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Trophic niche differentiation and phenotypic divergence among cryptic species of Japanese ninespine sticklebacks

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ABSTRACT

Background: Morphologically similar cryptic species often differ in ecological niches. Three cryptic species of ninespine stickleback (genus *Pungitius*), *P. tymensis* and the freshwater and brackish types of the *P. pungitius–P. sinensis* species complex are found in eastern Hokkaido, Japan. All three co-occur in river catchments, inhabiting the upstream (*P. tymensis*), midstream (freshwater *P. pungitius–P. sinensis* type), and downstream (brackish *P. pungitius–P. sinensis* type).

Questions: Are differences in trophic ecology among these species persistent? Do the three species differ in geometric body shape? Which ecological and morphological traits best distinguish these three cryptic species?

Methods: We used stable isotope analysis to estimate trophic position and different source contributions in order to quantify trophic niche differentiation among three species collected from eastern Hokkaido, Japan. We characterized body shape variation using geometric morphometrics. We conducted exchangeability analysis to test how well different traits could distinguish all three species.

Results: Isotopic source and niche overlap estimates clearly indicated a greater contribution of marine prey items to the brackish type than to the other two species and showed little differences between the freshwater type and *P. tymensis*. Body shape substantially differed between the freshwater type and *P. tymensis*, which had a deeper, elongated shape, whereas the brackish type was intermediate. Based on morphological and trophic traits, the brackish type and *P. tymensis* could be clearly distinguished. In contrast, misclassification of *P. tymensis* as the freshwater type based on stable isotope data and trophic morphology was high, indicating substantial overlap in trophic niche between these species.

Keywords: diet, ecological speciation, exchangeability analysis, resource, sexual dimorphism, stickleback.

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INTRODUCTION

Morphologically similar cryptic species are prevalent across diverse taxa (Bickford *et al.*, 2007). Because morphological traits are often linked to ecology and foraging behaviour (Schluter, 2000), morphologically similar species may exploit similar ecological niches. However, recent studies have shown that cryptic species often exploit different ecological niches (Rissler and Apodaca, 2007; Ashrafi *et al.*, 2011; Fišer *et al.*, 2015; Scriven *et al.*, 2015). In some cases, ecological divergence may be driven by differences in physiological rather than morphological traits (Wellborn *et al.*, 2004; Derycke *et al.*, 2008; DeMeester *et al.*, 2011). Therefore, such ecological information may help distinguish otherwise phenotypically cryptic species. The extent of ecological divergence between species is generally associated with their divergence time, such that more closely related species tend to occupy similar niches (Wiens and Graham, 2005; Losos, 2008; Pyron *et al.*, 2015), although divergence driven by strong competition between closely related sympatric species can confound this pattern (Losos *et al.*, 2003). Therefore, investigation of patterns of ecological differentiation among cryptic species can help us to establish methods for distinguishing them with high confidence and potentially understand better the evolutionary mechanisms underlying ecological differentiation.

Eastern Asia, in particular the Japanese archipelago, contains a high diversity of *Pungitius* species, many of which are cryptic (Takahashi *et al.*, 2001; Tsuruta and Goto, 2007; Wang *et al.*, 2015). Three species, *P. tymensis* and the freshwater type and brackish-water type of the *P. pungitius–P. sinensis* species complex occur in Hokkaido, Japan (Fig. 1A). Based on mitochondrial data, divergence between *P. tymensis* and the other two ninespine sticklebacks is estimated to have occurred 2.0–4.4 million years ago, most probably as a result of Pleistocene or Late Quaternary sea-level change (Takahashi and Goto, 2001; Aldenhoven *et al.*, 2010; Wang *et al.*, 2015). Because these three species are morphologically similar, it is difficult to distinguish them based only on their appearance (see figure 1a in Ishikawa *et al.*, 2013). Confusion about the taxonomy of the *P. pungitius–P. sinensis* species complex is also derived from the discrepancy between armour plate phenotypes and genotypes at neutral markers. Ninespine sticklebacks other than *P. tymensis* in Hokkaido were originally classified into *P. pungitius* and *P. sinensis* based on armour plate morphology (Wootton, 1976). However, recent genetic studies indicate that lateral plate morphs do not correspond to genetically distinct species (Takata *et al.*, 1987; Ishikawa *et al.*, 2013), so the *P. pungitius–P. sinensis* species complex is currently tentatively classified into the freshwater type and the brackish-water type based on their preferred habitats (Takata *et al.*, 1987). Despite the difficulty in defining species based on morphological traits, *P. tymensis* and the freshwater and brackish-water types mainly inhabit the upstream, midstream, and downstream of a river respectively, although they sometimes occur in sympatry (Tsuruta *et al.*, 2008; Ishikawa *et al.*, 2013), suggesting that these three cryptic species with relatively old divergence times may differ in ecological niches.

Previous work on these three Japanese *Pungitius* species has shown that they differ in neutral genetic markers, trophic morphology, salinity tolerance, and stomach contents (Ishikawa *et al.*, 2013). Gill raker morphology is strongly divergent among all three species and is also consistent with species differences in stomach content data. *Pungitius tymensis* has the shortest and fewest gill rakers and feeds primarily on benthos (i.e. terrestrial insects) and also zooplankton (e.g. Copepoda); the brackish type has the most numerous and the longest gill rakers and feeds mainly on planktonic crustaceans such as Mysidacea (Ishikawa *et al.*, 2013). The freshwater type has intermediate foraging morphology, but is benthic, feeding on items such as Isopoda. Genetic data showed that only a few hybrids were identified even in

sympatry (Takata *et al.*, 1987; Ishikawa *et al.*, 2013), suggesting that there is strong reproductive isolation among these three species. Habitat isolation, seasonal isolation, and hybrid incompatibility may contribute to reproductive isolation (Kobayashi, 1959; Takahashi *et al.*, 2005; Tsuruta *et al.*, 2008; Ishikawa *et al.*, 2013), but their relative contributions to total isolation are unknown.

