Animal Conservation

Animal Conservation. Print ISSN 1367-9430

An emerging infectious pathogen endangers an ancient lineage of common carp by acting synergistically with conspecific exotic strains

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Keywords
biodiversity loss; common carp; *Cyprinid herpesvirus 3*; emerging infectious diseases; koi herpesvirus; reservoir.

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Editor: Karina Acevedo-Whitehouse
Associate Editor: Paddy Brock

Received 10 April 2012; accepted 17 September 2012

doi:10.1111/j.1469-1795.2012.00604.x

Abstract

Anthropogenic introduction of non-native species and the subsequent spread of their accompanying pathogens can cause serious declines in native wildlife populations. The common carp *Cyprinus carpio*, which is distributed worldwide due to intensive human introduction, is of little concern for conservation. However, the recent discovery of its ancient lineage endemic to the Japanese Archipelago has brought awareness of the conservation significance of the Japanese strain because this strain appears to be threatened by non-native Eurasian strains that heavily colonize Japanese freshwater bodies. Another threat has been recently posed by a lethal emerging pathogen, *Cyprinid herpesvirus 3* (CyHV-3), which has spread to Japanese populations of common carp since 2003. Here, we report a significant decline in the frequency of Japanese native carp mtDNA haplotypes in two of the five subpopulations in Lake Biwa, one of the major habitats of the Japanese strain, after mass mortality caused by an outbreak of CyHV-3. It is likely that the less susceptible non-native strains may have competitively eliminated the native strain. Our results suggest that the emerging pathogen and invasion of Eurasian strains may pose synergistic threats to Japanese native common carp.

Introduction

Emerging infectious diseases can lead to the extinction and decline of wildlife populations. The occurrence of emerging infectious diseases has increased over the past several decades (Daszak, Cunningham & Hyatt, 2000), multiplying the threat to biodiversity. This increased incidence can be attributed to anthropogenic activities that promote the introduction and spread of novel infectious agents (Vitousek *et al*., 1996), similar to the way in which biological invasions, another major threat to biodiversity, emerge. Sharing similar causal mechanisms, emerging infectious diseases can interact with invasions of host species. In fact, the spread of novel infectious diseases carried by invaders has sometimes caused the native species population to crash. For example, two endemic rat species in Christmas Island went extinct when a pathogenic trypanosome was introduced with exotic black rats (Wyatt *et al*., 2008), and native red squirrel populations in the UK have declined substantially with the spread of a poxvirus brought by exotic grey squirrels (Tompkins, White & Boots, 2003). A theoretical study predicted that shared infectious diseases accelerate the replacement of native species by exotic species when the native species have lower disease resistance (Bell *et al*., 2009). Consequently, it is crucial to clarify the combined effects of biological invasions and emerging infectious diseases to better understand the driving forces of biodiversity loss.

Common carp *Cyprinus carpio* is a freshwater fish originating from the Eurasian continent. Human activity has spread this carp globally, and it is currently rated in the top 100 worst invasive species by the International Union for Conservation of Nature. Recent molecular studies discovered mitochondrial DNA (mtDNA) haplotypes unique to Japanese common carp populations, which represent an old lineage that diverged from Eurasian lineages *c.* 1.7–2.5 million years ago (Mabuchi *et al*., 2005). At the same time, several mtDNA haplotypes of conspecific Eurasian strains were found in all of the lakes and rivers examined, indicating extensive invasion by the non-native strains throughout Japan (Mabuchi, Senou & Nishida, 2008). The import of common carp from the Eurasian continent to Japan was first documented in 1905. The imported fish were intensively crossbred to produce better domesticated strains (Maruyama *et al*., 1987) and huge numbers of domesticated strains have been released to Japanese freshwater bodies for stock

enhancement (National Statistics Center, http://www.e-stat.go.jp), which would have resulted in the current widespread distribution of the Eurasian strains.

In Lake Biwa, Japan, common carp have been distinguished as 'wild type' and 'domesticated type' by their body shapes since the introduction of domesticated strains (Shiga Prefectural Fisheries Experiment Station, 1915); the wild type, considered indigenous to the lake, has a slender body, whereas the domesticated type has a stumpy body with greater body depth. This knowledge was verified by molecular evidence: the mtDNA haplotypes of common carp having a typical slender body were unique to Japanese populations (Mabuchi *et al.*, 2005), suggesting that the wild type is the Japanese native strain. The native haplotypes were more dominant in the Lake Biwa population than any other populations examined (Mabuchi *et al.*, 2008), highlighting the conservation importance of this site as a hot spot of the native strain.

