of cold 70% ethanol, dried, re-suspended in 50 µl of 0.1 mM TE pH 8.0 and stored at –70°C. FastPCR v. 3.1.32 beta software (Kalendar, 2004) was used to design primers from the complete mtDNA sequence of *P. ochraceus* (GeneBank Accession No. NC 004610; Smith *et al.*, 1990), targeting a 1386 base pair (bp) fragment of CO I and a 739 bp fragment of CO III. Primer sequences were: PocCOI-f 5'-tgagctggcat gataggcacc 3' and PocCOI-r 5'-ttcagggtgataggggttcg-3'; and PocCOIII-f 5'-accaacatcataccactgg-3' and PocCOIII-r 5'-agttagacaacatctacgaagtgc-3'. PCR reactions were performed in 50 µl of a mixture of 22 mM Tris–HCl, 55 mM KCl, 220 mM dNTPs, 1.55 mM MgCl₂, 150 nM forward primer, 150 nM reverse primer, 0.5u of Taq polymerase and 15 ng of DNA template. Mitochondrial partial CO I and CO

Table 1. Code, location, dates and size of the samples collected for the phylogeographical study of *Pisaster ochraceus*. The number of *P. giganteus* specimens used in the analysis is shown in parentheses.

Code	Sampling location	Sample size	Geographical position		Sampling dates	
			Latitude N	Longitude W		
1	VAN	Vancouver, British Columbia, Canada	16	49° 18.2'	122° 57.4'	10 May, 2004
2	VIC	Victoria, British Columbia, Canada	3	48° 26.9'	123° 20.9'	12 May, 2004
3	TB	Tillamook Bay, Oregon, USA*	4	45° 35.0'	123° 57.0'	30 June, 1994
4	CF	Cape Ferrello, Oregon, USA*	2	42° 06.0'	124° 20.0'	2 July, 1991
5	CY	Cayucos, California, USA*	6	35° 26.8'	120° 54.5'	18 & 19 December, 1998
6	DC	Diablo Canyon, California, USA	2	35° 12.5'	120° 51.4'	22 June, 1997
7	JA	Jalama, California, USA	11	34° 29.9'	120° 29.5'	24 September, 2003
8	COP	Coal Oil Point, California, USA	11	34° 29.3'	119° 52.8'	23 September, 2003
9	PVP	Palos Verdes Peninsula, California, USA*	2	33° 42.8'	118° 18.9'	18 January, 1999
10	SNI	San Nicolas Island, California, USA*		33° 12.8'	119° 27.5'	No information
11	WB	Westward Beach, California, USA*	1	33° 59'	118° 48'	30 December, 2000
12	SM	San Miguel, Baja California, Mexico	12	31° 53.7'	116° 43.9'	17, 23 & 24 October, 2003
13	CK	Campo Kennedy, Baja California, Mexico	13	31° 46.6'	116° 43.6'	20 August & 29 October, 2003
14	ER	Erendira, Baja California, Mexico	4	31° 16.5'	116° 22.9'	24 October, 2003
15	CH	La Chorera, Baja California, Mexico	10	30° 28.1'	116° 02.7'	21 February, 2004
16	PB	Punta Baja, Baja California, Mexico	16	29° 56.9'	115° 48.7'	19 December, 2003
17	PSC	Punta San Carlos, Baja California, Mexico	13	29° 37.5'	115° 28.9'	21 February, 2004

*donation samples.

