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Stable isotope and molecular analyses indicate that hybridization with non-native domesticated common carp influence habitat use of native carp

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Hybridization between native and non-native species has consequences for survival and growth rates of hybrid offspring, but the influences on their functional roles such as habitat use are little studied and poorly understood. The Japanese native common carp *Cyprinus carpio* coexist and hybridize with non-native domesticated carp in natural Japanese lakes. We have combined stable isotope and molecular information to examine whether habitat use of carp varies depending on the degree of hybridization between native and non-native carp. We sampled 69 carp from Lake Kasumigaura where hybrid swarms between native and non-native carp are advancing, evaluated the degree of hybridization for each individual by genotyping five single nucleotide polymorphism (SNP) markers, and analyzed their carbon and nitrogen stable isotopes. Although we did not find any genetically pure native carp in the lake, the results showed that carp $\delta^{13}C$ increased with increasing frequency of non-native alleles but that $\delta^{15}N$ did not change. This indicates that non-native carp use the littoral zone more frequently than native carp. This difference in habitat use was supported by a multisource mixing model, showing that the contribution of limnetic primary consumers to the diets of non-native carp was lower than that of individuals with the highest frequency of native alleles. By combining two very different methods, our results thus suggest that multiple-generation hybridization can influence habitat and resource use. Habitat partitioning should be considered when evaluating the genetic impacts of invasive species and races on native species and ecosystem processes.

Biological invasions of non-native species are widely recognized as a key to the loss of biodiversity (Vander Zanden et al. 1999, Sala et al. 2000, Kolar and Lodge 2001). Hybridization between differentiated lineages can threaten the genetic integrity and persistence of native species, and act as a force driving genetic homogenization and extinction (Allendorf et al. 2001, Perry et al. 2002, McDonald et al. 2008). A primary concern of many conservation biologists is that hybridization may lead to rapid displacement of native species by hybrid swarms or largely non-native admixtures (Fitzpatrick and Shaffer 2007).

Both empirical and theoretical studies suggest that hybridization between differentiated lineages can have many different consequences depending on variation in fitness among hybrid offspring (Rhymer and Simberloff 1996, Huxel 1999, Ellstrand and Schierenbeck 2000). It is well documented that the hybrids can have lower or greater fitness relative to that of either parental species (outbreeding depression or heterosis, respectively). However, the study of hybridization has been dominated by a focus on effects on survival and growth rates (McGinnity et al. 2003), and the impact of hybridization on functional roles through genetic character changes has been received much less attention until now. Because functional roles can relate fitness to genotypes and play important roles in ecosystem processes (Kareiva and Levin 2003), ignoring the impacts on functional roles can result in underestimating genetic impacts of invasive species on native species and ecosystem processes.

The functional roles of species can be categorized by their habitat use, trophic status, and ecosystem function (Mumby et al. 2008). To our knowledge, only one empirical study, though in laboratory-controlled conditions, demonstrated the possibility that the hybrids (F_1 and backcross) between non-native *Cyprinodon variegatus* and endemic *C. pecosensis* displayed greater swimming performances than purebred *C. pecosensis*. This suggests hybrids can have different functional roles from either parental species (Rosenfield et al. 2004). However, under natural conditions where hybrid

swarms occur, there is a considerable probability that multi-generation hybridization could change functional roles of advanced generation hybrids (F_3 and later generations after further recombination).

Freshwater fish face a considerable threat of hybridization with non-native taxa, and their number, origins, methods of introduction and impacts have been the most extensively documented of all aquatic organisms (Richter et al. 1997, Perry et al. 2002). Common carp *Cyprinus carpio* are among the most widely translocated species in the world for human consumption and sports fishing, though its natural range was restricted to temperate Eurasia (Barus et al. 2001). Even in Japan where the common carp is native, the Japanese native carp strain is suffering from cryptic multiple invasions by morphologically similar non-native domestic strains (Mabuchi et al. 2008). According to Maruyama et al. (1987), Japanese institutes have imported several foreign domesticated strains from some European and Asian countries since 1905, and large numbers of domesticated stocks have been introduced into Japanese natural waters frequently. Most importantly, a recent development of nuclear DNA markers provides evidence of hybridization between native and non-native carp, and has shown that some populations become hybrid swarms (Mabuchi unpubl.). In these cases, the functional roles of native carp may change through multi-generational hybridization with non-native carp.