The first aim of the present study was to characterize trophic niche differentiation and phenotypic divergence among the three cryptic ninespine stickleback species in eastern Hokkaido, Japan (Fig. 1). Although we have previously characterized divergence in stomach contents (Ishikawa *et al.*, 2013) and stomach content analysis is useful for high-resolution identification of prey items, it provides only a snapshot of trophic ecology – that is, what an individual has ingested in the previous 24 hours (Grey, 2006). This is a particularly important consideration when ontogenetic shifts occur in foraging behaviour over the life history of individuals, as these will not be reflected by a dietary snapshot such as stomach contents (Grey, 2001; Ravinet *et al.*, 2010). Stomach content analysis may also be biased towards overrepresentation of hard-bodied prey items and does not necessarily reflect energy intake – that is, gut contents may include items ingested but not assimilated (Hyslop, 1980; Jackson *et al.*, 1987). Additionally, analyses based on stomach contents alone are often not sufficient to resolve trophic position with high precision (Vander Zanden and Rasmussen, 1999; Post, 2002). Stable isotopes make it possible to quantify contributions from different dietary sources and estimate trophic level and an integrated signal of dietary preferences over a

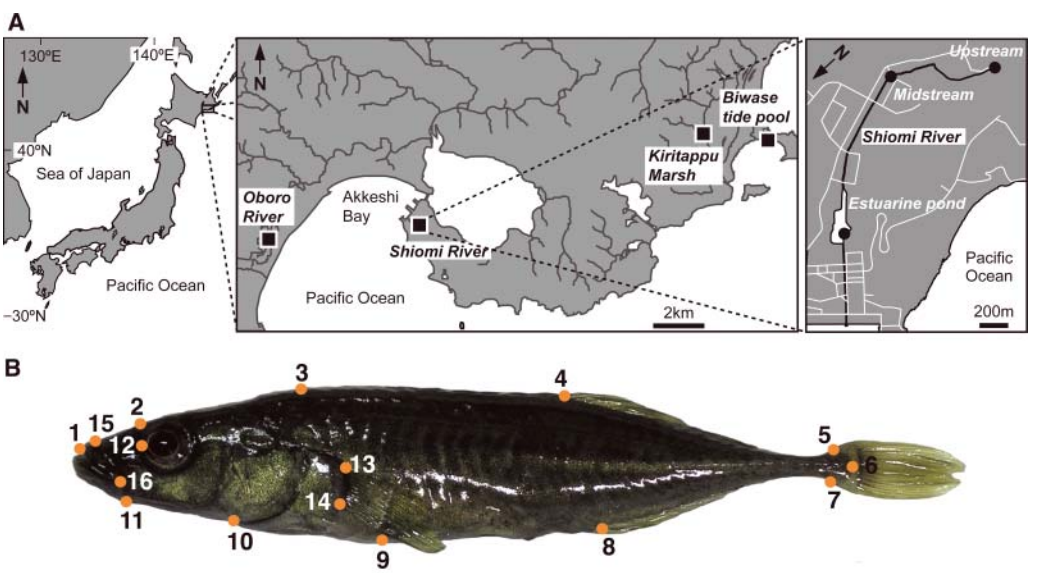

Fig. 1. (A) Map of sampling sites. (B) Landmark positions used in geometric morphometric analysis. Landmarks are: (1) anterior extent of premaxilla, (2) posterior extent of supraoccipital, (3) anterior insertion of first dorsal spine, (4) anterior insertion of dorsal fin, (5) dorsal insertion of caudal fin, (6) posterior extent of caudal peduncle, (7) ventral insertion of caudal fin, (8) anterior insertion of anal fin, (9) insertion point of pelvic spine to pelvic girdle, (10) posteroventral extent of preopercular, (11) anterorventral extent of preopercular, (12) anterior extent of orbit, (13) anterodorsal insertion of pectoral fin, (14) posterodorsal insertion of pectoral fin, (15) anterior extent of maxilla, (16) posterior extent of maxilla.

longer time period [i.e. 3–4 months with fish muscle tissue (Perga and Gerdeaux, 2005)]. Combining both stomach content and stable isotope analysis provides a much more robust alternative for quantifying trophic ecology than relying on a single method (Berner *et al.*, 2008; Bolnick and Paull, 2009; Mathews *et al.*, 2010; Ravinet *et al.*, 2014).

Geometric morphometrics is a powerful method for analysing body shape variation (Zelditch *et al.*, 2012). In *Gasterosteus*, body shape is substantially associated with habitat (e.g. Walker, 1997; Aguirre *et al.*, 2008; Sharpe *et al.*, 2008; Hendry *et al.*, 2011). For example, body shape varies along coastal rivers; downstream fish are typically more slender compared with a deeper shape seen in fish from upstream (Taylor and McPhail, 1986; Ravinet *et al.*, 2013a). A shallow body depth may be suitable for planktivorous fish, which perform prolonged swimming to feed in open water, while deeper body shape can improve manoeuvrability when feeding in more complex upstream benthic habitats (Taylor and McPhail, 1986; Hendry *et al.*, 2011). Given the fact that cryptic ninespine stickleback species inhabit different reaches of a river, they may vary in body shape.

The second aim of the study was to perform exchangeability analysis using these new data (stable isotope and geometric morphometrics) as well as previously reported data (genotypes at neutral microsatellite markers and morphological measurements) (Ishikawa *et al.*, 2013) in order to find the traits that can best distinguish these three species. Exchangeability analysis examines how easily individuals can be exchanged among species based on composite traits from multivariate phenotypic values (Hendry *et al.*, 2013). Exchangeability analysis differs from other methods using standard statistical tests for differences between trait means or significant variance terms, in that it makes use of the probability distribution of individual classifications to assess exchangeability between populations or species (Hendry *et al.*, 2013). This type of analysis can tell us the extent of phenotypic and niche overlap among cryptic species as well as the best diagnostic composite trait useful for defining species.

MATERIALS AND METHODS

Sample collection

Fish captured for this study have been described previously (Ishikawa *et al.*, 2013). Briefly, fish were collected with minnow traps or hand nets from four different watersheds in May 2011: Shiomi River, Biwase tidepool, Kiritappu Marsh, and Oboro River (Fig. 1, Table 1). The number of species present differed between sites with only the brackish type found at Biwase tidepool and *P. tymensis* and the freshwater type occurring at Kiritappu Marsh and the Oboro River (Table 1). All three species were present in the Shiomi River (Table 1) and this site was sampled at three points: upstream, midstream, and estuary (see the right upper panel of Fig. 1). Morphological cues such as lateral plate number are not reliable for species identification because of overlap between species (Takahashi *et al.*, 2001; Ishikawa *et al.*, 2013; Wang *et al.*, 2015). All fish were therefore genotyped with eight microsatellite markers (*Pun212*, *Pun134*, *Pun19*, *Pun68*, *Pun117*, *Stn433*, *Pun171*, *Pun78*) on different linkage groups to confirm species identification; these results are reported elsewhere (Ishikawa *et al.*, 2013).

Benthic macroinvertebrates were collected from the Shiomi river system and Akkeshi Bay by hand nets or from local suppliers (for further details, see Ravinet *et al.*, 2014). Samples were taken to represent putative prey items and also in order to represent the isotopic baseline of benthic and pelagic food webs for trophic level calculation (Post, 2002) (see 'Stable isotope analysis').