A mass die-off of more than 100 000 common carp occurred in Lake Biwa in spring 2004, which was caused by an emerging pathogen *Cyprinid herpesvirus 3* (CyHV-3) (Fujiwara, 2006). CyHV-3, which emerged less than two decades ago, was introduced into Japanese carp farms in 2003 (Pokorova *et al.*, 2005) and spilled over to Japanese wild common carp populations (Iida & Sano, 2005). In Lake Biwa, the mass mortality showed two noticeable patterns. One was that 93% of the dead carp were adults (> 350 mm in length, 4–10 years old), and the death of non-adults (< 350 mm, 1–4 years old) was very rare (< 8%; Fujiwara, 2006). The other was, to our surprise, that approximately 90% of the dead carp had Japanese native mtDNA haplotypes; the frequency of native haplotypes was lower in surviving fish, ranging from 34% to 88%, depending on the lake region (Mabuchi *et al.*, 2010). The latter pattern implies that the native strain would have been preferentially eliminated from the population by the CyHV-3 outbreak.

Here, we report a significant decline in the frequency of native mtDNA haplotypes in two of the five subpopulations examined in Lake Biwa after the CyHV-3 outbreak. Our data suggest that the recent introduction of CyHV-3 accelerated the replacement of the Japanese native strain by the Eurasian strains that invaded Japan a century earlier.

Materials and methods

Sample collection

We purchased common carp that were captured by local fishermen from the coastal waters of Lake Biwa, including inflowing rivers and satellite lakes (Fig. 1): 461 fish from April to August 2006, 130 fish from March to November 2008, and 65 fish from March to August 2009. After measuring standard length (SL) and body depth (BD), that is, the lateral body from the anterior base of the dorsal fin to the anterior base of the pelvic fin, we collected gill tissues for determination of mtDNA haplotypes. We also used the published data on mtDNA haplotypes and SL for 606 common carp captured from the same waters as ours between June 2004 and July 2005 (Mabuchi *et al.*, 2010). We did not use data of the dead fish in the 2004 outbreak because we sought to compare the change in haplotype frequency in the surviving common carp.

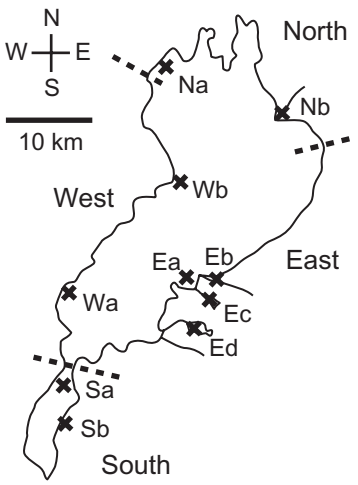

Figure 1 Sampling locations in Lake Biwa. The site names correspond to those in Supporting Information Appendix S1.

Identification of mtDNA haplotypes

We extracted DNA from gill tissues using the Wizard® SV 96 Genomic DNA Purification System (Promega, Madison, WI, USA) or Gentra Puregene Mouse Tail Kit (QIAGEN, Hilden, Germany) according to the respective manufacturer's instructions and performed restriction fragment length polymorphism (RFLP) analysis as follows. We polymerase chain reaction (PCR)-amplified a partial fragment of mtDNA (~1100 bp) including the complete D-loop region with primers L15927-Thr (Miya & Nishida, 2000) and H690-12S (Miya & Nishida, 1999) using the PCR conditions originally described in Mabuchi *et al.* (2008) and digested the product with the restriction enzyme *Dde*I (TOYOBO, Osaka, Japan). According to Mabuchi *et al.* (2008), who reported 19 Japanese native and nine non-native haplotypes, the native haplotypes have two *Dde*I restriction sites in the D-loop region (three in the amplified sequence), whereas the non-native haplotypes have one (two in the amplified sequence). We determined haplotypes by the RFLP band patterns.