The purpose of our study is to examine whether habitat use of native carp changes with the degree of hybridization with non-native carp. Specifically, we examined natural carp populations in Lake Kasumigaura where hybrid swarms occur due to extensive stocking for aquaculture since 1969. Habitat and resource use by carp was estimated using stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$) and mixing models. We predicted that $\delta^{13}C$ and/or $\delta^{15}N$ values of carp would change with degree of hybridization, which was quantified using neutral nuclear DNA markers.

Conventional methods (telemetry, direct dietary analyses) for habitat and resource use have several limitations, including small sample size, high cost, and the rapid digestion of soft-bodied prey, thus providing only a snapshot of an individual's diet. Animals with high degree of omnivory compound these problems. In contrast, stable isotope ratios of consumer tissues integrate dietary variation over an extended period offering a powerful tool for characterizing trophic pathways (Vander Zanden et al. 1999, Post 2002, Rubenstein and Hobson 2004). Consumer $\delta^{13}C$ values can be used to determine ultimate sources of dietary carbon, because there is little trophic enrichment (<1.3‰; Post 2002). $\delta^{15}N$ values become enriched by 2–4‰ between prey and predator tissues, thereby providing an estimated of consumer trophic position (Vander Zanden and Rasmussen 1999, Vanderklift and Ponsard 2003). In addition, animals incorporate isotopic signatures into their tissues that reflect the food source or their environment. Provided environmental or food-web isotope signatures vary spatially or across habitat gradients, animal migrations can be inferred using isotope techniques (Hobson 1999). In particular, in freshwater food webs, $\delta^{13}C$ values of primary consumers become gradually more enriched from limnetic (pelagic) to littoral, because benthic (attached) algae are typically enriched in $\delta^{13}C$ values relative to phytoplankton (France 1995, Hecky and Hesslein 1995, Post 2002). Furthermore, recent advances in mixing models (Phillips and Gregg 2003, Phillips et al. 2005) allows us to calculate feasible range of contributions of multiple food sources to a consumer's diet (Hicks et al. 2005, Inger et al. 2006, Caut et al. 2008).

Methods

Study lake

Lake Kasumigaura (approximately 60 km northeast of Tokyo), the second largest lake in Japan, is a shallow lake with a maximum water depth of 7 m. The lake area 220 km^2 and its catchment total 2157 km^2. In the lake, extensive carp farming using floating-net-cage culture had been carried out since 1969. The maximum production of 8800 tonnes $year^{-1}$ of carp in whole basin of the lake was recorded in 1978, and about 5000–7000 tonnes of carp had been produced annually before the spread of the koi herpes virus (KHV) in 2004 (Statistics Dept, Ministry of Agriculture, Forestry and Fisheries of Japan 2003). Carp farming and stocking have been not permitted since then. Mabuchi et al. (2008) demonstrated that carp populations in the lake were predominated by the non-native haplotypes, probably because intentional releases and accidental escapees from the net pens used for aquaculture. Unfortunately, there is no reliable information when a hybrid swarm formed. Considering carp aquaculture history, it is reasonable to assume that hybridization and backcrossing have been occurring for about 40 years, or an estimated 10–20 generations, because carp reach sexual maturity at the age of 2–4 years (Barus et al. 2001).

Sample collection

A total of 69 carp were caught in Kita-ura, the north bay of Lake Kasumigaura (36˚04'N, 140˚32'E) in June, July, August and November 2006. Carp sampling was carried out by commercial stationary nets in cooperation with local professional fishermen, whose nets select for carp of TL > 200 mm. Because the nets were set for several consecutive days, we could not quantify the stomach contents of carp adequately. Fish were kept in a cool box until they were processed in the laboratory. In the laboratory, fish standard length (SL, ± 1.0 mm) and wet weight (± 1.0 g) were measured.