Table 1. Sample sizes used in geometric morphometric and stable isotope analyses

Site	Sex	Morphometrics			Stable isotopes		
		Brackish	Freshwater	*P. tymensis*	Brackish	Freshwater	*P. tymensis*
Biwase tidepool	Male	3	0	0	3	0	0
	Female	12	0	0	12	0	0
	Unknown	0	0	0	0	0	0
	Total	15	0	0	15	0	0
Shiomi River	Male	3	2	5	11	2	7
	Female	9	8	7	35	8	10
	Unknown	0	2	0	34	12	19
	Total	12	12	12	80	22	36
Kiritappu Marsh	Male	0	8	0	0	8	0
	Female	0	4	0	0	4	0
	Unknown	0	2	0	0	0	0
	Total	0	14	0	0	12	0
Oboro River	Male	0	14	4	0	15	4
	Female	0	15	10	0	17	10
	Unknown	0	1	1	0	1	0
	Total	0	30	15	0	33	14

Stable isotope analysis

We dissected dorsal muscle from each fish ($n = 212$ in total: $n = 50$ for *P. tymensis*; $n = 67$ for the freshwater type; $n = 95$ for the brackish type; see Table 1 for a full breakdown of sample sizes for each species at each site) and dried it for 48 hours at 60°C. Dried muscle tissue was then ground and weighed into micro-tin capsules for analysis. Samples were analysed for $\delta^{13}C$, $\delta^{15}N$, %C and %N on a Carlo Erba Elemental Analyser and a Thermo Finnigan Delta Plus XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) at the Duke Environmental Isotope Laboratory (DEVIL) at Duke University, North Carolina, USA. Prior to analysis, fish muscle was lipid-normalized using C:N ratios (Kiljunen *et al.*, 2006).

Since we were primarily interested in differences between species, interspecies differences in $\delta^{13}C$, $\delta^{15}N$, and estimated trophic position were tested using general linear mixed models (GLMMs) with site as a random effect using the nlme package. However, we further investigated intraspecific differences in trophic ecology among sites using standard GLMs. Only a subset of the 212 individuals used for stable isotope analysis were sexed by gonad inspection and measured for standard length. Therefore, to test for differences in diet between the sexes and dietary variation with body size, we used this subset ($n = 142$) in GLMMs with sex and species as full effects, standard length as a covariate, and site as a random effect. As with our morphometric analyses, we first fit this full model before performing stepwise model selection with AIC using the *stepAIC* function from the MASS R package (Venables and Ripley, 2002).

Stable isotope analysis can also be used to quantify isotopic niche as a proxy for the ecological niche that organisms inhabit (Layman *et al.*, 2007; Newsome *et al.*, 2007; Jackson *et al.*, 2011). However, accurate estimation of isotopic niche should ideally account for sampling and measurement error (Jackson *et al.*, 2011). To account for this and to formally test differences in

isotopic niche overlap, we used a Bayesian method to estimate standard ellipse area (SEA_B) implemented in *siber* (Stable Isotope Bayesian Ellipses in R) (Jackson *et al.*, 2011).

We additionally estimated the relative dietary source contributions of putative marine and freshwater prey items to each of the ninespine stickleback species. To do so, we used a Bayesian isotopic mixing model implemented in *siar* (Stable Isotope Analysis in R) (Parnell *et al.*, 2010). Assuming isotopic values represent a composite signal of diet, the mixing model approach makes use of known fractionation rates and isotopic concentrations in order to estimate the proportion of diet made by each source. We ran *siar* using standard trophic fractionation rates for fish muscle (mean ‰ ± SD: $\delta^{13}C$, 1.63 ± 0.63; $\delta^{15}N$, 3.54 ± 0.74) (Minagawa and Wada, 1984; France, 1995), both of which work well for stable isotope analysis on sticklebacks (Ravinet *et al.*, 2013b, 2014). Similar to *siber*, the *siar* model produces posterior probability distributions of estimates that can account for error. Furthermore, Bayesian analysis of source contributions and niche size make it possible to perform exact tests for differences between groups by directly comparing posterior probability distributions.

Geometric morphometrics

Pictures were taken from the left sides of euthanized fishes. A total of 16 landmarks were chosen to capture overall body shape (see Fig. 1B). Digitized coordinates were placed on photographs using tpsDig2 (Rohlf, 2006). Initial shape analysis was performed in MorphoJ (Klingenberg, 2011). We quantified shape by performing a Procrustes fit using all individuals before correcting any incorrectly placed or swapped landmarks from any individuals exhibiting extreme deviations from the mean shape across the entire data set using the MorphoJ 'Find Outliers' feature (http:www.flywings.org.uk/MorphoJ_guide/). Outliers are identified by Procrustes distance deviations in the observed data set from an expected multivariate normal distribution (http:www.flywings.org.uk/MorphoJ_guide/). Following manual correction for erroneously placed landmarks, we performed principal component analysis (PCA) to summarize shape variation among all individuals. For all morphometric analyses, centroid size measured from the digital images was used as a proxy for body size. Shape variation between the sexes and species for all principal components was tested using a multivariate analysis of covariance (MANCOVA) with centroid size as a covariate. Following this, univariate general linear mixed models (GLMMs) were used to test PCs individually explaining >10% of the total variance. For each GLMM, we first fit a full model with sex and species as fixed factors, centroid size as a covariate, and sampling site as a random effect using the package nlme in R (Pinheiro *et al.*, 2016). We then used the function *stepAIC* from the R package MASS to perform stepwise model selection based on AIC scores (Venables and Ripley, 2002); in short, terms were removed from the model sequentially to find the nested model with the lowest AIC score.

Exchangeability analysis

To determine what measurements best distinguish *Pungitius* species, we used exchangeability analysis – that is, how easily individuals can be exchanged among species based on trait values (Hendry *et al.*, 2013). In their paper proposing this method, Hendry *et al.* (2013) focused on conspecific exchangeability, but here we used the method to assess which trait measurements best distinguish each of the three species studied: *P. tymensis*, the freshwater type and brackish type.

We examined the extent of exchangeability between each of the three species based on five categories of measured values: diet (using $\delta^{13}C$ and $\delta^{15}N$ values), defence traits (first dorsal spine length, second dorsal spine length, the most posterior dorsal spine length, anal spine length, pelvic spine length, and pelvic girdle length), trophic morphology (gill raker length, number of gill rakers on upper gill arch, and number of gill rakers on lower gill arch), body shape (Procrustes coordinates), and neutral genetic markers (*Pun212*, *Pun134*, *Pun19*, *Pun68*, *Pun117*, *Stn433*, *Pun171*, and *Pun78*). Morphological measurements and neutral microsatellite genotypes have been reported previously (Ishikawa *et al.,* 2013), and other traits were taken from the current study (see 'Results').