To confirm the accuracy of the RFLP results, we sequenced the complete D-loop region for 10 subsamples from each of the native and non-native haplotypes determined by RFLP. We also sequenced two samples that had unexpected band patterns to determine the haplotypes. The TaKaRa Bio Dragon Genomics Center (Yokkaichi, Japan) performed the sequencing analysis. A phylogenetic analysis was performed using the complete nucleotide sequences of the D-loop region. We aligned all 22 sequences in this study and the 28 in Mabuchi *et al.* (2008) together with an outgroup sequence from goldfish (accession number:

AB111951) and constructed a phylogenetic tree using the neighbor-joining method with 1000 bootstrap replicates using ClustalX (2.0). The tree was visualized using NJplot (2.3). Two major clades, that is, native and non-native, were highly supported by bootstrap values > 90%. The phylogenetic analysis classified all 10 sequences from each of the native and non-native haplotypes determined by RFLP into the relevant haplotypes. All 10 native sequences and 9 out of 10 non-native sequences were identical to those in Mabuchi *et al*. (2008). The other non-native haplotype was close to c1 but contained three substitutions and a single deletion (accession number: AB690263). Two samples that showed unexpected RFLP band patterns were determined non-native haplotypes; both sequences had a substitution from T to C at the 173rd base in the D-loop region (AB690264, AB690265), which introduces an additional recognition site for *Dde*I.

Comparison of haplotype frequency

Prior to statistical analysis, we categorized the common carp into groups using the following criteria: (1) breeding year when fish were captured, starting from March to the next February, because common carp start breeding from around March to April in Lake Biwa; (2) size class, that is, fish ≥ 350 mm or < 350 mm in SL, with the former assumed to be adults and the latter to be non-adults based on the size at which wild common carp start breeding (Miyadi, Kawanabe & Mizuno, 1976), because fish <350 mm rarely died in the 2004 CyHV-3 outbreak; (3) the lake coast area where the fish were sampled, that is, east, north, south and west, divided by province (Fig. 1) because Mabuchi *et al*. (2010) reported significant regional variation in haplotype frequency. We compared the haplotype frequencies separately among groups from the same breeding year to detect regional and size-dependent variations in each year. To detect temporal changes in haplotype frequency, we also compared the frequencies separately for non-adults and adults in each lake region. In each set of groups, we performed pairwise exact tests with a Markov chain of 100 000 steps (Raymond & Rousset, 1995) using the ARLEQUIN 3.5 software (Excoffier & Lischer, 2010). We excluded groups that contained fewer than 10 samples to eliminate statistical uncertainty. We used a false discovery rate control procedure to control the type I error rate at < 0.05 in each series of multiple comparisons (Benjamini & Hochberg, 1995).

Morphological analysis of native and non-native haplotypes

The difference in BD relative to SL is a major phenotypic characteristic discriminating the native and non-native strains, with the BD/SL of adult fish defined as 0.21–0.31 for the native strain and 0.30–0.35 for the non-native strains in Lake Biwa shortly after the introduction of domesticated strains (Shiga Prefectural Fisheries Experiment Station, 1915). To assess the distribution of phenotypes in each of the native and non-native haplotypes, we produced histograms of the BD/SL values of adult fish (≥ 350 mm in SL) captured in 2006–2009 separately for the native and non-native haplotypes. BD data were missing for one sample from 2006.

Results

Regional and size-dependent variations in haplotype frequency

Size-dependent differences were found shortly after the CyHV-3 outbreak. Adults had significantly lower native haplotype ratios than the non-adults by 26% in the east and 35% in the south in 2004 and by 33% in the east in 2005 (Table 1). In contrast, no such differences were found in any region in 2006, 2008 and 2009. Significant regional variation was detected in non-adults shortly after the CyHV-3 outbreak, where the non-adults in the north had a significantly higher ratio of native haplotypes than those in the east (by 28%) and south (by 37%) in 2004 or than that in the south in 2005 (by 32%; Table 1). However, no such variations were detected in non-adults in 2006 or in adults of any year.

Temporal changes in haplotype frequency

The proportion of native haplotypes decreased significantly in the non-adults in the north by 25% from 2004 to 2006 and by 37% from 2005 to 2006 (Fig. 2c). Non-adults in the east showed no significant change in haplotype frequency between 2004 and 2008. However, a significant reduction in the ratio of native haplotypes was detected in 2009 compared to 2005 by 45% (Fig. 2b). No significant change occurred in the haplotype frequency in adults in the east (Fig. 2a). Adults in the south showed a significant increase in the frequency of native haplotypes in 2005 and 2006, which was 31% greater than in 2004 (Fig. 2d), while non-adults in the south showed no significant changes (Fig. 2e).