We collected potential carp food items for stable isotope analysis from around the fish collection site during summer 2006. Major food items obtained included shrimps *Macrobrachium nipponense*, zooplankton (mainly *Diaphanosoma*.sp and copepods), chironomid larvae (mainly *Chironomus plumosus*), and periphyton and detritus. This choice was based on earlier studies of dietary analyses (Garcia-Berthou 2001, Khan 2003). Shrimps were collected together with carp using the stationary nets. From the deep part of the lake (limnetic zone, depth 4–5 m), bulk zooplankton were filtered from the water using a 500-μm mesh net and visually inspected to remove particulate contaminants. At the same location, chironomid larvae were collected simultaneously using an Ekman grab sampler. Based on Post (2002), we

collected a mixture of periphyton (attached algae) and detritus from the littoral zone (depth < 2 m). Periphyton and detritus were brushed from buoy ropes and emergent macrophytes that had been placed near the bottom using a soft toothbrush.

Genetic analysis

We assessed the degree of hybridization of each individual using nuclear DNA markers. According to the result of the Mabuchi et al.'s (2008) mitochondrial DNA analysis, the main genetic split within common carp is between Japanese and Eurasian (European and Chinese) strains. Thus, it is expected that also in nuclear DNA the main genetic difference is between the Japanese native and non-native (Eurasian) strains. Unfortunately, no pure native population of common carp has so far been found in Japanese natural waters: in every Japanese waters so far analyzed (including Lake Kasumigaura), more than or nearly half of the haplotypes originated from Eurasian strains (Mabuchi et al. 2008). However, a recent detailed mitochondrial DNA survey in Lake Biwa, Japanese's largest lake, revealed that more than 80% of specimens from the deep off-shore waters (30–70 m) had native Japanese haplotypes. Furthermore, >90% of the specimens that had washed ashore in the lake during mass mortality caused by koi herpes virus (KHV) in 2004 had native Japanese haplotypes (Mabuchi et al. in press). To develop nuclear DNA markers discriminating between Japanese native and non-native strains, we used the following 31 specimens as those of the hypothetical Japanese native strain: 19 specimens from the deep waters of the lake, and 12 KHV-killed specimens. These specimens were compared with 30 specimens from Eurasia (Europe, China, Taiwan and Indonesia). Within the five DNA markers used in this study (for details see below), differences between the Eurasian and hypothetical Japanese native strains were almost completely fixed (Mabuchi et al. unpubl.).

In the first step of nuclear DNA marker development, a microsatellite-enriched genomic library from the Lake Biwa wild strain of common carp (Mabuchi et al. 2006) was used for isolation of HapSTR makers (Mabuchi et al. unpubl.). A HapSTR, a conjunction of 'haplotype' and 'short tandem repeat' maker, consists of a microsatellite region and its flanking sequences (Hey et al. 2004). In this study, single nucleotide polymorphisms (SNPs) in the flanking sequences were used as markers, because significant levels of homoplasy have been reported for microsatellite allele length in various animals (van Oppen et al. 2000). Five SNPs within different five HapSTR loci were used as diagnostic markers for Japanese native versus non-native populations. Primers for four of the five HapSTR loci (c20, 25, 37, 52) are described in Mabuchi et al. (unpubl.). The fifth locus (Koi 57-58) used primers from David et al. (2001).

Five SNPs were genotyped by direct-sequencing of the flanking regions for 69 individual carp from Lake Kasumigaura. Total DNA was extracted from a fin clip preserved in ethanol, using an AquaPure genomic DNA purification system according to the manufacturer's protocol. Multiplex PCR was performed using a thermal cycler and reactions were carried out using 40 cycles of a 10 μl reaction volume containing 4.5 μl of distilled water, 1.0 μl of 10× PCR buffer, 1.0 μl of dNTP (4 mM), 0.3 μl of 0.5 unit Ex Taq, 1.0 μl of template, and 0.25 μl each primer (10 μM) (following 10 primers included: c20-F, -R, c25-F, -R, c37-F, -R, c52-F, -R, Koi 57-58-F, -R). The thermal cycle profile was as follows: denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s. The PCR products were electrophoresed on a 1.0% L 03 agarose gel column and stained with ethidium bromide for band characterization via ultraviolet transillumination. Double-stranded multiplex PCR products, purified using a presequencing kit, were subsequently used for direct cycle sequencing using dye-labeled terminators. Primers used were five of the ten primers used for the multiplex PCR: c20-F, c25-R, c37-R, c52-R and Koi 57-58-F. All sequencing reactions were performed according to the manufacturer's instructions. Labeled fragments were analyzed using a DNA sequencer. Sequence editing and SNP genotyping was performed using EditView ver. 1.01.