For each trait category, we performed discriminant analysis on principal components (DAPC) (Jombart *et al.* 2010). This straightforward method first requires a principal component analysis and then a discriminant function analysis on principal components in order to classify individuals back to the species level. For diet, defence, trophic and body shape, PC and discriminant function analyses were conducted using the *prcomp* and *lda* functions in R v.3.2.2 (R Development Core Team 2015), while neutral microsatellite markers were analysed using the *dapc* function in adegenet (Jombart *et al.* 2010). Following Hendry *et al.* (2013), we calculated assignment probability and cross-classification probability. Assignment probability is the probability of classification to the correct species out of all three included in the analysis. For the cross-classification analysis, the self-classification category was omitted and the probability of classification to the other two species was calculated. Then, both assignment and cross-classification probabilities were divided by the random expectations (33.3% for assignment probability and 50% for cross-classification probability). Our approach made one small departure from the original DAPC and exchangeability formulation (Jombart *et al.*, 2010, Hendry *et al.*, 2013). Namely, we did not substitute missing measurements with mean trait values as some measurements such as body shape were only performed on a subset of individuals; this also meant that the number of samples used in each category differed. To account for this, we calculated proportions assigned to each individual trait category rather than focusing on the absolute number of individual assignments.

RESULTS

Trophic niche differentiation estimated by stable isotope analysis

Mean $\delta^{13}C$ and $\delta^{15}N$ values of macroinvertebrates did not differ between the Akkeshi Bay and Biwase tidal sites (GLMMs with species as random factor: $\delta^{13}C$, $P = 0.66$; $\delta^{15}N$, $P = 0.44$); thus values for invertebrates sampled at these locations were pooled and treated as a marine signal. Freshwater prey showed a strong depletion of $\delta^{13}C$ (GLM: $R^2 = 0.96$, $F_{3,34} = 6.66$, $P = 0.001$; Fig. 2A), indicating a transition from a marine to freshwater benthic carbon source. Similarly, $\delta^{15}N$ values were lower in freshwater prey relative to marine food items, suggesting a more depleted $\delta^{15}N$ baseline ($R^2 = 0.96$, $F_{3,34} = 11.17$, $P < 0.0001$; Fig. 2A).

Mean $\delta^{13}C$ differed among the three *Pungitius* species ($R^2 = 0.81$, $F_{2,206} = 363.28$, $P < 0.0001$; GLMMs with site as random effect and size as a covariate). The brackish type had a less depleted mean $\delta^{13}C$ value (15.25 ± 3.19‰; mean ± SD) than either the freshwater type or *P. tymensis* (-26.90 ± 2.02 and 25.52 ± 2.43‰, respectively; Tukey HSD $P < 0.001$; Table 2, Fig. 2A). The similar, more depleted $\delta^{13}C$ signal in both *P. tymensis* and the freshwater type suggested predominately freshwater foraging in these species.

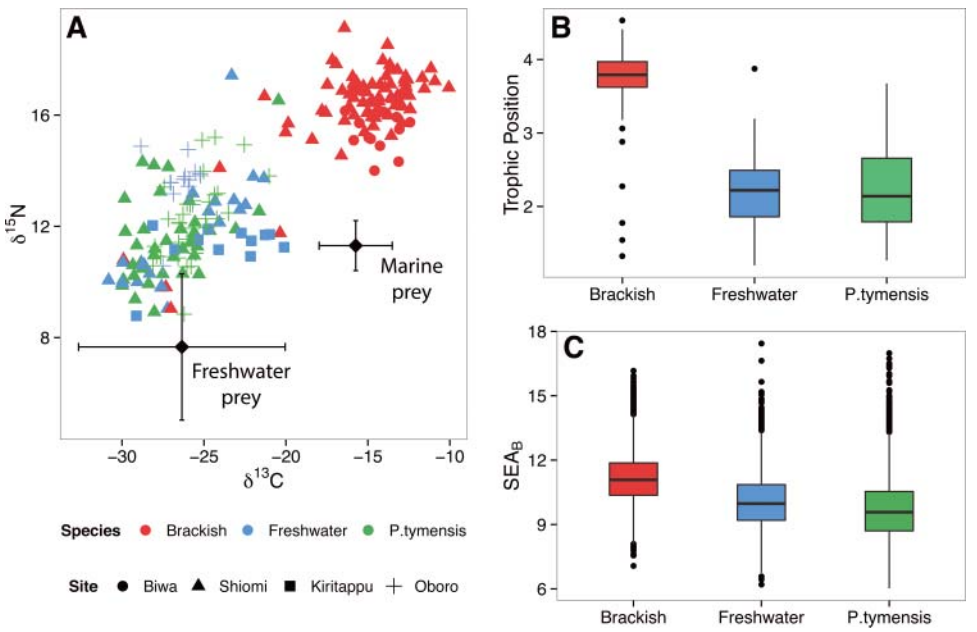

Fig. 2. Stable isotope analysis. (A) Isotopic biplot showing $\delta^{13}C$ and $\delta^{15}N$ variation among ninespine stickleback species. Black diamonds indicate mean values for prey items, horizontal and vertical error bars represent standard deviation around the mean. Boxplots showing species distributions of estimated trophic position (B) and isotopic niche size measured as Bayesian standard ellipse area (SEA_B) (C).

Table 2. Mean stable isotope values, trophic position, and estimates of standard ellipse area (SEA_B) for three ninespine stickleback species found in the Shiomi River, Hokkaido

	$\delta^{13}C$		$\delta^{15}N$		Trophic position		SEA_B		
Species	Mean	SD	Mean	SD	Mean	SD	Median	L95 HPD	U95 HPD
Brackish type	−15.26	3.20	16.23	1.54	3.70	0.51	11.09	9.12	13.67
Freshwater type	−25.52	2.44	11.91	1.56	2.20	0.49	9.99	7.93	12.80
P. tymensis	−26.91	2.02	12.08	1.68	2.22	0.51	9.56	7.33	12.73

Note: L95 HPD = lower 95% highest posterior density, U95 HPD = upper 95% highest posterior density (HPD).