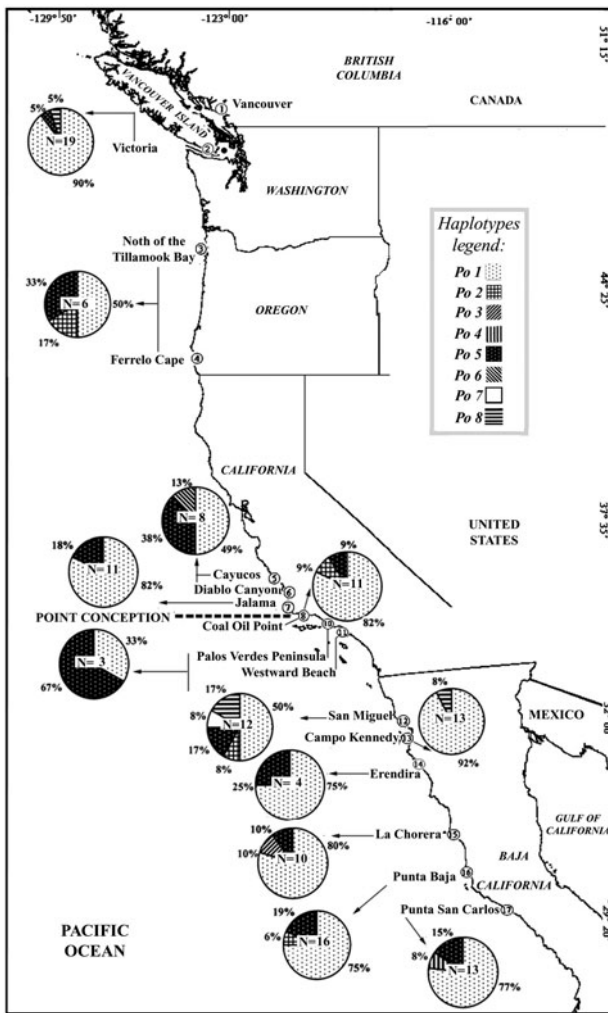


Fig. 1. Study sites, sample size (N), and haplotype frequencies distribution along the north-eastern Pacific Coast for *Pisaster ochraceus*.

III genes were amplified in a BIO-RAD iCycler 3.021 thermocycler through the following profile: initial denaturalization at 95°C for 4 minutes; 35 cycles of denaturalization at 94°C for 45 seconds, annealing at 59°C for 45 seconds, extension at 72°C for 1.5 minutes and a final extension step at 72°C for 7 minutes. Amplification products of both partial genes were sequenced for a single specimen in an ABI-Prism 3100 capillary automatized sequencer and deposited in the GenBank (Accession Nos. DQ021905 and DQ021906) and used to select the potential endonucleases for RFLP analysis. The endonucleases selected were *Mbo I*, *Bfa I*, *Nci I* and *Mnl I* for CO I, and *Nla III* and *Tsp509 I* for CO III. Digestion reactions of PCR products were carried out in a final volume of 10 µl following the manufacturer’s instructions. The products of enzymatic digestions were analysed through 10% PAGE against a 50 bp molecular weight marker.

Statistical analysis of the compound haplotypes distribution was performed through an analysis of molecular variance (AMOVA) to obtain the fixation indices *Fst* and ϕ_{st} , using 1000 permutations. For this analysis, localities were grouped into provinces, with Point Conception as the limit between them. The geographical homogeneity in haplotype frequencies was also evaluated with Monte Carlo

simulations as described by Roff & Bentzen (1989). According to this method, a large number of randomizations (in this case 1000) of the original data set must be generated, subject to the constraint that the original row and column totals remain equal to the original data matrix; the *P* value is equal to the frequency of χ^2 statistics that exceed the original statistic. A MATLAB procedure was developed for this purpose. The phylogeographical pattern was assessed through the construction of a minimum spanning network (MSN) using the Jukes and Cantor method to compute genetic distances; this and the AMOVA tests were conducted with Arlequin version 2.0 software (Schneider *et al.*, 2000).

RESULTS

The RFLP patterns obtained from the digestion of the two partial gene amplicons from 126 *Pisaster ochraceus* specimens consisted of eight compound haplotypes and six *Pisaster giganteus* specimens of three compound haplotypes (Table 2; Figure 1). Frequency distribution of haplotypes pointed out two predominant haplotypes: *Po1* and *Po5*; the former was present at all localities and the latter was absent only at Campo Kennedy. *Po2* and *Po8* are the haplotypes following in importance, since they are present at both sites north and south of Point Conception. The rest of the haplotypes (*Po3*, *Po4*, *Po6* and *Po7*) were specific to some locality and rare. San Miguel, in Baja California, exhibited the largest number of haplotypes, where *Po7* was specific.

For the hierarchical AMOVA, localities were grouped into north and south populations, with Point Conception as the limit between them, thereby representing each biogeographical province. Most of the molecular variance was explained within province differences (98.86% for *Fst* and 97.87% for ϕ_{st}). Therefore, no genetic differentiation was evident between provinces (*Fst* = 0.011, *P* = 0.217 and ϕ_{st} = 0.021, *P* = 0.133). The Roff & Bentzen (1989) algorithm confirmed the homogeneity in the haplotype frequencies between provinces (*P* = 0.369). The *P* values for the Oregonian and the Californian provinces were 0.069 and 0.479 respectively. At α = 0.05, the null hypothesis of no significant geographical

Table 2. Number of composite haplotypes found per location or couple of locations (abbreviations as in Table 1).

