Freshwater Biology

Freshwater Biology (2012) **57,** 1823–1838 doi:10.1111/j.1365-2427.2012.02841.x

The signal crayfish is not a single species: cryptic diversity and invasions in the Pacific Northwest range of *Pacifastacus leniusculus*

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SUMMARY

1. We used historical sources, morphology-based taxonomy and mtDNA sequence data to address questions about the signal crayfish *Pacifastacus leniusculus*. These included evaluating unrecognised cryptic diversity and investigating the extent to which *P. leniusculus* may have been introduced within its presumed native range in the Pacific Northwest region of North America. Our study builds and expands on Pacific Northwest phylogeographic knowledge, particularly related to patterns of glacial refugia for freshwater species.

2. Extensive collections (824 specimens) from British Columbia (Canada), Idaho, Nevada, Oregon and Washington (United States) were used to characterise *P. leniusculus* at the mitochondrial 16S rRNA gene. Genetic groups within the species were elucidated by phylogenetics and AMOVA; evolutionary relationships within the most common and diverse group were investigated using a statistical parsimony haplotype network, a nested AMOVA, and tests of isolation by distance. Morphological measurements were used to relate findings of molecular analyses to three historically recognised *P. leniusculus* subspecies and characterise cryptic diversity by morphology.

3. We found substantial cryptic diversity, with three groups highly distinct from *P. leniusculus* in discrete geographic regions: the Chehalis River glacial refugium, Central Oregon and the Okanagan Plateau. Disjunct distributions of *P. leniusculus* relative to these cryptic groups and known patterns of Pleistocene glaciation and landscape evolution cast doubt on whether *P. leniusculus* is native to some areas such as coastal drainages of northern Washington and southern British Columbia. Morphological traits previously used to characterise *P. leniusculus* subspecies still persist but may be incapable of distinguishing *P. leniusculus* from newly discovered cryptic groups.

4. Cryptic diversity found within *P. leniusculus* highlights the pressing need for a thorough investigation of the genus *Pacifastacus* using data based on more extensive gene and taxon sampling. It also warrants conservation attention, as introductions of *P. leniusculus* within the Pacific Northwest may carry risks of hybridisation and introgression for cryptic groups. Owing to high genetic diversity and limited dispersal capacity relative to more vagile organisms like freshwater fish, crayfish of the genus *Pacifastacus* offer powerful potential insights into the geological history and phylogeography of the Pacific Northwest region. Finally, by shedding light

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on the long-neglected native range of *P. leniusculus*, our results should also better inform our understanding of potential source populations for, and the ecology of, this important invasive species in regions including Europe, Japan and elsewhere in North America.

Keywords: crayfish, mtDNA, *Pacifastacus leniusculus*, Pacific Northwest, phylogeography

Introduction

The Pacific Northwest of North America has been a focus of phylogeographical research, because of the region's complex but well-studied geological history and location straddling the last glacial maximum (Soltis *et al*., 1997; Brunsfeld *et al*., 2001). Despite more than one hundred peer-reviewed phylogeographical studies conducted in the Pacific Northwest over the past decade (Schafer *et al*., 2010), considerable taxonomic bias is evident, with only four studies on freshwater fish and one on a freshwater invertebrate (*Daphnia*). Little work has addressed the phylogeography of crayfish in this region (but see Sonntag, 2006), even though crayfish are among the most globally endangered taxonomic groups, numerous molecular studies have found high levels of cryptic diversity within crayfish and crayfish have proved useful in reconstructing glacial refugia, palaeodrainages and stream capture events (Hughes & Hillyer, 2003; Trontelj, Machino & Sket, 2005; Apte, Smith & Wallis, 2007).

Although species-poor relative to crayfish diversity hotspots like Australia and the southeastern United States (Crandall & Buhay, 2008), the Pacific Northwest is native to four extant species in the endemic genus *Pacifastacus* (Astacidae, Decapoda). This includes the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), a widespread and commercially harvested crayfish that is also a major invasive species in Asia, Europe and elsewhere in North America (Lodge *et al*., 2000; Larson, Olden & Usio, 2010). Despite its prominent profile as an invader, *P. leniusculus* is poorly known from its native range (Larson & Olden, 2011). We propose that *P. leniusculus* may be a valuable organism for exploring freshwater phylogeography in the Pacific Northwest owing to its large geographic range and highly restricted dispersal ability relative to more vagile organisms like fish or aquatic insects with flying adults (Hughes & Hillyer, 2003; Bentley, Schmidt & Hughes, 2010). The combination of restricted dispersal ability and the isolated, dendritic nature of freshwater ecosystems promotes high levels of genetic structure and diversity. It is important to characterise this diversity not only for conservation purposes but also for the insights it provides into geological history and its effects on contemporary ecological communities (Hughes, Schmidt & Finn, 2009). Furthermore, common but understudied organisms like *P. leniusculus* can offer valuable and divergent insights into ecology and historical biogeography relative to well-studied model organisms (Whiteley, Spruell & Allendorf, 2006).

We used extensive field collections throughout the Pacific Northwest, morphological measurements and mtDNA sequence data to investigate the native range phylogeography of *P. leniusculus* in relation to its historically ambiguous taxonomy and distribution. We sought to elucidate genetic structure within this species, evaluate morphologically based taxonomy and examine the possibility that *P. leniusculus* may have been widely transported and introduced within its presumed native range. In addition, our work broadens the taxonomic portfolio applied to phylogeographic questions in the Pacific Northwest on issues like glacial refugia (Schafer *et al*., 2010) and aimed to provide a broad foundation for future research on this important species.

Methods

Pacifastacus leniusculus

Pacifastacus leniusculus was described initially as three separate species – *P. leniusculus*, *Pacifastacus trowbridgii* (Stimpson, 1857) and *Pacifastacus klamathensis* (Stimpson, 1857) – which confounded taxonomists for a century with their variable and occasionally similar morphology. In a landmark study, Miller (1960) aggregated these species into subspecies on the basis of distinct morphologies at or near type localities and the presence of intergrades elsewhere, a conclusion favoured over a concurrent and contradicting study (Riegel, 1959) by subsequent taxonomic keys (Hobbs, 1972). Miller's (1960) conclusions have persisted into the molecular era without re-evaluation, although the subspecies are widely ignored in contemporary research (but see Sonntag, 2006).

Miller (1960) reported considerable morphological variability both within and between the *P. leniusculus* subspecies that is difficult to summarise briefly (see Appendix S1). To represent the complex and multivariate

nature of these results, we duplicate here the discriminant function axis used by Miller (1960) in characterising seven *P. leniusculus* populations, including those at or near the *P. leniusculus*, *P. trowbridgii* and *P. klamathensis* type localities (Fig. 1; Appendix S2). Miller's (1960) results demonstrated considerable morphological variability separating *P. leniusculus* populations along a distinct *P. l. klamathensis* to *P. l. leniusculus* gradient with an intermediate region of *P. l. trowbridgii* or intergrade morphologies (Fig. 1; Appendix S1). Molecular methodologies offer a powerful capacity to resolve such long-standing taxonomic ambiguities while simultaneously identifying cryptic diversity that can go unrecognised by morphology alone (Bickford *et al.*, 2007; Sweeney *et al.*, 2011).

Miller (1960) also documented the known distributions for all *Pacifastacus* species from early naturalist reports and museum records (Fig. 1). Distributional patterns for the *P. leniusculus* subspecies are apparent, with *P. l. klamathensis* changing from a coastal distribution in northern California and southern Oregon to a distribution east of the Cascade Mountains from northern Oregon to southern British Columbia. *Pacifastacus l. leniusculus* was predominantly reported from the lower Columbia River and its tributaries with a few peripheral populations, and *P. l. trowbridgii* occurred in western Oregon and Washington with erratic occurrences east of the Cascade Mountains.