Variation in $\delta^{15}N$ values showed a similar pattern of enrichment in the brackish type but lower values in the freshwater type and *P. tymensis* ($R^2 = 0.67$, $F_{2,206} = 171.98$, $P < 0.0001$; see Table 2, Fig. 2A), while the freshwater type and *P. tymensis* had very similar values. A more enriched mean $\delta^{15}N$ value in the brackish type (16.22 ± 1.54‰) suggests foraging at a higher trophic level. Estimates of trophic level following baseline correction showed that this was indeed the case ($R^2 = 0.71$, $F_{2,206} = 205.23$, $P < 0.0001$); the brackish type had a mean trophic level (± SD) of 3.70 ± 0.50, a level above that of *P. tymensis* and the freshwater type (2.20 ± 0.49 and 2.22 ± 0.51, respectively; $P < 0.0001$; Table 2, Fig. 2B). Analysis

on a subset of individuals that were sexed and measured for standard length ($n = 142$) indicated that $\delta^{13}C$, $\delta^{15}N$, and trophic position did not differ significantly between males and females (GLMMs with site as a random effect, size as a covariate, and sex and species as fixed effects; $P = 0.09$ for the effects of sex on $\delta^{13}C$; sex term removed from the best-fit model for $\delta^{15}N$ and trophic position). Size was a significant overall positive term for all three dietary models ($\delta^{13}C$: $R^2 = 0.89$, $F_{1,133} = 99.31$, $P < 0.0001$; $\delta^{15}N$: $R^2 = 0.78$, $F_{1,135} = 67.29$, $P < 0.0001$; trophic position: $R^2 = 0.81$, $F_{1,135} = 87.15$, $P < 0.0001$) but was only significant within species for $\delta^{15}N$ and trophic position ($P < 0.0001$ in both cases); there were no species × size interactions. In short, $\delta^{15}N$ and trophic position increased with standard length.

Although our main focus was to investigate isotopic differences between species, some intraspecific variation between sites was observed. For the freshwater type, mean $\delta^{13}C$ was slightly less depleted for individuals from Kiritappu (-23.97 ± 2.92‰) compared with the Oboro River and Shiomi River (-26.21 ± 2.94 and -25.63 ± 1.54‰, respectively; $R^2 = 0.07$, $F_{2,64} = 3.61$, $P = 0.033$), suggesting more freshwater foraging or a more depleted freshwater baseline in the latter two populations. The mean $\delta^{15}N$ value for *P. tymensis* was slightly higher in the Oboro River (13.54 ± 1.01‰) compared with the Shiomi River (11.51 ± 1.54‰; $R^2 = 0.29$, $F_{1,48} = 20.64$, $P = 3.74 \times 10^{-5}$) and was reflected by a slight increase in estimated trophic level in the Oboro River (2.66 ± 0.30 vs. 2.05 ± 0.48 in Shiomi River).

Isotopic niche size did not differ significantly between the species based on posterior comparisons of SEA_B estimates ($P > 0.05$ based on posterior comparisons; Fig. 2C), with the distribution of difference between pairwise estimates always encompassing zero. There was no niche overlap at all between the brackish type and either of the other two species. However, niche overlap was high between the freshwater type and *P. tymensis*, with a mean overlap of 52% for the two species (51% and 53% of total isotopic niche area of each species, respectively).

Estimated dietary source contributions suggested a predominately putative marine diet for the brackish type (Fig. 3): putative marine dietary source contribution was greater for the brackish type than the other two species ($P < 0.001$ in both cases, based on posterior comparisons). Estimated dietary source contributions of *P. tymensis* were almost entirely freshwater (Fig. 3). Despite no obvious difference in diet between the freshwater type and *P. tymensis* using standard stable isotope analysis (see previous section), dietary source contributions indicated a greater proportion of putative marine dietary source in the freshwater type than in *P. tymensis* ($P < 0.002$).

Body shape variation revealed by geometric morphometrics

Analysis of centroid size revealed that the freshwater and brackish types showed larger body size than *P. tymensis* (GLMM with site as a random effect: $R^2 = 0.39$, $F_{2,96} = 7.62$, $P = 0.0008$ for species term; Fig. 4); centroid size also differed consistently between the sexes in all three species, with females being larger than males (same model, $F_{1,96} = 8.47$, $P = 0.0045$; Fig. 4). Multivariate analysis on all shape principal components revealed significant and large effects of species, body size, and sex on shape variation (see Table 3). Three major axes accounted for >10% of shape variation each, cumulatively explaining 57.2% of the total variance explained (PC1 = 22.9%, PC2 = 19.2%, PC3 = 14.5%; Fig. 5) and we therefore focus on these primarily here. Variation along PC1 contained some

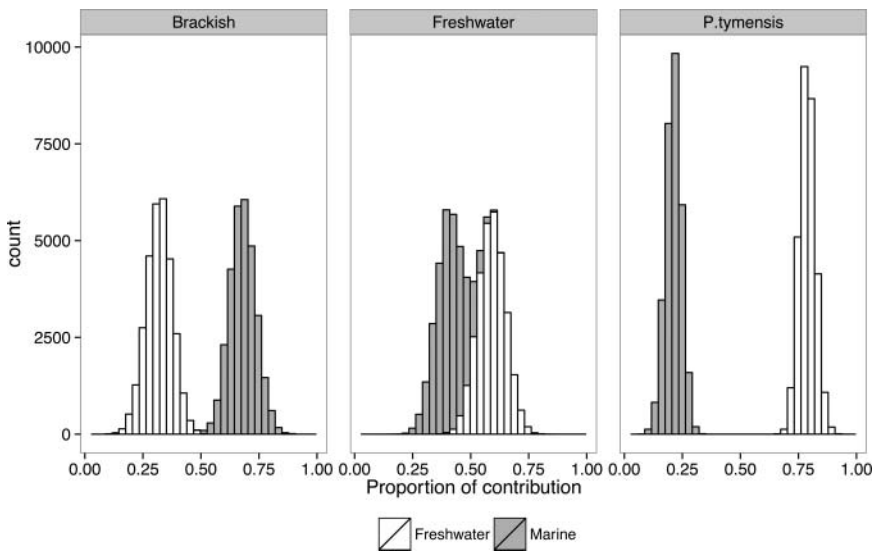

Fig. 3. Posterior probability distributions of mean dietary source contributions to each ninespine stickleback species estimated using *siar*.