Hybrid index (HI) for each individual was calculated by entering a score of 0 for each non-native SNP and 1 for each Japanese native SNP. Scores for all five loci thus ranged from 0 (all non-native SNPs) to 10 (all Japanese native SNPs). SNP frequencies are available from the authors.

Stable isotope analysis

For stable isotope analysis, dorsal muscle tissue was taken from individual carp. The use of white muscle tissue for stable isotope analysis has lower variability in $\delta^{15}N$ values compared to other tissues, and does not require acidification to remove inorganic carbonates (Pinnegar and Polunin 1999). Shrimps were dissected, and only muscle tissue was removed and used for isotope analysis. Fish and shrimp muscle samples, and bulk zooplankton samples were dried at a constant temperature (50°C) for 48 h and ground to a fine powder with an agate pestle and mortar. We then extracted lipids from all animal tissues using a 2:1 chloroform:methanol solution because lipids are depleted in ^{13}C compared with whole organism (Peterson and Fry 1987). Periphyton and detritus samples were prefiltered through a 100-μm mesh net to remove large invertebrates, followed by filtration onto precombusted glass-fiber filters (GF/C) and drying at 50°C for 48 h.

Stable isotope analyses were performed on a continuous flow isotopic ratio mass spectrometer. The standard errors of the replicates of all our analyses were 0.03‰ for $\delta^{13}C$ and 0.11‰ for $\delta^{15}N$. All stable isotope values are reported in the δ notation: $\delta^{15}N = ([(^{15}N_{sample}/^{14}N_{sample})/(^{15}N_{standard}/^{14}N_{standard})] - 1) \times 1000$, where the global standard is atmospheric nitrogen, and $\delta^{13}C = ([(^{13}C_{sample}/^{12}C_{sample})/(^{13}C_{standard}/^{12}C_{standard})] - 1) \times 1000$, where the global standard is PeeDee Belmnite (Peterson and Fry 1987).

Statistical analysis

We were interested in the effect of hybridization index (HI) on $\delta^{13}C$ and $\delta^{15}N$ values of carp, but we considered other two possible factors; standard length of carp (Size) and sampling month (Season). The weight of carp were not included because there was a significant relationship between carp size and weight ($r^2 = 0.91$). We examined the effects of HI, Size and Season on $\delta^{13}C$ and $\delta^{15}N$ values of carp using

generalized linear models (GLMs) with Gaussian distribution and identity link. We carried out simple regressions among the predictor variables to test for multicollinearity. Following Burnham and Anderson (2002), we used an information-theoretic model selection approach to rank and evaluate the models. The first step of the model selection procedure was to calculate the Akaike's information criterion corrected for small sample sizes (AIC_c) for the full set of candidate models. The Akaike weights (w_i) were then calculated for each model to identify the models that best explained our data. The Akaike weight reflect the weight of evidence in support of a particular model relative to the entire model set, and varied from 0 (no support) to 1 (complete support) (Burnham and Anderson 2002). The 'null' model was included as an intercept only. The candidate model with the highest Akaike weight was selected as the best model. Models with $\Delta AIC \leqslant 2$ were considered to have substantial support as candidate models (Burnham and Anderson 2002). We conducted a likelihood ratio test between all models with substantial support and the null model. Standardized parameter estimates and SE were calculated to evaluate the relative importance of individual variables in the best-performing models. The statistics program R 2.7.1 (R Development Core Team 2008) was used for all the analyses.