Miller's (1960) results provide a historical baseline for evaluating *P. leniusculus* subspecies with molecular approaches and identifying patterns of potential human introductions within the Pacific Northwest. As early as 1895, *P. leniusculus* had been introduced within North America to Lake Tahoe (Abrahamsson & Goldman, 1970) and as early as 1912 was being stocked outside its native range in Californian coastal streams and the Sacramento River (Riegel, 1959). These authors refer to Oregon's lower Columbia River and its tributaries as one source for introduced *P. leniusculus*, an intuitive origin owing to the large commercial fishery for this species in Oregon that was active as early as 1893 and peaked with

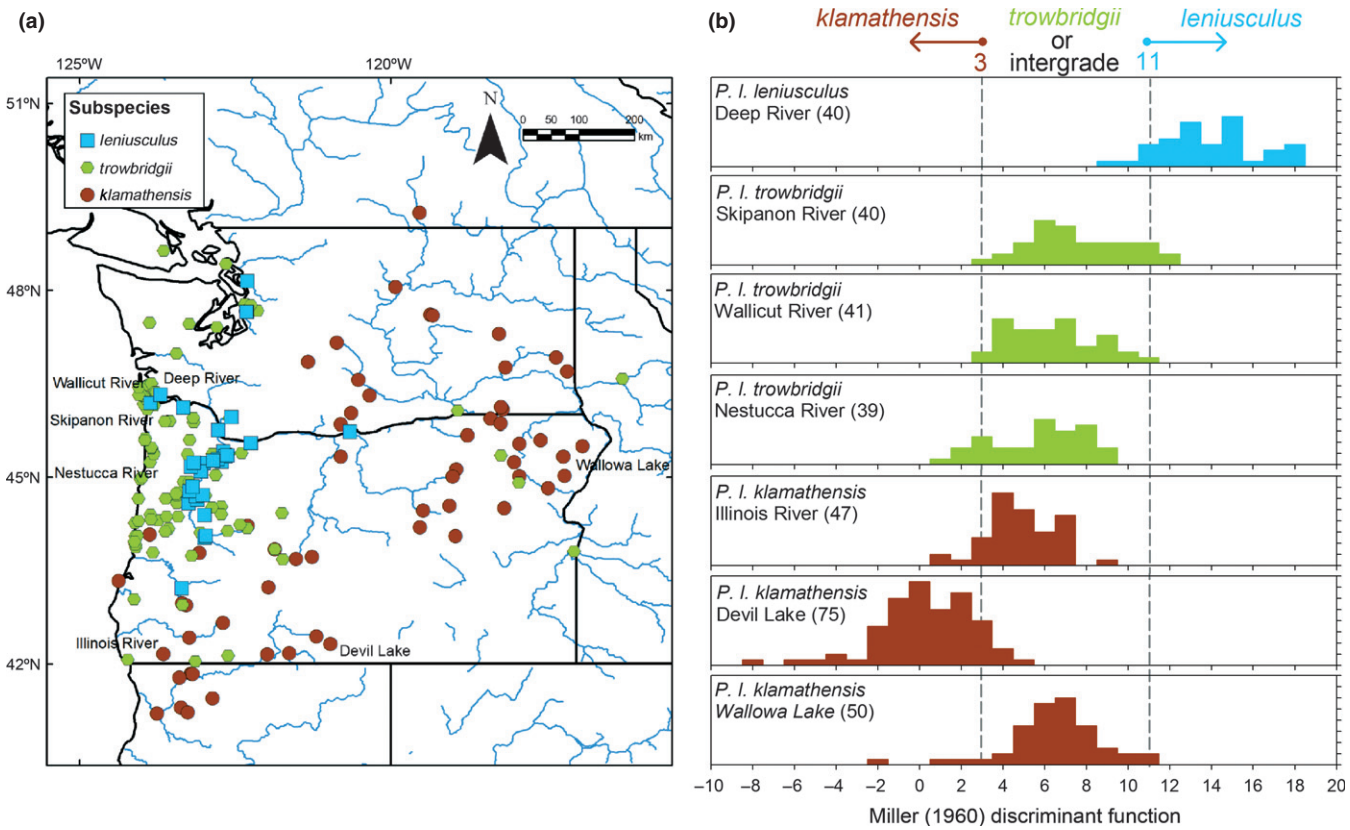

Fig. 1 Distribution of *Pacifastacus leniusculus* subspecies in the Pacific Northwest (a) prior to 1960 reported in Miller (1960), and (b) first axis of a discriminant function analysis (DFA; Appendix S2) classifying *Pacifastacus leniusculus* to subspecies by total length, carapace length, rostrum length and areola length for seven sites in the Pacific Northwest (labelled in a). Miller (1960) DFA values ≤3 are referenced as *P. l. klamathensis*-like morphology and Miller (1960) DFA values ≥11 are referenced as *P. l. leniusculus*-like morphology throughout the manuscript, with intermediate values either *P. l. trowbridgii*-like morphology or ambiguous intergrades.

79 832 kg year^{-1} crayfish harvested by 1930 (Miller & Van Hyning, 1970).

Based on this invasion history, it seems plausible that *P. leniusculus* is also widely introduced within its presumed native range in the Pacific Northwest (Larson & Olden, 2011). Possible introduction pathways include live fishing bait, stocking for harvest, and lake or river management (Lodge *et al.*, 2000). Such invasions near the true native range of a species are common in crayfish and can be as problematic as long distance invasions (Larson & Olden, 2010). In the Pacific Northwest, the presence of *P. leniusculus* in the historically glaciated Canadian province of British Columbia could perhaps be explained by human introductions. For example, Carl & Guiguet (1957) report that *P. leniusculus* was introduced from Oregon to Shawnigan Lake on Vancouver Island between 1908 and 1929, and that crayfish from this Vancouver Island population were subsequently stocked (reportedly unsuccessfully) into Paul Lake near Kamloops on the British Columbia mainland in 1935.

This attempted introduction into the Fraser River drainage may indicate that *P. leniusculus* was not historically widespread on the lower British Columbia mainland. Carl & Guiguet (1957) cite the presence of crayfish in other Vancouver Island rivers as evidence that *P. leniusculus* was native to British Columbia, but Miller's (1960) records for this crayfish in Canada are exceedingly sparse and no *P. leniusculus* specimens from the province at the Royal British Columbia Museum predate 1935 (K. Kuchnow, pers. comm.). Miller (1960) described Vancouver Island *P. leniusculus* as 'intergrade crayfish between *P. trowbridgii* and *P. leniusculus*' that were 'believed to be progeny of these two species or intergrade forms imported from the Columbia River'. Accordingly, it seems plausible that crayfish on Vancouver Island reported by Carl & Guiguet (1957) could represent further stocking and spread from an introduced Shawnigan Lake population. Typical 'invasive' impacts of *P. leniusculus* are even evident on Vancouver Island, where this crayfish is implicated in the collapse of a federally listed three-spined stickleback (*Gasterosteus aculeatus* Linnaeus) species pair in a lake where the crayfish was known to be historically absent (Behm, Ives & Boughman, 2010).

Alternatives to human introductions that could explain *P. leniusculus* occupancy of post-glaciation British Columbia include persistence in a northern coastal glacial refugium (Schafer *et al.*, 2010) or colonisation of the province during and following glacial retreat via stream capture events from southern refugia like the Columbia and Chehalis rivers (McPhail & Lindsey, 1986). Such post-glacial range expansions should produce phylogeographic patterns distinct from those generated by human introductions, which can cause low genetic diversity geographically disjunct from evident source populations (Hughes & Hillyer, 2003; Johnson *et al.*, 2011). Distinguishing patterns of species introductions using molecular information can be difficult (Fitzpatrick *et al.*, 2012), but our combination of historical literature and hypotheses based on regional geological history provides a robust framework for evaluating native and hypothesised invasive ranges.