Table 3. MANCOVA on all body shape principal components with centroid size as a covariate

Factor	d.f.	Wilks' λ	F	P	η^2
Body size	1,28	0.241	7.62	<0.0001	0.76
Sex	2,56	0.164	3.56	<0.0001	0.99
Species	2,56	0.008	24.19	<0.0001	0.58
Size × Sex	2,56	0.387	1.48	<0.05	0.55
Size × Species	2,56	0.417	1.33	0.09	0.84
Sex × Species	3,84	0.139	2.27	<0.0001	0.61
Size × Species × Sex	2,56	0.448	1.20	0.2	0.86

Note: η^2 represents the partial variance explained by each factor and is calculated as 1 – Wilks' λ. Note that for multivariate models, partial variance explained can sum to greater than 1 and is intended as a relative means of comparing factors.

specimen-bending artefacts but also represented an increase in depth in the caudal peduncle, elongation of the snout, and elongation of the premaxilla, as well as a slight posterior shift in the position of the pectoral fin (lower right panel in Fig. 5). PC2 clearly captured an overall deepening of the body and a slight elongation of both the snout and caudal peduncle (lower right panel in Fig. 5); PC3 also showed evidence of posterior deepening of the body and the caudal peduncle (lower right panel in Fig. 5).

GLMMs with site as a random effect indicated that centroid size contributed to shape variation on all three axes and significant shape differences occurred between species for PC1 and PC3 but not PC2 (Table 4). Nonetheless, there was a significant size × species interaction for PC2, indicating that shape variation with size for this axis differed between species (Table 4). Significant sex effects were present only for PC1 and PC2, although a significant sex × species interaction was present for PC3; the sex × species interaction was

Table 4. General linear mixed models for differences in body morphology summariszd by three major principal components (PC)

PC axis	Factor	d.f.	*F*	*P*
PC1	Size	1,93	5.64	0.020
	Sex	1,93	28.10	<0.0001
	Species	1,93	13.81	<0.0001
	Size × Species	1,93	2.30	0.106
PC2	Size	1,90	11.94	<0.001
	Sex	1,90	13.75	<0.001
	Species	1,90	2.06	0.133
	Size × Sex	1,90	1.32	0.253
	Size × Species	1,90	4.27	0.017
	Sex × Species	1,90	2.93	0.059
PC3	Size	1,91	7.80	0.006
	Sex	1,91	1.24	0.269
	Species	1,91	79.98	<0.0001
	Size × Species	1,91	1.76	0.178
	Sex × Species	1,91	3.24	0.044

Note: Terms differ because in all cases a full model was fitted (i.e. size as covariate, sex and species as fixed factors, all interactions permitted) and then the final model was chosen using stepwise analysis of AIC.

close to significant for PC2. Taken together, these data suggest that morphological differences between the sexes are not consistent among species (Fig. 5, Table 4). PC2 appears to reflect primarily sex differences with an increased body depth in males versus females (Fig. 5). Focusing on PC1 and PC3 as the main axes describing shape variation between the species (Fig. 5), there is considerable morphological divergence between *P. tymensis* and the freshwater type: *P. tymensis* individuals are characterized by an elongated (larger PC1) and typically deeper body shape (larger PC3), whereas the freshwater type has a shorter (smaller PC1) and thinner body shape (smaller PC3) (Fig. 5). In contrast, the brackish type was intermediate in body shape. In all species, males tend to have larger PC1 (more compressed body and larger head) and larger PC3 (deeper body shape) than females.

Species classification based on exchangeability analysis

Classification to the correct species (i.e. self-classification) was typically high (i.e. 2–3 times the random expectation) for all three species for all five trait categories (Fig. 6, upper panel), except that *P. tymensis* had the lowest rates of correct classification for both diet and trophic morphology (1.4 and 1.7 times the random expectation, respectively). *Pungitius tymensis* showed a high rate of misclassification to the freshwater type for these categories (1.25–1.5 times above the random expectation; see Fig. 6, upper panel), suggesting that foraging ecology offers a poor means of distinguishing these species.

For cross-classification analyses, the freshwater type showed a high rate of cross-classification to the brackish type for all categories (2–3 times the expected amount), except for diet and trophic morphology, for which cross-classification was highest to *P. tymensis*

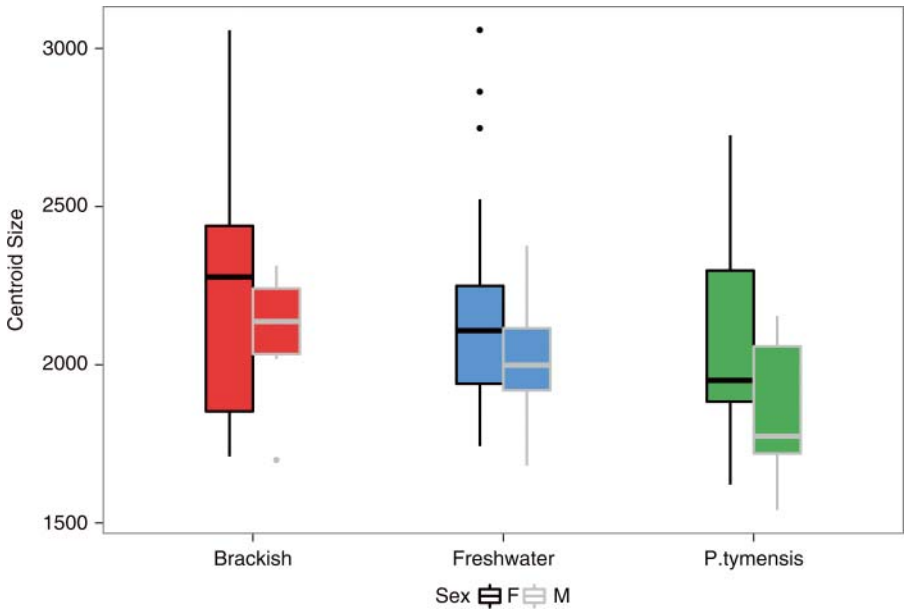
3000
2500
2000
1500
Centroid Size
Brackish
Freshwater
P.tymensis
Sex F M

Fig. 4.