Mixing model

We used the program IsoSource (Phillips and Gregg 2003) to estimate carp diets. This program allowed us to calculate feasible range of contributions of multiple possible sources to consumer's diet, when the number of sources was too large to permit unique solutions from general mass balance mixing models. We used source increments of 1% and a mass balance tolerance of ± 0.1‰. We evaluated contribution of each food item (shrimps, chironomid larvae, zooplankton, and periphyton and detritus) to carp diet. The mean $\delta^{13}C$ and $\delta^{15}N$ values for each food source were used in the analysis. To correct the food items for trophic fraction; we added + 0.4‰ to $\delta^{13}C$ based on the carbon trophic fraction determined from literature sources of aquatic animal studies (Post 2002, McCutchan et al. 2003). Following the meta-analysis of Vanderklift and Ponsard (2003), we employed a mean $\delta^{15}N$ enrichment of 2.54 ‰, which is similar to enrichment estimated by the laboratory experiment using carp (Sakano et al. unpubl.).

The range of potential contributions from each source in some case is narrow and constrained, allowing for easy interpretation, while in other cases the range is broad and diffuse, which limits meaningful conclusions about source contributions. For more constrained and interpretable results, a posteriori aggregation method was applied to pool the frequency distributions generated by IsoSource for food sources that share common attributes, for example, primary producers with similar $\delta^{13}C$ values (Phillips et al. 2005). We chose to combine the fractions of zooplankton and chironomid larvae as one food item (limnetic primary consumers). This is because chironomid larvae in our study site were depleted in ^{13}C, indicating that they are supported by limnetic phytoplankton-derived detritus (Vander Zanden et al. 2006). After the mixing analysis, the IsoSource output was read into Excel software, where a new variable was created, i.e. the sum of sources to be combined (zooplankton + chironomid larvae = limnetic primary consumers). Thus, we calculated the mean and 1st to 99th percentiles of three possible source contributions.

Results

Degree of hybridization and stable isotopes ratios in carp

The mean score of the hybrid index (HI) for total 69 carp was 2.0 (range 0–8), and there were no genetically pure Japanese native carp with a HI score of 10. Three models had substantial support for explaining the variation in carp $\delta^{13}C$ values (Table 1). The model containing both size and HI had the most support (likelihood ratio test; DF = 1, p = 0.02). HI had a larger standardized parameter estimate than size (–0.12 ± 0.06 SE, –0.10 ± 0.06 SE, respectively). The fitted model showed a negative relationship between $\delta^{13}C$ values and HI (Fig. 1). The model including only HI also had substantial support (likelihood ratio test; p = 0.03), indicating that degree of hybridization is a significant variable in predicting carp $\delta^{13}C$ values.

Only one model including size had substantial support for explaining the variation in carp $\delta^{15}N$ values (Table 1, likelihood ratio test; DF = 1, p = 0.02). The parameter estimate for size was 0.02 ± 0.01, indicating that $\delta^{15}N$ values are expected to increase with size. The model including both size and HI was only moderately supported, but was more likely than the null model. $\delta^{15}N$ values tended to

Table 1. Model comparison for testing the effects of hypothesized independent variables (a) $\delta^{13}c$ and (b) $\delta^{15}N$. All possible combinations variables were compared.

Model	K	AIC_c	Δ_i	w_i
(a)$\delta^{13}C$				
Size + HI	4	108.14	0.00	0.27
HI	3	108.47	0.33	0.23
Size	3	109.28	1.13	0.15
Size + HI + Season	7	110.46	2.32	0.08
Size + Season	6	110.47	2.32	0.08
HI + Season	6	110.66	2.51	0.08
Null	2	111.04	2.90	0.06
Season	5	111.70	3.56	0.05
(b)$\delta^{15}N$				
Size	3	228.23	0.00	0.56
Size + HI	4	230.48	2.25	0.18
Null	2	231.17	2.94	0.13
Size + Season	6	232.96	4.73	0.05
HI	3	233.07	4.85	0.05
Size + HI + Season	7	235.44	7.21	0.02
Season	5	235.49	7.26	0.01
HI + Season	6	237.74	9.52	0.00

Definitions of abbreviations used are as K = number of estimated parameters in the model; = Akaike's information corrected for small sample; Δ_i = difference in AIC_c between best model (model with smallest value of AIC_c and model; w_i = Akaike weight, which indicates the weight of evidence in favour of model i.