Finally, while attempting to collect *Pacifastacus connectens* Faxon and *Pacifastacus gambelii* Girard for use as outgroups, we unexpectedly found *P. leniusculus* widespread in the closed desert basins of eastern Oregon and Snake River tributaries of southern Idaho and northern Nevada. These occurrences represent areas from which *P. leniusculus* was historically absent or rare (Miller, 1960; Larson & Olden, 2011), with the occurrence above Shoshone Falls representing an unambiguous introduction, as this is a well-documented barrier in Pacific Northwest freshwater biogeography beyond which this crayfish was never previously known (Miller, 1960; McPhail & Lindsey, 1986). This alternative region of hypothesised *P. leniusculus* introductions provides a useful contrast for evaluating patterns of genetic diversity and structure in British Columbia and the adjacent and similarly glaciated Puget Sound of Washington (hereafter referred to as Salish Sea drainages), as these two remote areas should harbour genetically distinct *P. leniusculus* owing to their wide geographic isolation and disparate geologic histories (glaciated versus non-glaciated).

Sample collection and mtDNA sequencing

We collected 824 *P. leniusculus* specimens between 2006 and 2010 from 63 sites throughout British Columbia, Oregon, Washington, southern Idaho and northern Nevada. We also collected three *P. connectens* individuals from one site in southern Idaho for use as our *a priori* outgroup. DNA was extracted from muscle tissue dissected from the abdomen or walking legs using the DNeasy Tissue kit (Qiagen, Hilden, Germany), with elution buffer pre-heated to 70 °C. DNA was diluted 1 : 10 in ddH_2O, and the 16S rRNA mtDNA gene was PCR-amplified and sequenced using primers 16Sar-L and 16Sbr-H from Imai *et al.* (2004). Genetic work was carried out at the National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan (390 samples) and the Pacific Biological Station, Nanaimo, BC, Canada (427 samples), following standard methods for mtDNA analysis.

Genetic analyses

Our molecular data set consisted of mitochondrial 16S rRNA gene sequences of lengths 437–440 bp from 824 *P. leniusculus* and three *P. connectens* individuals. Sample sizes used in analyses varied with the nature of the technique and the question being addressed, as described in relevant sections below. Sequence quality and base-calling accuracy were evaluated by viewing chromatograms using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). Sequences were aligned in BioEdit (Hall, 1999) using the Clustal W multiple alignment option with default settings; the resulting alignment was verified by visual inspection and imported into GenAlEx version 6.41 (Peakall & Smouse, 2006). Descriptive genetic diversity measures including haplotype diversity, number of polymorphic sites, average number of pairwise differences and gene diversity were generated using Arlequin version 3.5 (Schneider *et al.*, 2000).

Initial exploratory analyses of the whole data set (n = 827) in GenAlEx included a principal co-ordinates (PCO) analysis of pairwise individual-by-individual haploid genetic distances, which uncovered five seemingly distinct genetic clusters that were characterised by subsequent analyses. A minimum evolution phylogenetic tree was constructed in Mega 4 (Tamura *et al.*, 2007) after all identical sequences had been removed from the alignment. This analysis used Kimura's two-parameter model (K2P; Kimura, 1980) with pairwise gaps omitted and robustness testing by bootstrapping (1000 replicates). Total sampled molecular variance was partitioned into four groups suggested by the PCO, excluding *P. connectens* because of small sample size, using an analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) as performed in GenAlEx. Pairwise and global F_{ST} (which is equivalent to the Φ_{PT} given by GenAlEx for haploid data) estimates generated by the AMOVA were used to evaluate the genetic distinctiveness of the clades, with probability values for F_{ST} derived from null distributions generated from 999 random permutations among groups.

The group identified as *P. leniusculus* based on morphology and proximity to the *P. l. leniusculus* type locality (Fig. 1) had the highest genetic diversity of the PCO groups. Excluding *P. connectens*, the other three observed groups were named Chehalis, Central Oregon and Okanagan based on their geographic distributions. We explored evolutionary relationships within the *P. leniusculus* group by constructing a statistical parsimony network using a 95% confidence limit in the program TCS version 1.21 (Clement, Posada & Crandall, 2000). Loops in the network were removed manually following rules based on coalescent theory as described by Pfenninger & Posada (2002).

We used information on geological history of the Pacific Northwest, the natural history of *P. leniusculus* and records of known successful and attempted human translocations to propose introduced regions for this species (see *Pacifastacus leniusculus* section). We hypothesised that these proposed introduced regions would possess low and similar genetic diversity relative to each other in contrast to high and unique genetic diversity of the *P. leniusculus* native range, despite the two introduced regions being separated by over 1000 km and having disparate glaciated (Salish Sea drainages) versus unglaciated geologies. We characterised genetic structure among the native and proposed introduced regions, as well as among and within individual sampled sites, using a nested AMOVA in GenAlEx, excluding sites with fewer than five crayfish because of sample size limitations. We also contrasted our native to proposed introduced regions with isolation by distance, an analysis anticipated to represent better the spatial extent of the two disjunct introduced regions. We predicted trivial isolation by distance for the proposed introduced regions relative to pronounced isolation by distance within the native range. We evaluated isolation by distance with Mantel tests (999 random permutations) in GenAlEx as the haploid genetic distance by linear geographic distance, which we compared between native and proposed introduced ranges using analysis of covariance (ANCOVA).

Morphological analyses

Because of a logistical decision to store specimens grouped by site rather than individually (following tissue dissection for molecular analyses), morphological results could not be paired to genetic results for each crayfish. Consequently, we evaluated our crayfish at the site level for the *P. leniusculus* subspecies morphologies as characterised by Miller (1960; Fig. 1). Five sites that contained both *P. leniusculus* and the Chehalis cryptic group in sympatry were excluded from morphological analysis; at no other sites did we detect our *Pacifastacus* groups co-occurring. We matched Miller's (1960) methodology by making the same set of morphological measurements (Appendix S1) with vernier callipers to the nearest 0.01 mm and only conducted morphological-based analyses on undamaged male crayfish larger than 20 mm carapace length. We used Miller's (1960) formula (Appendix S2) for his single reported discriminant function axis (Fig. 1) to characterise crayfish on the morphological

gradient from *P. l. leniusculus* to *P. l. klamathensis*. We then compared Miller's (1960) model with our own discriminant function analysis on 16 morphological ratios (Appendix S2), which we used to classify crayfish as *P. leniusculus*, Chehalis, Central Oregon or Okanagan. Our contemporary discriminant function analysis was analysed in SPSS (IBM Statistics Version 19, Chicago, IL, U.S.A.) and used morphological measurements evaluated individually by Miller (1960) but excluded from the historical discriminant function analysis, probably due to computational limitations of the time (Appendix S2). Finally, the majority of crayfish specimens used in both molecular and morphological analyses were deposited in the invertebrate collection at the Royal British Columbia Museum, Victoria, Canada.

Results

The 16S mtDNA sequence alignment analysed here was 440 bp in length and contained gaps at three positions. Fifty-seven variable sites defined 82 haplotypes among the 827 sequences generated, three of which were from the putative outgroup species *P. connectens* (GenBank accession JX077131) and the remainder from *P. leniusculus* and cryptic groups (GenBank accession numbers JX077132–JX077955). A minimum evolution tree placed all sequences into five clades (Fig. 2a), similarly supported by a PCO that depicted all groups as unequivocally distinct (Fig. 2c). With respect to putative 'ingroup' clades (i.e. excluding *P. connectens)*, monophyly was supported by bootstrap values for the Chehalis, Central Oregon and Okanagan clades based on a significance threshold of ≥70% as suggested by Hillis & Bull (1993), whereas whether the *P. leniusculus* clade was monophyletic or paraphyletic remained unresolved (Fig. 2a). The branching order among the four ingroup clades and the outgroup was not well resolved; thus, monophyly of the ingroup taxa was not supported or excluded by the current data set. We present a collapsed version of the tree because there was minimal resolution within the major clades (Fig. 2a).

The three sequences and single haplotype from *P. connectens* were excluded from descriptive measures and AMOVA because of the prohibitively low sample size for this species. Descriptive measures indicated a high degree of genetic divergence among the remaining groups (Table 1). AMOVA attributed a large and statistically significant portion of total sampled genetic variance to among-group differences (80%; $P \leq 0.001$); indeed, there were no shared haplotypes among groups, and the average number of pairwise sequence differences between groups was much higher than that within groups (Tables 1 and 2). Pairwise F_{ST} estimates were large and highly significant ($P \leq 0.001$), ranging from 0.72 (between *P. leniusculus* and Chehalis) to 0.95 (between Okanagan and Chehalis; Table 2).