Sites	Haplotypes							
	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8
Oregonian								
VAN + VIC	17	0	0	0	1	0	0	1
TB + CF	3	1	0	0	2	0	0	0
CY + DC	4	0	0	0	3	1	0	0
JA	9	0	0	0	2	0	0	0
Californian								
COP	9	1	0	0	1	0	0	0
PVP + SNI	1	0	0	0	2	0	0	0
SM	6	1	0	0	2	0	1	2
CK	12	0	0	0	0	0	0	1
ER	3	0	0	0	1	0	0	0
CH	8	0	1	0	1	0	0	0
PB	12	1	0	0	3	0	0	0
PS	10	0	0	1	2	0	0	0

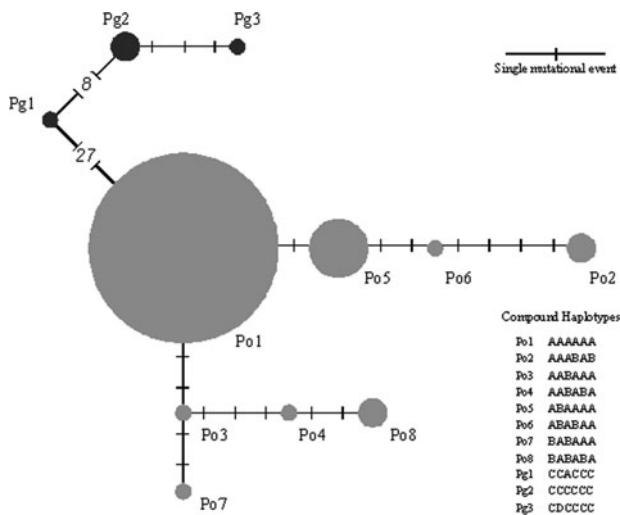


Fig. 2. Minimum spanning network for the polymerase chain reaction-restriction fragment length polymorphism analysis of CO I and CO III mitochondrial gene fragments for *Pisaster ochraceus*. The area of circle of each haplotype is proportional to the frequency of this haplotype. Genetic distances were computed according to the Jukes and Cantor method. Circles in black represent the *P. giganteus* haplotypes.

variation within each province cannot be rejected, but the Oregonian province shows a higher genetic difference, mainly due to the frequency distribution of *Po1*.

Minimum spanning network topology for *P. ochraceus* haplotypes is shown in Figure 2. Haplotype *Po1* showed the highest frequency and occupied a central position, from which the rest of the haplotypes derive by one to four mutational events. *Pisaster giganteus* haplotypes are also linked to the most common *P. ochraceus* haplotype, but through 27 mutational events, representing a clear polarized and independent group.

Therefore, we found no evidence suggesting a phylogeographical pattern along the sampling zone. Haplotypes *Po1*, *Po2*, *Po3*, *Po4*, *Po5*, *Po7* and *Po8* were present south of Point Conception, whereas *Po1*, *Po2*, *Po5*, *Po6* and *Po8* were found north of it, indicating a slightly larger diversity for the Californian province.

DISCUSSION

Pisaster ochraceus populations were found to exhibit high genetic homogeneity along the study area. Similar patterns have been described for other echinoderm species, such as the purple urchin *Strongylocentrotus purpuratus*, where the most common haplotypes were shared by all populations throughout its geographical range (Flowers *et al.*, 2002). Such homogeneity could be explained by high rates of genetic flow among populations of these species as a consequence of the dispersal capacity of their planktotrophic larvae. Therefore, the chance for dispersal, settlement, and effective recruitment into other areas besides that of origin will be greater for larvae of the most common haplotypes. Dispersal capabilities of larvae are associated with the time of planktotrophic larvae in the water column before their settlement, the fertility rate of the adult organisms, the spawning season, and the area where the latter takes place, coupled to physical factors, such as oceanic currents, upwellings, and

local or regional gyres (Dawson, 2001; Wares *et al.*, 2001; Cowen *et al.*, 2002; Hohenlohe, 2004).