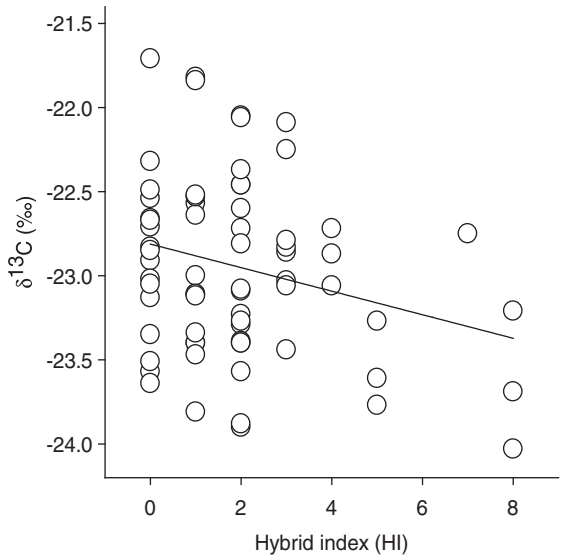

Figure 1. Observed (filled circles) and predicted (gird line) $\delta^{13}C$ values of carp in relation to their body size and hybrid index (HI). HI values close to 0 indicate a fish with all non-native single nucleotide polymorphisms (SNPs), while individuals with values close to 10 indicate a fish with all Japanese native SNPs.

increase with HI, but the estimate reported above was not significant (0.005 ± 0.08, i.e. included zero within the confident interval) (Fig. 2).

IsoSource modelling

Because we found the effect of HI on stable isotope ratios, we performed mixing model using the mean isotopic data of two contrasting carp groups; individuals with HI of zero (n = 17, mean $\delta^{13}C$ = –22.9 ‰, $\delta^{15}N$ = 17.8 ‰) and individuals with HI of eight (n = 3, mean $\delta^{13}C$ = –23.6 ‰, $\delta^{15}N$ = 17.9 ‰). For both groups, a 'constrained solution'

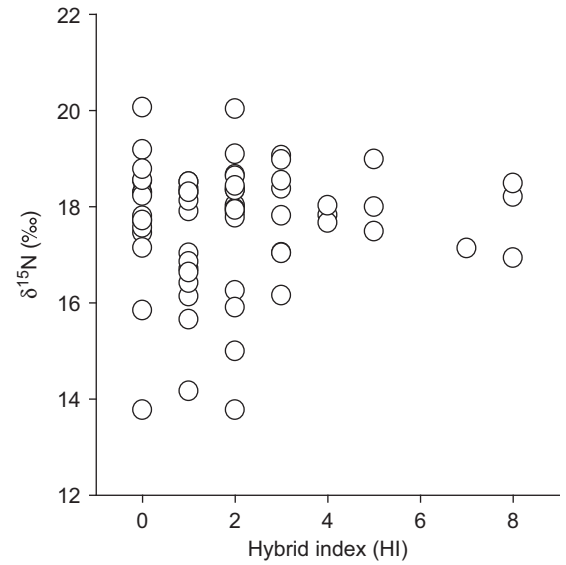

Figure 2. Relationship between $\delta^{15}N$ values for carp and their body size.

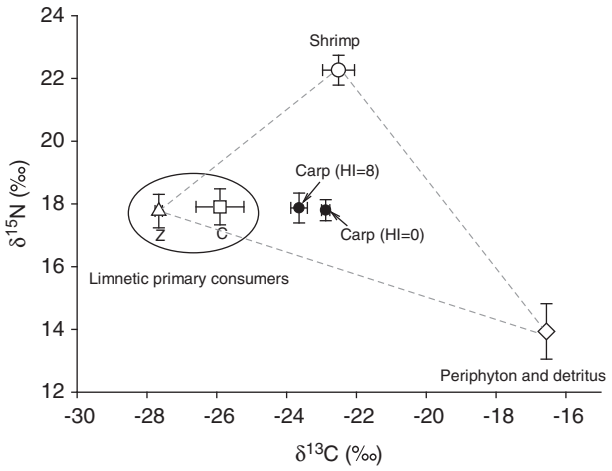

Figure 3. $\delta^{13}C$ and $\delta^{15}N$ (Mean ± SE) of the four food sources (open symbols, after correction for trophic enrichment). Chironomid larvae (C) and zooplankton (Z) are limnetic primary consumers. The polygon is circumscribed by the isotopic values of the end-member food sources, with the signatures of the carp with HI = 0 and HI = 8 in its centre (filled circles).