Owing to its central position in both the minimum evolution tree and PCO ordination, *P. connectens* appeared equally divergent from the other four groups as the latter were to each other, suggesting no definable difference between this expected outgroup taxon and other sample groups (Fig. 2). Mindful of their different sample sizes, the Okanagan group appeared to have relatively low diversity whereas the Central Oregon group had relatively high diversity (Table 1). The PCO and haplotype diversity measures show the *P. leniusculus* group to be the most genetically diverse clade. This does not appear to be driven solely by sample size; average pairwise sequence difference within this group was twice as high as for the next highest group, which had the smallest sample size (Central Oregon, $n = 39$), and the frequency of the most common haplotype was lowest in the *P. leniusculus* group (Table 1).

The parsimony network of the *P. leniusculus* group (Fig. 3a) suggests a reasonably close relationship among the majority of haplotypes sampled, with few unsampled haplotypes intervening in most areas of the network. Some notable branches on the network include the following: (i) haplotypes in light orange, collected from the Willamette River and tributaries in the vicinity of Corvallis, Oregon, which are separated from the next closest sample by five unsampled haplotypes; (ii) haplotypes in dark red, collected from Umpqua and Klamath River tributaries, which are separated from other samples by seven unsampled haplotypes; and (iii) haplotypes in light to dark green, collected predominantly from Columbia River tributaries east of the Cascade Mountains and separated from other samples by one to four unsampled haplotypes (Fig. 3).

Nested AMOVA found a significant difference in genetic variance between the native and proposed introduced regions for *P. leniusculus* (11%; $P \leq 0.001$), but the largest proportion of genetic variance occurred among sample sites (73%; $P \leq 0.001$). Mantel tests demonstrated isolation by distance for both native ($r = 0.413$, $P \leq 0.001$) and hypothesised introduced ($r = 0.262$, $P \leq 0.001$) *P. leniusculus* sites. However, ANCOVA revealed that both genetic diversity ($F_{1, 493} = 52.208$, $P \leq 0.001$) and the slope between genetic diversity and geographic distance ($F_{1, 493} = 37.203$, $P \leq 0.001$) were significantly different between native and hypothesised introduced ranges (Fig. 4). Mean haploid genetic distance separated by

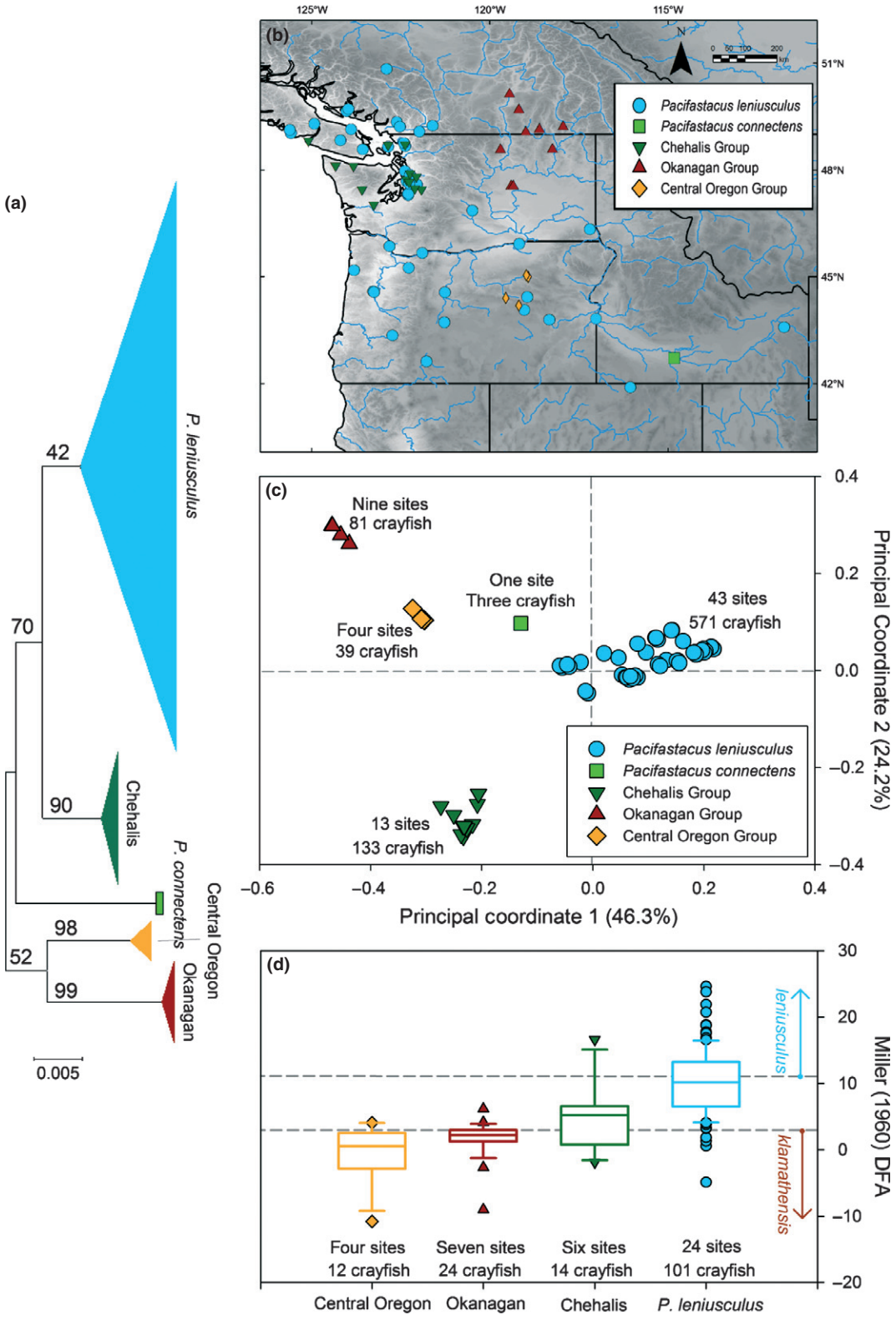

Fig. 2 Minimum evolution tree (a) for *Pacifastacus* crayfish collected in the Pacific Northwest, labelled as *Pacifastacus leniusculus*, the *a priori* outgroup *Pacifastacus connectens* and cryptic groups Chehalis, Central Oregon and Okanagan. The distribution of these *Pacifastacus* groups in the Pacific Northwest region of North America (b). Principal co-ordinates analysis of pairwise genetic distances between all *Pacifastacus* individuals collected in the study region (c). Values for the four *Pacifastacus* groups (d) on Miller's (1960) DFA axis (Fig. 1) with number of sites and individual crayfish available for morphological analysis (see text) provided on the *x*-axis.

Table 1 Number of individuals (with sampled sites in parentheses) and descriptive genetic diversity statistics for four *Pacifastacus* crayfish groups described in this study, excluding *Pacifastacus connectens* because of small sample size

	Pacifastacus leniusculus	Chehalis	Central Oregon	Okanagan
n (sites)	571 (43)	133 (13)	39 (4)	81 (9)
No. haplotypes	56	14	5	6
Most common haplotype (%)	39%	54%	56%	93%
No. polymorphic sites	36	12	7	5
Mean no. pairwise differences:				
Within group (%)	3.38 (0.77%)	0.91 (0.21%)	1.66 (0.38%)	0.17 (0.04%)
With all other groups (%)	12.11 (2.77%)	10.35 (2.37%)	13.52 (3.09%)	14.60 (3.34%)
Gene diversity*	0.83	0.66	0.62	0.14
SD gene diversity	0.01	0.04	0.06	0.05

*Gene diversity is the probability that two randomly chosen haplotypes are different in a given sample.