Particularly, *P. ochraceus* is a highly fertile species (Lambert, 2000), with a spawning season from March to May (Farmanfarmanian *et al.*, 1958; Boolotian, 1966; Pearse & Eernisee, 1982). The planktotrophic larvae may remain in the water column for more than six months (Strathman, 1978), a period that allows them to initiate metamorphosis only when they find the appropriate conditions of substrate and/or nourishment (George, 1999; Crawford & Jackson, 2002).

Therefore, it is very likely that these larvae have a dispersal capacity ranging from a few metres up to hundreds of kilometres along their distribution, if the oceanographic and environmental conditions are favourable. Coinciding with the spawning season, the California Current system presents, in spring, a marked southward tendency (Strub & James, 2000). The previous scenario may explain the high degree of homogeneity found in the *P. ochraceus* populations north and south of Point Conception, from Vancouver to Baja California.

Dawson (2001), in his compilation of phylogeographical studies carried out along the coast of California, published an important number of invertebrate taxa with larva stages remaining for more than 4 weeks in the plankton and with high fertility rates, whose populations do not present genetic differentiation through Point Conception. In particular, no phylogeographical structure has been found for the *Colisella digitalis* and *C. austrodigitalis* (Murphy, 1978) limpets, the mussel *Mytilus californianus* (Levinton & Suchanek, 1978), the sea star *Ecasterias troschelli* (Stickle *et al.*, 1992) and the coral *Paracyathus stearsii* (Beauchamp & Powers, 1996; Hellberg, 1996).

Nevertheless, there are examples of species whose populations do present a strong phylogeographical signal along the coast of the north-eastern Pacific, as in the case of the sea stars *Leptasterias hexactis* (Kwast *et al.*, 1990) and *Leptasteria epichlora* (Stickle *et al.*, 1992), and the gastropod *Nucella emarginata* (Wares *et al.*, 2001). These species have larval stages that are incubated by the adults or that cannot disperse large distances due to their permanence time in the plankton.

Analysing the minimum spanning network, one may assume that the haplotypes *Po1* and *Po5* are the oldest, since they are the ones that have had the opportunity to accumulate the greater number of copies (Donnelly & Tavaré, 1986; Crandall & Templeton, 1993; Excoffier & Smouse, 1994), whereas the new haplotypes are derived from the haplotypes with larger frequencies. Particularly, *Po1* seems to be the ancestral haplotype, since it is the one that connects with the external group. The presence of specific alleles (*Po3*, *Po4*, *Po6* and *Po7*), found in some populations, might be due to their recent genetic differentiation; however, more specific analyses must be performed to test this assumption.

In summary, the results of this study seem to fit the second hypothesis proposed by Avise (2000, p. 136), which states that 'species with life histories conducive to dispersal generally have less phylogeographic population structure'. The high planktonic duration stage, high fertility, and the spawning season of the species, coupled to the circulation pattern of the California Current System, are the main factors determining the homogeneity in the genetic structure of populations. Given the genetic continuity, as shown through the RFLP

study of a 2125 bp fragment of the mitochondrial DNA of *P. ochraceus*, we propose to discard the supposed subspecies *P. ochraceus ochraceus* and *P. ochraceus segnis*. Therefore, results of this study recognize *P. ochraceus* as a single species, as established also by Stickle *et al.* (1992) and Lambert (2000).

ACKNOWLEDGEMENTS

The collection of starfish tissue in their diverse stages was achieved with the help of many people. The authors are deeply grateful to: A. Giles, V. Rodríguez, M. Neocoechea, A. Ortíz, D. Guzmán, K. Selkoe, R. Beas, H. Serrano and A.L. González, from Ensenada, Baja California; to Dr Blanchette and A. Wyndham from the PISCO team of the University of California at Santa Barbara; to B. Leighton from the Simon Fraser University, and to Dr P. Lambert, curator of the Royal British Columbia Museum, Canada. We also thank Dr G. Hendler, curator of the Natural History Museum of Los Angeles, for his kind donation and Leslie Harris for her hospitality. This project was financed by CONACYT (AMELIS Project J37689 and SEP-CONACYT project J50046 to L.B.L.) and UC MEXUS (Key Intertidal Species Project to L.B.L.); the first author received a grant from CONACYT (No. 168156). We gratefully acknowledge the two anonymous referees for their helpful comments on the manuscript.

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