(Phillips et al. 2005) was produced, indicating that the range of possible contributions for the three candidate sources is well constrained. As a result, the models showed differences in the range of contributions of three food sources between the carp groups. Carp individuals with HI of eight had higher mean proportions of the limnetic primary consumers and lower mean proportions of shrimps, and periphyton and detritus than those of carp with HI of zero (Fig. 3, Table 2).

Discussion

By using two very different approaches, stable isotope and molecular analyses, we clearly demonstrate that the degree of hybridization influence on carp $\delta^{13}C$ values, and by inference, diet and habitat (Fig. 1). Recent decades have seen tremendous progress in invasive species research. To our knowledge, there have been very few detailed studies focused on the impacts of multi-generational hybridization, and our study is the first to demonstrate changes in functional roles of native species through hybridization with non-native species in natural environments. However, we stress that this potential impact through hybridization should be carefully considered because our observed pattern is based on small sample sizes with carp individuals with HI > 4, although statistically significant.

In freshwater systems, $\delta^{13}C$ values have proven beneficial for discriminating between two major pathways of carbon production (France 1995). The $\delta^{13}C$ values of limnetic organisms are generally more depleted as a result of reduced isotopic discrimination between dissolved inorganic carbon and benthic algae during carbon fixation than the open water phytoplankton (France 1995, Hecky and Hesslein 1995, Post 2002). In the present study, carp showed lower $\delta^{13}C$ values with higher HI score, indicating that carp with higher HI score utilize limnetic zone as their feeding habitats more frequently. This difference in habitat

Table 2. Mean proportions and 1st–99th percentile ranges of possible contributions to the diet of carp with HI = 0 and HI = 8, which were caluculated by using IsoSource program (Phillips and Gregg 2003).

	Food		
Carp	Pelagic primary consumers† (%)	Shrimp (%)	Periphyton and detritus (%)
HI = 0	48 (42–56)	24 (19–28)	28 (25–31)
HI = 8	59 (53–66)	19 (15–23)	21 (18–25)

†Following the method descirbed by Phillips et al. (2005), the fraction of zooplankton and chironomid larvae sampled in pelagic zone are combined into pelagic primary consumers.

use is supported by the results from IsoSource. Although the mean HI = 8 used in the mixing model was calculated based on only three individuals, carp individuals with the highese HI score relied on a higher proportion of limnetic primary consumers (zooplankton and chironomid larvae) and a lower proportion of periphyton and detritus in the littoral zone, compared to individuals with the lowest HI scores (HI = 0; Fig. 3, Table 2).

Our observed $\delta^{13}C$ pattern appears to represent differences in habitat and resource use, but variation in carp $\delta^{13}C$ values and diet proportions estimated from IsoSource between carp individuals with HI = 0 and HI = 8 were not large. It remains unclear that to what degree $\delta^{13}C$ difference can reflect significant different habitat use, but some previous studies have shown that even small differences in $\delta^{13}C$ value, which cannot be addressed in traditional stomach content analysis, could reflect significant partial dietary segregation and difference in feeding microsite among sex or ecological similar species (Genner et al. 1999, Forero et al. 2002, Cherel et al. 2008). Alternatively, lake morphology could make it hard to detect difference in habitat use among hybrid carp individuals. A recent isotopic study has demonstrated that lake morphology can change the degree of littoral habitat use in a mobile predator lake trout *Salvelinus namaycush* (Dolson et al. 2009). In the case of shallow lakes such as Lake Kasumigura, the boundary between the limnetic and littoral zones is typically not clear. In contrast, in Lake Biwa (mean depth 41 m; maximum depth 104 m) with higher habitat complexity of littoral habitats where genetically nearly pure native carp remain in Japan, non-native domesticated carp are typically only observed in shallower habitats in the lake (Mabuchi et al. in press). Several earlier field observations in Lake Biwa showed that wild carp (probably a native strain but not determined by genetic analysis) used pelagic and deep habitats throughout the year except spawning season (Furukawa 1966). For these reasons, we infer that habitat use of the littoral and limnetic zone by carp in Lake Kasumigaura overlaps, but is at least partly influenced by the degree of hybridization.