Table 2 Descriptive statistics of genetic differences between four *Pacifastacus* crayfish groups. Upper diagonal is the average number of sequence differences between groups (% sequence differences in parentheses) and lower diagonal is the pairwise F_{ST} values between groups. All F_{ST} values are statistically significant at $P \leq 0.001$

	Pacifastacus leniusculus	Chehalis	Central Oregon	Okanagan
P. leniusculus		9.7 (2.2%)	14.0 (3.2%)	15.1 (3.5%)
Chehalis	0.72		12.8 (2.9%)	13.5 (3.1%)
Central Oregon	0.78	0.92		11.4 (2.6%)
Okanagan	0.82	0.95	0.94	

1000 km at proposed introduced sites was low relative to genetic haploid distance separated by 200 km in the native range. Average haploid distance was two to four times higher in the native range at the same geographic distances relative to the proposed introduced range (Fig. 4), a split in genetic diversity between these two regions supported by descriptive statistics (Table 3).

Subspecies assignments from Miller's (1960) discriminant function analysis (Fig. 1; Appendix S2) were not evenly distributed among *P. leniusculus* and our three cryptic groups. Central Oregon and Okanagan groups possessed predominantly *klamathensis*-like morphology, the Chehalis group had predominantly *trowbridgii*-like or intermediate morphology, and *P. leniusculus* spanned all three subspecies but most often resembled *trowbridgii* or *leniusculus* (Fig. 2). Our new discriminant function analysis assigned individual crayfish to the four molecular groups with a 90% correct classification rate (Fig. 5a). Common misclassifications included 10% of *P. leniusculus* assigned to Chehalis or Okanagan groups by morphology, and 30% of Chehalis individuals misclassified as *P. leniusculus*. Many morphological attributes identified as important by Miller (1960), like the ratio of acumen length to rostrum width or palm length relative to claw length, were major contributors to our discriminant function analysis (Fig. 5b). Accordingly, the first axis of our discriminant function analysis closely resembled Miller's (1960) single discriminant function axis despite incorporating a greater range of morphological traits (Fig. 5c; Appendix S2).

Discussion

Our study is the first to discover cryptic diversity within *P. leniusculus*, previously unrecognised by both morphology (Miller, 1960) and in past molecular investigations of this species (Agerberg & Jansson, 1995; Sonntag, 2006). We also propose that widespread introductions of *P. leniusculus* may have occurred within the presumed native range of this species, ranging from southeastern Idaho to the Salish Sea drainages of Washington and British Columbia. We confirmed that the range of morphological variability characterising three historical *P. leniusculus* subspecies persists, but that the morphology of some subspecies (*P. l. klamathensis*, *P. l. trowbridgii*) spans both *P. leniusculus* and cryptic groups, while another subspecies morphology (*P. l. leniusculus*) occurs predominantly within *P. leniusculus*. We discuss these results below in relation to Pacific Northwest biogeography and geological history, identify data limitations and future research needs, and conclude with an emphasis on conservation and management implications.

Cryptic diversity

Our discovery of considerable cryptic diversity within *P. leniusculus* is not entirely surprising given the large geographic range of this historically recognised species, the complex geologic history of the Pacific Northwest and similar findings in other crayfish (Trontelj *et al.*, 2005; Apte *et al.*, 2007; Bentley *et al.*, 2010). Admittedly, our use of a single mtDNA marker is a limitation, as nuclear DNA

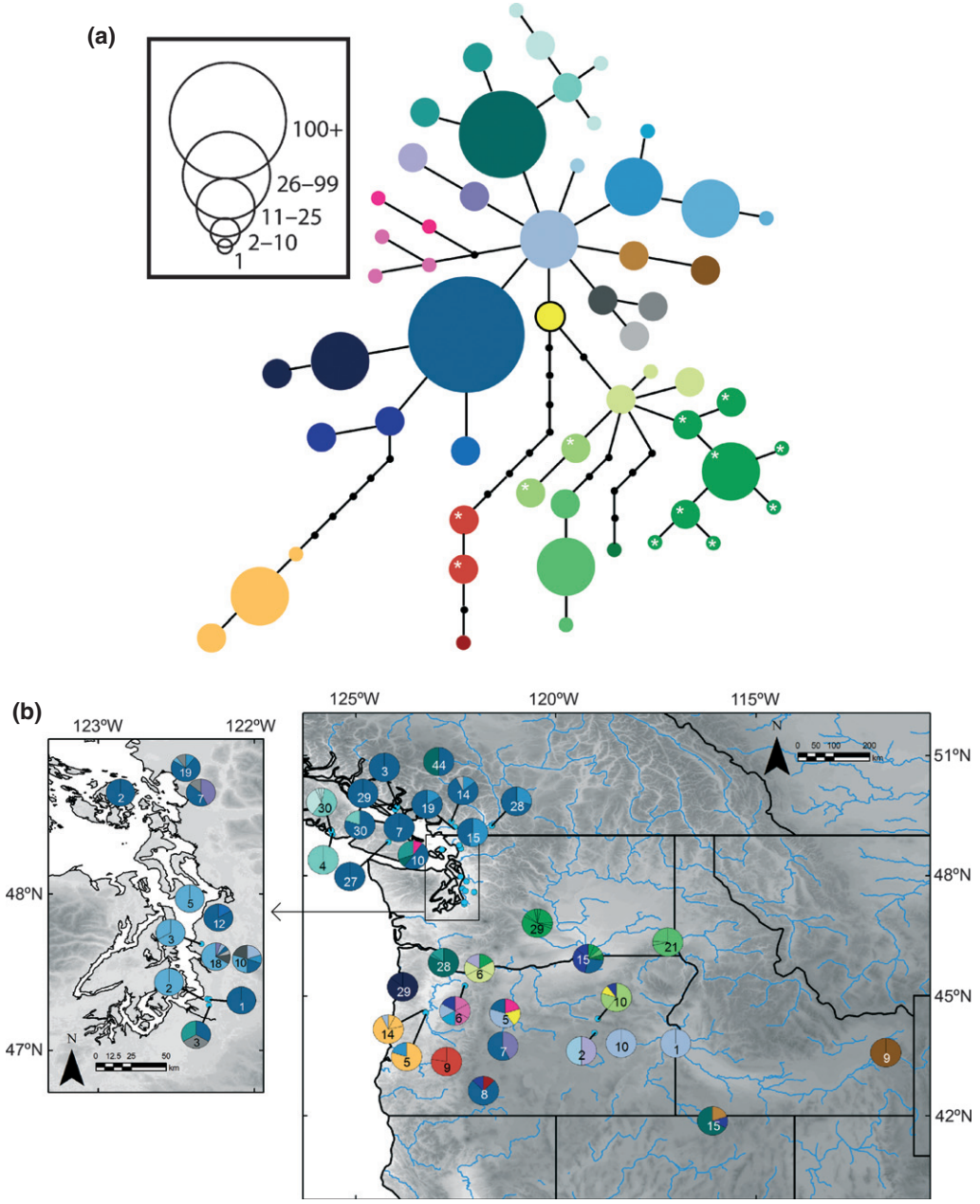

Fig. 3 TCS statistical parsimony haplotype network (a) for the *Pacifastacus leniusculus* group (Fig. 2) with distributions of haplotypes by geographic locations (b), where numbers indicate total crayfish collected at that site. Asterisks (a) are referenced in morphological analysis (Fig. 5).

can produce discordant phylogenetic results (Sota & Vogler, 2001; Keck & Near, 2008); however, our findings provide hypotheses for further studies. In particular, our discovery of cryptic groups that are more distinct from *P. leniusculus* than our *a priori* outgroup *P. connectens*, despite the fact that *P. leniusculus* and *P. connectens* are assigned to separate subgenera (Bouchard, 1977), warrants further investigation with additional molecular markers and expanded taxon sampling. Confirming the validity of, and characterising the relationships among, our identified cryptic groups and recognised *Pacifastacus* species is an important next step for advancing our understanding of the phylogeography of these organisms.