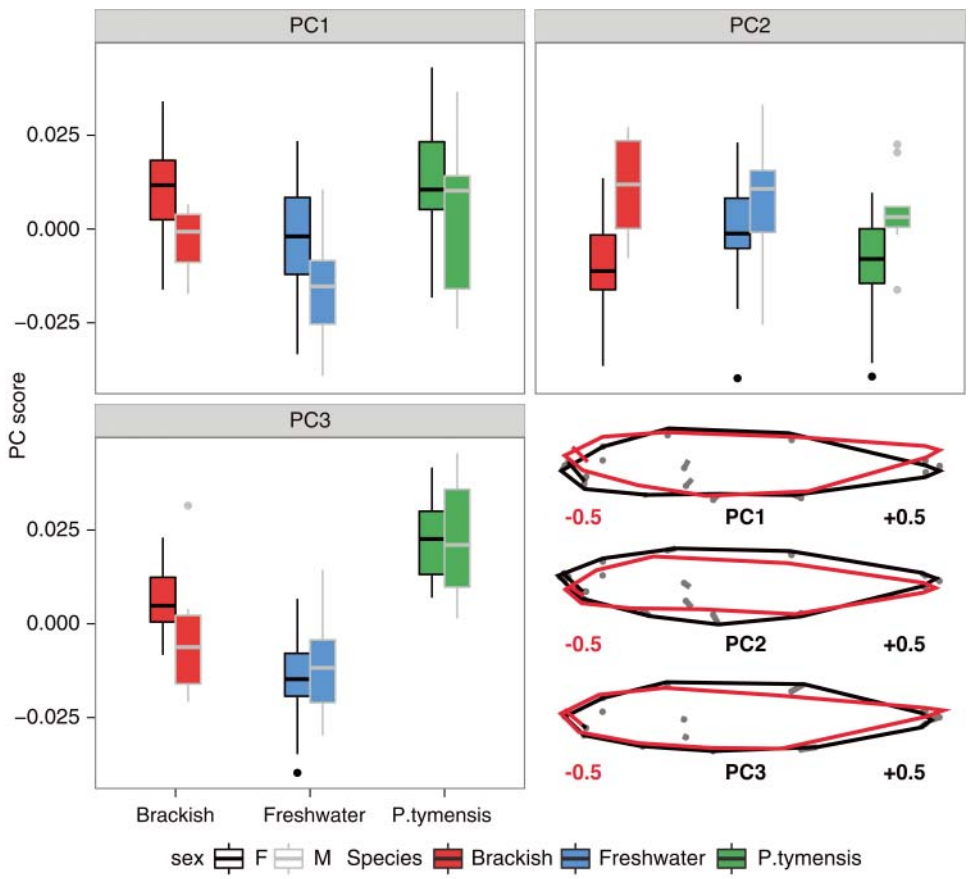
PC1
PC2
PC3
0.025
0.000
−0.025
PC score
-0.5
PC1
+0.5
-0.5
PC2
+0.5
-0.5
PC3
+0.5
Brackish
Freshwater
P.tymensis
sex F M Species Brackish Freshwater P.tymensis

Fig. 5.

(2.1–2.7 times the expectation; see Fig. 6, lower panel). Both brackish type and *P. tymensis* have high rates of cross-classification to the freshwater type for all traits, except that *P. tymensis* was cross-classified to not only the freshwater type but also the brackish type based on body shape.

DISCUSSION

Trophic niche differentiation amongst the three species

Our results demonstrate that the three *Pungitius* cryptic species show trophic niche divergence, but that there is a considerable niche overlap between the freshwater type and *P. tymensis*. Overall, our stable isotope data are consistent with our previous stomach content data that these three cryptic ninespine stickleback species exploit divergent prey resources along a marine–freshwater gradient (Ishikawa *et al.*, 2013). Source contributions suggested a greater proportion of putative marine prey in the brackish type than the other two species. Standard stable isotope analysis and Bayesian estimates of niche overlap suggested a similar benthic, freshwater diet for both the freshwater type and *P. tymensis*. However, the results of our mixing model analysis suggest subtle source contribution differences between the freshwater type and *P. tymensis* – specifically, a greater freshwater dietary contribution for the latter. Previous stomach content data also suggest some subtle niche differentiation between these two species; *P. tymensis* fed on benthic larvae, terrestrial insects, and some zooplankton, whereas the freshwater type fed primarily on benthic isopods in the midstream (Ishikawa *et al.*, 2013).

Stable isotope analysis is complementary to stomach content analysis. The long-term integration of diet is a potentially useful property of stable isotope analysis: for $\delta^{13}C$ and $\delta^{15}N$, fish muscle tissue typically reflects dietary assimilation over the previous 3–4 months (Hesslein *et al.*, 1993; Grey, 2000). This may be extended even further if sampling is conducted after overwintering, as growth typically ceases and nutrients are used to sustain basal metabolic processes (Perga and Gerdeaux, 2005). As the fish included in this study were collected in May, stable isotope signals likely mainly reflect growth from the previous summer. While it is possible that our isotopic data do not fully reflect trophic ecology after overwintering, the isotopic signal observed here might represent dietary preference over a considerable proportion of the life history of these species – an important consideration given that many fish species exhibit ontogenetic dietary shifts (Grey, 2001; Ravinet *et al.*, 2010), including sticklebacks (Sillett and Foster, 2000; Ravinet *et al.*, 2014). Indeed, a positive relationship between standard length and trophic position suggests some dietary shifts occur with growth in all three species. Furthermore, stable isotope analysis can give us information about trophic position. Higher $\delta^{15}N$ in the brackish type indicates foraging at a higher trophic level than either of the other

Fig. 4. Body size (measured as centroid size) differences among ninespine stickleback species.

Fig. 5. Principal component (PC) analysis of size-corrected Procrustes residuals produced by geometric morphometrics results in three major axes of divergence (PC1, PC2, and PC3). Boxplots show variation among species and sexes – full-colour boxes to left are female, opaque boxes to right are male. The lower right panel indicates outlines showing how body shape varies along each axis. Black outlines represent shapes with a lower value (–0.5); red outlines represent shapes with a higher value (+0.5); grey dots indicate the positions of consensus body shape.