Using neutral nuclear SNP markers, we found a gradual change in habitat use of carp from limnetic to littoral with increasing non-native alleles. However, carp individuals with lower HI seemed to increase variances in $\delta^{13}C$ values (Fig. 1). One possible reason for this result is differences in adaptive traits to the wild environments among feral non-native domesticated carp. Because a large feral population has coexisted for a long time with native carp in many natural Japanese freshwaters (Mabuchi et al. 2008), carp individuals even with lower HI scores are likely to include feral strains that fully adapted to the wild environments (i.e. re-adaptation). The presence of the adapted strain is supported by our experimental previous study, demonstrating a feral strain with low mean HI score has some morphological and behavioral traits which seem to adapt to the wild environment, in spite of being reared in captive environment (Matsuzaki et al. 2009). We suspect that the habitat use of carp might be partly influenced by feralization and naturalization, as well as by degrees of hybridization itself.

The relationship between carp $\delta^{15}N$ values and HI was not clear, but carp $\delta^{15}N$ values tended to be slightly higher with increasing HI (Fig. 2). Kafuku (1966) reported that the Japanese wild strain have fewer gill rakers and a shorter intestine than domesticated carp, although genetic identification was not performed. If the wild strain used in Kafuku (1966) was genetically pure or nearly pure native carp, their results suggest that native carp are more carnivorous and have higher trophic level. Thus, the moderate increase of carp $\delta^{15}N$ values with increasing HI could result from difference in feeding behaviour, but this has not yet resolved and remains as a future challenge.

Although there is little recognition of changes in functional roles such as through hybridization, these changes can result in alterations of ecosystem functioning (Olden et al. 2004). In aquatic systems, it is known that fishes play particularly important roles as habitat couplers (e.g. littoral – limnetic) as a result of their high mobility and flexible foraging tactics (Karlsson and Byström 2005, Schindler and Scheuerell 2002). Since carp are highly mobile feeders, they are likely to utilize a larger proportion of the available habitat area (Crook et al. 2001). Our results using a mixing model demonstrate that the proportions of limnetic and littoral food items in the diet of carp were significantly different between individuals with HI = 0 and HI = 8. We believed that hybridization and possibly feralization could influence on the magnitude of habitat coupling, resulting in a change in ecosystem functioning of the lakes.

Conservation implications

Our study also showed that the Lake Kasumigaura carp population is largely a hybrid swarm with most samples containing high frequencies of introduced alleles. Although five loci are relatively few to use to detect variation within the swarm, analysis of more complex swarms in fish and salamander has used similar numbers of loci (Kanda et al. 2002, Ryan et al. 2009). Hybrid swarms make conservation and recovery of endangered species much difficult. Allendorf et al. (2001) suggests that in cases where nearly all populations have apparently become a hybrid swarm, any remaining non-hybridized populations should be given priority. Hybrids should be protected according to the degree of retention of native alleles. Although few non-hybridized populations probably remain in Lake Kasumigaura, our results suggest that remaining hybrids with higher HI could fill the ecological role of the native carp. Any management undertaken should be applied cautiously because ecological information is incomplete. To conserve or restore not only genetically pure native carp but also hybrid individuals with higher HI, therefore, not only establishment of a supportive

breeding program using remaining non-admixed individuals but also the adequate regulation of continued and repeated release of non-native domesticated carp by fishery managers, is urgently needed.

Finally, rates of hybridization are increasing worldwide across wide range of taxa through human-mediated translocations. To understand the effects of hybridization on unique functional roles of native species, quantitative approach combining molecular and stable isotope analyses as in this study would be promising for other taxa.

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