A complex array of Pacific Northwest geological events may have contributed to the distribution of our cryptic *Pacifastacus* groups, ranging from the Miocene flood basalts of the Columbia Plateau to the Pliocene orogeny of the Cascade Mountains to Pleistocene glaciation. We

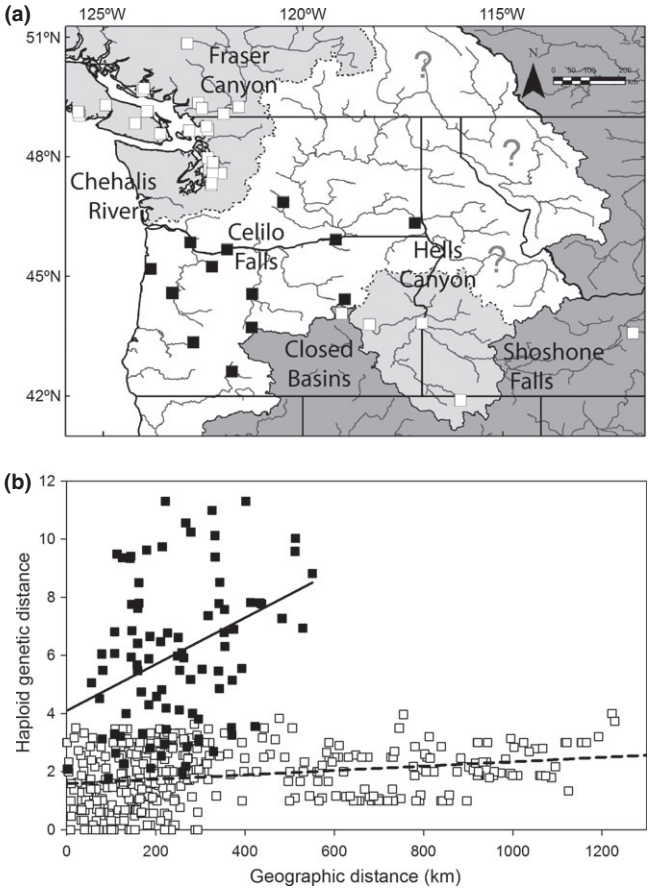

Fig. 4 The Pacific Northwest region of North America (a) coded to represent regions that are native (white), non-native (dark grey), and proposed here as non-native (light grey) for *Pacifastacus leniusculus* (Fig. 3 only, excluding Fig. 2 cryptic groups) with study sites coded as native (■) and hypothesised introduced (□). The map (a) includes names and locations of some geographic features discussed as potential historical barriers to dispersal and gene flow for Pacific Northwest crayfish. The Salish Sea drainages of coastal Washington and British Columbia are separated from the Columbia River glacial refugium by the separate Chehalis River glacial refugium and its distinct cryptic *Pacifastacus* group (Fig. 2). Biplots of mean haploid genetic distance by geographic distance are given separately for pairwise comparisons of native and proposed non-native sites (b).

speculate that the Okanagan group may have re-colonised deglaciated British Columbia from a sub-Okanagan Lobe glacial refugium in the Columbia River via north–south running rivers, and that a small founding population, perhaps impacted by the catastrophic glacial Lake Missoula floods, might account for the relatively low genetic diversity there. Additional field sampling may reveal that the Central Oregon and Okanagan groups are more widespread than reported here. For example, our sample sites are inadequate to evaluate the eastern range extent of Okanagan *Pacifastacus* into the Columbia River headwaters of British Columbia, Idaho and Montana.

Table 3 Number of individuals (with sampled sites in parentheses) and descriptive genetic diversity statistics for native and proposed introduced ranges (Fig. 4) of *Pacifastacus leniusculus* in the Pacific Northwest

	Native	Proposed introduced
n (sites)	192 (14)	379 (29)
No. haplotypes	41	23
Most common haplotype (%)	13%	54%
No. polymorphic sites	30	13
Mean no. pairwise differences within group	5.77	1.63
Gene diversity*	0.94	0.69
SD gene diversity	0.01	0.03

*Gene diversity is the probability that two randomly chosen haplotypes are different in a given sample.

The distribution of the Chehalis group is consistent with past recognition of the Chehalis River and adjacent Olympic Peninsula as a freshwater glacial refugium distinct from the Columbia River. This refugium harbours morphologically and genetically unique fish populations and an entirely endemic fish species, the Olympic mudminnow *Novumbra hubbsi* Schultz (McPhail & Lindsey, 1986; Taylor, Pollard & Louie, 1999). Notably, *N. hubbsi* has a distribution spanning the Olympic Peninsula, Chehalis River and some drainages of the Puget Sound region that resembles our observations for Chehalis group haplotypes (Trotter, McMillan & Kappes, 2000). Colonisation of southern Puget Sound rivers by these Chehalis endemics may be attributable to the historical drainage connection of Puget Sound to the Chehalis River by glacial outwash (Bretz, 1913; McPhail & Lindsey, 1986). A survey of lake occupancy by crayfish (unpublished) similarly supports the distribution of Chehalis group morphology, as assigned by our discriminant function analysis, in the southern half of the Puget Sound region (Appendix S3).

Intermittent occurrences of Chehalis haplotypes farther north in the Puget Sound region, and at one site on Vancouver Island, may represent either northward dispersal of this group with glacial retreat, made difficult by inundation of lowlands with salt water during deglaciation as well as the east–west orientation of Puget Sound rivers, or alternatively the introduction of these crayfish via hypothesised widespread human stocking (see next section). Our detection of the Chehalis group at a single, remote stream on the west coast of Vancouver Island adds another organism to the list of plant and animal endemics shared between the Olympic Peninsula and this island (e.g. Ogilvie & Ceska, 1984; McKey-Fender, Fender & Marshall, 1994), and may contribute to unresolved