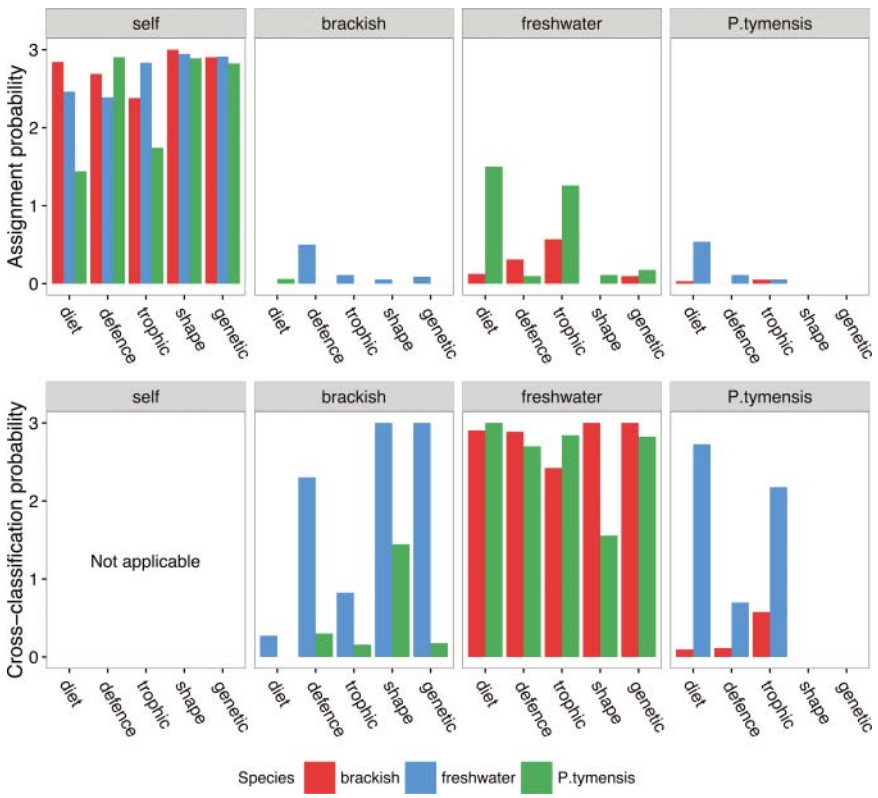

Fig. 6. Exchangeability analysis. Ratio of proportion assigned to each category to the expected proportion assigned by chance is shown. Upper panels show assignment to correct species (i.e. self-classification) or misclassification to one of the two alternative species; here, random expectation is 0.33. Lower panels show cross-classification where individuals are assigned to the next highest-probability following correct classification; here, random expectation is 0.5. Individual panels represent species assigned to; bar colours indicate 'true' species.

two species. The difference in trophic levels likely reflects the presence of cryptic trophic levels within the marine planktonic food-web (Harrod and Grey, 2006; Santer *et al.*, 2006).

It should be noted that our Bayesian mixing model analysis suggests some contribution of putatively marine prey resources to *P. tymensis* (median 21%, 14–27% highest posterior density). We argue that this is mostly due to the fact that the putatively marine prey resource we used in this analysis does not represent a complete marine signal – this would also explain why the majority of brackish individuals are more $\delta^{13}C$ enriched than their putative marine prey (Fig. 2A).

Body shape divergence among the three species

Our geometric morphometric analysis showed considerable body shape variation among ninespine stickleback species. Compared with the patterns of body shape variation in three-spine sticklebacks, we found a somewhat mixed pattern of convergence in body shape

between *Pungitius* and *Gasterosteus*. Benthic foraging *P. tymensis* has an increased body depth and a compressed caudal peduncle compared with the planktivorous brackish type, whereas the similarly benthic freshwater type has a much more shallow body depth and a more slender, elongated caudal peduncle than the brackish type. In threespine sticklebacks, a shallower, more slender body shape is associated with open-water, planktivorous foraging, whereas a deeper bodied shape is associated with benthic feeding (Schluter, 1995; Berner *et al.*, 2008; Willacker *et al.*, 2010). Increased body depth suggests more manoeuvrability in complex environments, which is likely linked to greater foraging efficiency on benthic prey (Walker, 1997; Hendry *et al.*, 2011). Our data therefore indicate that there are some differences in patterns of morphological variation between threespine and ninespine sticklebacks.

In addition to morphological differences among species, we also found significant sex differences in body shape within species. In all cases, males tended to have larger heads and deeper body shapes, while females tended to have longer bodies. This pattern of sexual dimorphism is similar to that observed in threespine sticklebacks (Kitano *et al.*, 2007, 2012; Spoljaric and Reimchen, 2008; Aguirre and Akinpelu, 2010). However, body shape sexual dimorphism appears to be more pronounced in threespine sticklebacks than ninespine sticklebacks: there is very little overlap between male and female body shape in the former (Kitano *et al.*, 2007, 2012; Aguirre *et al.*, 2008; Cooper *et al.*, 2011), whereas substantial overlap between the sexes is observed in the latter. Although adaptation to divergent resources between the sexes is one of the possible driving forces of the evolution of sexual dimorphism in threespine sticklebacks (Bolnick and Lau, 2008; Reimchen *et al.*, 2008; Cooper *et al.*, 2011), we did not find any significant differences in stable isotopes between the sexes in the ninespine stickleback species analysed. These results suggest that the patterns and driving forces of sexual dimorphism may differ between threespine and ninespine stickleback lineages despite the fact that directions of shape differences are often shared.

Evolutionary inferences from exchangeability analysis

Our exchangeability analysis indicated that multiple trait measurements enable us to distinguish these three cryptic species with a high degree of confidence. However, both diet and trophic morphology proved to be relatively poor distinguishing traits between the freshwater type and *P. tymensis*. Despite the niche similarity between the freshwater type and *P. tymensis*, no hybrids were found where these two species are sympatric (Ishikawa *et al.*, 2013), suggesting that trophic divergence is unlikely to play an important role in maintaining reproductive isolation. However, there is habitat isolation, with *P. tymensis* inhabiting more upstream habitats than the freshwater type (Tsuruta *et al.*, 2008; Ishikawa *et al.*, 2013). Divergence in some other physiological traits, including salinity tolerance (Ishikawa *et al.*, 2013), may contribute to habitat isolation.

Reflecting the differences in preferred habitats (the most upstream for *P. tymensis* and the most downstream for the brackish type), misclassification of the brackish type and *P. tymensis* into each other was generally low except for body shape. The brackish type and the freshwater type could also be confidently classified based on the majority of traits, although some brackish individuals were misclassified into the freshwater type based on trophic morphology and defence morphology. Because the frequencies of hybrids were very low between the brackish type and the freshwater type (Ishikawa *et al.*, 2013), a combination of isolating barriers, including ecological divergence (Ishikawa *et al.*, 2013 and this study) and hybrid male sterility (Takahashi *et al.*, 2005), likely contribute to total reproductive isolation. It should be

noted that we analysed only wild-caught fish in this study, so we cannot exclude the possibility that phenotypic divergence may be caused by phenotypic plasticity (Wund *et al.*, 2008; Svanbäck and Schluter, 2012). Further work is necessary to characterize physiological divergence and isolating barriers among these three species and also understand their genetic basis.

Finally, the high level of accuracy exhibited by our exchangeability analysis suggests this method, which incorporates variation at the individual level (Hendry *et al.*, 2013), could be used to identify cryptic *Pungitius* species. Although identification of species was conducted using neutral genetic markers in this study, our data set could be feasibly used as a training set for further analyses, removing the need for genotyping new samples. By distinguishing cryptic species non-lethally at low cost (i.e. using body shape and defence trait measurements only), exchangeability analysis could be used in conservation efforts to preserve rare ninespine stickleback populations.

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