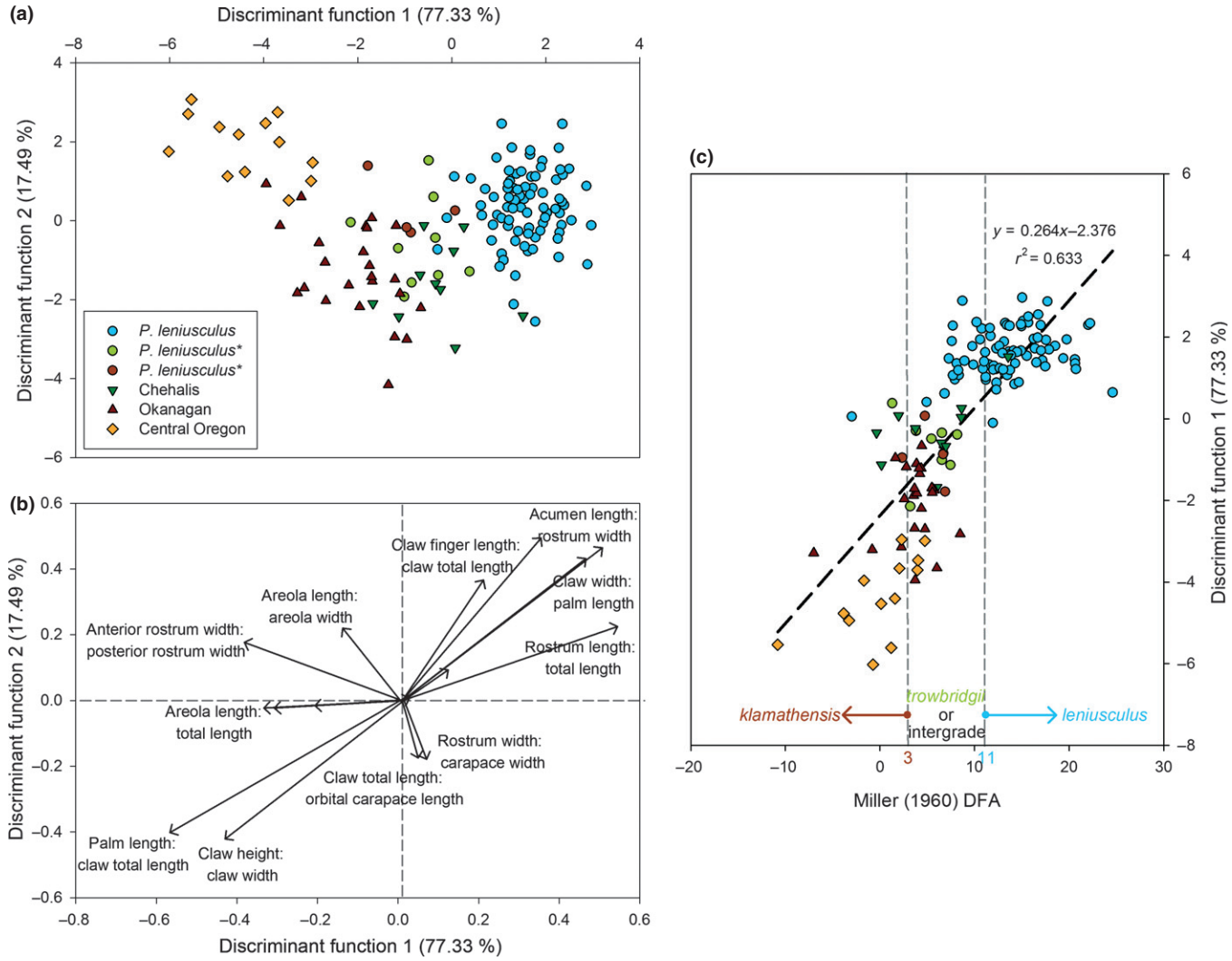

Fig. 5 Discriminant function analysis (DFA) assigning individual crayfish (a) to four *Pacifastacus* groups (Fig. 2) based on 16 ratios from morphological measurements (b; Appendix S2). Individual crayfish scores are plotted (a) on the first two DFA axes and coded by the *Pacifastacus* groups (Fig. 2) and two within- *P. leniusculus* haplotype branches (*) that were not used for DFA assignments (Fig. 3). The first DFA axis from this analysis is plotted (c) against Miller's (1960) DFA axis (Fig. 1).

debates on the validity of Pacific Northwest coastal glacial refugia (Schafer *et al*., 2010).

Hypothesised introductions

The human role in species invasions spans a gradient from cases of absolute certainty to those of high ambiguity. For some sites in California, Japan and Sweden, permitted introductions of *P. leniusculus* provide high certainty with regard to the origin and initial invasion pathway of this crayfish (Abrahamsson & Goldman, 1970). However, crayfish invasions usually occur through their undocumented and increasingly illegal uses as live bait, releases of aquarium organisms or through stocking for harvest (Lodge *et al*., 2000; Larson & Olden, 2011). Such introduction pathways leave little or no paper trail, and in the absence of reliable historical data on species distributions, can be difficult to separate from previously unrecognised portions of native ranges or recent natural range expansions. Molecular approaches provide a powerful, if admittedly imperfect, tool for investigating human contributions to species range expansions (Johnson *et al*., 2011; Fitzpatrick *et al*., 2012). At a minimum, we anticipate our results will challenge pervading assumptions of the entire Pacific Northwest as the native range for ecologically or evolutionarily homogenous *P. leniusculus*.

We believe the presence of *P. leniusculus* in Idaho, southeastern Oregon and Nevada represents unambiguous introductions because of an absence of historical records for this species over major freshwater barriers like

Shoshone Falls. Our isolation by distance analysis demonstrated that putatively introduced populations in far-removed (e.g. 1000 km) Salish Sea drainages share low and similar genetic diversity with these proposed introduced populations of the interior Pacific Northwest. Nested AMOVA supported significant genetic differences between native and proposed introduced regions, but assigned the majority of *P. leniusculus* genetic variance to the among site level, perhaps owing to the low vagility (e.g. limited terrestrial dispersal) and high overall genetic diversity of this species. Our nested AMOVA may have been limited by a sampling imbalance between native (14 sites, 192 crayfish) and proposed introduced regions (20 sites, 358 individuals), as well as the possibility of multiple introductions from different source populations maintaining some distinct genetic diversity at introduced sites. We hope that our study serves as a foundation for additional molecular investigations on the potential role of human introductions in the distribution of *P. leniusculus*, perhaps through future application of microsatellite markers only recently developed for this species (Azuma *et al*., 2012).

Our hypothesis that *P. leniusculus* has been widely introduced by humans to the Salish Sea drainages was also supported by observed disjunct distributions of this crayfish relative to our unexpected discovery of *Pacifastacus* cryptic diversity. Natural colonisation of the Salish Sea drainages by Columbia River-originating *P. leniusculus* would have necessitated either passage into the Fraser River headwaters via a stream capture across the Okanagan region or northward dispersal through the distinct Chehalis River refugium and up the Puget Sound glacial trough (McPhail & Lindsey, 1986). Both of these dispersal scenarios seem unlikely, as we failed to detect *P. leniusculus* from the intervening colonisation corridors (Chehalis, Okanagan) which were instead exclusively occupied by cryptic *Pacifastacus* groups. Our hypothesis of widespread human-mediated introductions into Salish Sea drainages may prove false if the number and distribution of our sampling sites was inadequate to detect *P. leniusculus* from these probable colonisation corridors. However, given the ubiquity of the most common *P. leniusculus* haplotypes in both the Salish Sea drainages and Columbia River refugium, we find it suspicious that we failed to detect any of these haplotypes from four sites and 74 crayfish in the Chehalis and Olympic glacial refugium, which we propose as the most likely northward dispersal route for *P. leniusculus*. At present, our morphologically based assessment of crayfish collected widely throughout lakes of the Puget Sound region supports a southern distribution of the Chehalis group and a predominantly disjunct northern distribution of *P. leniusculus*, although some *P. leniusculus* morphologies were intermittently detected in southern Puget Sound (Appendix S3).

The behaviour and ecology of *P. leniusculus* also inform our expectations for how this crayfish might colonise recently glaciated regions. Although observed occasionally to tolerate estuarine conditions (Miller, 1960), we do not anticipate that *P. leniusculus* can disperse long distances through salt water. Further, while crayfish species have varying desiccation tolerances and capacities to disperse overland, terrestrial dispersal has not been widely reported for *P. leniusculus* and has been found trivial in explaining patterns of genetic diversity for other freshwater crayfish over spatial scales smaller than those considered here (Bentley *et al*., 2010). Finally, steep stream gradients and high water velocity severely limit the dispersal and distribution of invasive *P. leniusculus* populations elsewhere (Light, 2003; Pintor & Sih, 2011), potentially precluding natural colonisation above hydraulic barriers like the fast-flowing Fraser River canyon (McPhail & Lindsey, 1986). Even features like Celilo Falls, prior to hydropower development and impoundment, may have inhibited gene flow between coastal and interior *Pacifastacus* crayfishes as suggested by the many *P. leniusculus* haplotypes unique to either east or west of the Columbia River gorge (Fig. 3) and the historical dominance of *P. l. leniusculus* morphologies west of the Cascade Mountains (Fig. 1).

Morphology and P. leniusculus subspecies

We found that the range of morphological attributes used by Miller (1960) to characterise *P. leniusculus* subspecies retains some capacity to distinguish among evolutionarily distinct *Pacifastacus*. Notably, the *leniusculus* subspecies morphology described by Miller (1960) was almost exclusively contained within the group we identified as *P. leniusculus*, the single exception being a lake occupied by the Chehalis group in the Puget Sound region in close proximity to many *P. leniusculus* occupied lakes (Fig. 2; Appendix S3; see next section for comments on hybridisation and introgression). Thus, we conclude that the long acumen and rostrum, prominent post-orbital spines, and wide claws with short, convex palms used by Miller (1960) to define *P. l. leniusculus* still reliably identify this species relative to cryptic *Pacifastacus*.

By contrast, the *trowbridgii* and *klamathensis* subspecies defined morphologically by Miller (1960) are ambiguous, spanning both *P. leniusculus* and the three cryptic groups. Although our discriminant function analysis separated these cryptic groups from *P. leniusculus* with high accu-

racy, some *P. leniusculus* individuals were misclassified. The paucity of *P. leniusculus* with *trowbridgii* or *klamathensis*-like morphology in our study may have been caused by limited sampling in some regions of the Pacific Northwest where such crayfish may be more prevalent (orange, red and green branches of haplotype network; Figs 3 and 5). Sonntag (2006) reported high fidelity between *P. leniusculus* subspecies morphology and phylogenetic groups identified with mtDNA in a study conducted exclusively within the coastal drainages of northern California and southern Oregon. Similarly, we found *klamathensis*-like morphology in *P. leniusculus* from the Umpqua River drainage of southern Oregon in association with highly distinct *P. leniusculus* haplotypes.

We propose that the *P. l. klamathensis* subspecies might be valid in and adjacent to its Klamath River drainage type locality, despite our Central Oregon and Okanagan groups sharing similar morphology. *Pacifastacus l. trowbridgii* or *klamathensis*-like morphology was also found at sites dominated by unique *P. leniusculus* haplotypes confined to Columbia and Snake River tributaries east of the Cascade Mountains. Morphological traits associated with historical *P. leniusculus* subspecies may therefore have some taxonomic value, but our discovery of cryptic *Pacifastacus* groups that physically resemble these subspecies will necessitate a more dedicated evaluation of links between *Pacifastacus* morphology and phylogeny. Future studies should investigate phenotypic plasticity of *Pacifastacus* morphology; traits like spine length or chelae shape could be plastic responses to environmental conditions. Finally, mtDNA will be a valuable 'barcode' for researchers and managers needing to identify our proposed cryptic groups while the capacity to discriminate among these crayfish by morphology is investigated further (Sweeney *et al.*, 2011).

Conservation and management

Western North America exemplifies the severe crisis in crayfish conservation, as one of five historically recognised *Pacifastacus* species has been declared extinct and a second is listed under the US Endangered Species Act (ESA) (Light *et al.*, 1995). Factors like habitat modification and destruction undoubtedly impact crayfish populations, but interactions with invasive crayfish are recognised as the leading cause of native crayfish declines (Lodge *et al.*, 2000). Our discovery of considerable cryptic diversity historically mistaken as *P. leniusculus* heightens the challenge and need for active conservation of western North American crayfish. As examples, the Chehalis group is adjacent to multiple invasions from a diverse portfolio of non-native crayfish; the known range of the Okanagan group has been widely invaded by the virile crayfish *Orconectes virilis* Hagen; and the Central Oregon group is directly threatened by a well-established population of rusty crayfish *Orconectes rusticus* Girard (Larson & Olden, 2011). Management responses are urgently needed to protect the unique *Pacifastacus* diversity documented here from the many invasive crayfish populations that have established in the Pacific Northwest over just the past decade.

Our results also underscore the need for management agencies from federal to local levels to discourage, both via regulation and education, the potential introduction of live crayfish regardless of origin or identity. Although the import and introduction of crayfish from outside the Pacific Northwest is generally prohibited, current policies are more permissive in regard to the live transport of *Pacifastacus* crayfish within the Pacific Northwest after harvest or for live bait (Larson & Olden, 2011). We propose that *P. leniusculus* has been and continues to be widely introduced within the Pacific Northwest, and we demonstrate a high degree of previously unrecognised and geographically structured *Pacifastacus* genetic diversity within this region that could be vulnerable to impacts from translocated *Pacifastacus*. The ecological impacts of invasive *P. leniusculus* in regions adjacent to the Pacific Northwest (e.g. California) are not trivial, and these same impacts should be anticipated in additional areas where we propose this species has been introduced (e.g. southern Idaho, Salish Sea drainages).

The transition to managing *Pacifastacus* crayfish with greater caution in the Pacific Northwest will require some adjustments, from researchers to managers to the public. Changes in the management of native salmonids in western North America over time may be an apt parallel. Species like the cutthroat trout *Oncorhynchus clarkii* Richardson have been found to harbour highly distinct genetic diversity, much of which has been lost because of biotic interactions with introduced salmonids. Some of these invasive salmonids originate from remote regions (Dunham *et al.*, 2002), while others were stocked from within western North America and affected previously isolated *O. clarkii* populations, not only through competition but also hybridisation and introgression (Hitt *et al.*, 2003). We propose a similar scenario for Pacific Northwest *Pacifastacus*, in which unique evolutionary groups are not only threatened by crayfish introduced from afar, but also by carelessness in regard to the transport and release of crayfish within their own genus. If *Pacifastacus* introductions within the Pacific Northwest continue or increase, we are at risk of not only losing a highly unique

evolutionary heritage, but also organisms that offer insights into the biogeography and geologic history of the Pacific Northwest through their genes.

Finally, our results have implications for the study and management of invasive populations of *P. leniusculus* elsewhere, including Europe and Japan (Lodge *et al*., 2000; Azuma *et al*., 2012). The broad extent of our phylogeographic sampling in the Pacific Northwest and archiving of genetic data should allow other researchers to identify native range sources for *P. leniusculus* populations in their regions (e.g. Filipová *et al*., 2011), as well as providing contrasts for investigations of genetic structure in the invaded range (but see Fitzpatrick *et al*., 2012 for limitations of such comparisons). Our narrowing of the probable native range for *P. leniusculus* also means that models predicting the global invasive distribution for this crayfish are likely to underestimate the extent of climatic niche shifts the species has actually experienced (Larson *et al*., 2010), while our suggestion that invasive populations of *P. leniusculus* originate from large, low-altitude rivers of the lower Columbia River catchment may explain the observed intolerance of these crayfish for steep stream gradients and high water velocity (Light, 2003; Pintor & Sih, 2011). In addition, our identification of diverse phylogenetic lineages and potential cryptic species within the crayfish historically recognised as *P. leniusculus* is invaluable for studies that seek to compare the ecology and behaviour of this species between native and introduced ranges (Pintor, Sih & Bauer, 2008; Larson *et al*., 2010). There may be no reason to expect equivalent ecological function between *P. leniusculus* and our newly identified *Pacifastacus* groups, and consequently, our work emphasises the potential value of incorporating phylogenetic information into increasingly common contrasts of ecology between native and introduced ranges.

Acknowledgments

This research was supported by a Grant-in-Aid for Scientific Research (C) (No. 20510219), the American Fisheries Society, ARCS Foundation, British Columbia Habitat Conservation Trust Foundation, National Science Foundation, NOAA Fisheries and NOAA Sea Grant, the Oregon Zoo, the University of Washington and the Washington Lake Protection Association. Some crayfish specimens were generously provided by N. Haddaway, M. Hill, E. Lowery and D. Shiozawa. Information on *P. leniusculus* specimens at the Royal British Columbia Museum was provided by M. Frey and K. Kuchnow. Geoff Lowe, T. Korenaga, A. Mahony and A. Tabata assisted with laboratory work; A. Wong assisted with morphological measurements; and H. Ishiniwa and S. Gilmore assisted with data analysis. William H. Clark and W.J. Gerth provided logistical support to N. Usio, and A. Hildrew, T.R. Maret, D.C. Rogers and two anonymous reviewers provided comments and suggestions that improved this work. The lead author would like to acknowledge George C. Miller for his exceptional contributions on Pacific Northwest crayfish.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Abbreviated summary of morphometric conclusions from Miller (1960) for the three signal crayfish *Pacifastacus leniusculus* subspecies, with diagrams from Miller (1960) for adult specimens (a), rostral insets (b), and young adult specimens (c).

Appendix S2. Measurements and ratios used in discriminant function analysis for *Pacifastacus* groups (Figs 2 and 5) with Miller's (1960) discriminant function formula used in classifying *P. leniusculus* subspecies (Fig. 1).

Appendix S3. Results of a 2007–2009 survey of crayfish occupancy in 100 lakes of Washington's Puget Sound region representing the spatial distribution of Chehalis (south) and *leniusculus* (north) morphology from the contemporary discriminant function analysis (Fig. 5).

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(Manuscript accepted 8 June 2012)