Phenotypic distributions of native and non-native haplotype groups

Figure 3 shows the distribution of the BD/SL for each of the native and non-native haplotype adults. Eighty-one per cent of the native haplotype individuals (100 out of 123) had morphological phenotypes of the native strain (Fig. 3a), and 46% of the non-native haplotype individuals (62 out of 134) had those of the non-native strains (Fig. 3b).

Discussion

We found a significantly decreased native haplotype frequency in adults compared with non-adults in the east and south in 2004, and in the east in 2005 (Table 1). These observations corresponded to the fact that the great majority of dead carp in the outbreak were native haplotype

Table 1 Haplotype frequency for each group classified by breeding year, size class and lake region. Ratios of native haplotypes were calculated for the groups containing > 10 samples

Year	Size class	Region	Number of individuals		Ratio of native haplotypes
			Native	Non-native	
2004	Non-adult	East	90	70	56%[a]
		North	93	18	84%[b]
		South	9	10	47%[ac]
		West	4	2	–
	Adult	East	13	30	30%[cd]
		North	1	2	–
		South	6	45	12%[d]
2005	Non-adult	East	18	5	78%[ab]
		North	43	2	96%[a]
		South	7	4	64%[bc]
	Adult	East	42	52	45%[c]
		South	17	23	43%[c]
2006	Non-adult	East	97	63	61%[ab]
		North	27	19	59%[ab]
		South	5	7	42%[ab]
		West	37	13	74%[a]
	Adult	East	62	61	50%[ab]
		North	4	7	36%[ab]
		South	20	27	43%[b]
		West	5	7	42%[ab]
2008	Non-adult	East	65	44	60%[a]
	Adult	East	9	12	43%[a]
2009	Non-adult	East	7	14	33%[a]
	Adult	East	23	21	52%[a]

The ratios of native haplotypes with different superscripts were significantly different after false discovery rate correction ($P < 0.05$) in pairwise exact tests, which were conducted separately for each breeding year. The data of 2004 and 2005 were cited from Mabuchi *et al.* (2010).

adults (Fujiwara, 2006; Mabuchi *et al.*, 2010), perhaps due to horizontal transmission of CyHV-3 between adults while mating (Ito *et al.*, 2007; Uchii *et al.*, 2011). Consequently, the haplotype frequency of the non-adults in 2004 and 2005, which would have hatched in 2000–2002 and 2001–2003, respectively [see Supporting Information Appendix S1; wild common carp grow a maximum of 150 mm in SL in the year after hatching in Japan (Miyadi *et al.*, 1976)], would represent the original frequency in the parent population a few years before the outbreak.

Although Mabuchi *et al.* (2010) reported that the north subpopulation had a much higher frequency of native haplotypes than the eastern or southern subpopulations based on the 2004 and 2005 data, which are cited in Table 1, the original frequency before the CyHV-3 outbreak remained unknown. However, assuming that the haplotype frequency of non-adults in 2004 and 2005 would be almost equivalent to that of the Lake Biwa population before the CyHV-3 outbreak, the northern subpopulation would originally have had the highest native haplotype frequency, *c.* 84–96%, among the regional subpopulations (Table 1). Such regional heterogeneity in haplotype frequency before the CyHV-3 outbreak may be attributed to different habitat preference of the native and non-native strains (Mabuchi *et al.*, 2010). Although their ecological differences are largely unknown, Hurukawa (1966) reported that 'wild-type' individuals preferentially inhabit deeper waters compared with the 'domesticated type', which tends to prefer shallow waters. As the northern coast of Lake Biwa is much deeper and has steeper slopes compared with the eastern and southern coasts, the northern coast may have favored the native strain, resulting in the conservation of the high native haplotype frequency in the north. However, the regional heterogeneity in haplotype frequency disappeared, either in adults or non-adults, in 2006 (Table 1), possibly because of the reduction in native haplotypes in the north, as explained below.

A rapid decline in the native haplotype frequency was observed in the northern non-adult subpopulation in 2006 compared to 2004 and 2005 (Fig. 2c). Because the native strain would be more susceptible to CyHV-3 and also transmit CyHV-3 more effectively [CyHV-3 multiplication was more than 10 times greater in the native strain than in non-native strains that were experimentally infected (National Research Institute of Aquaculture, 2006)], the northern adult subpopulation might have experienced greater mortality during the outbreak compared with the other regional subpopulations that had lower native haplotype frequencies. This drastic decrease in native haplotype adults would have been passed on to their descendants immediately, that is, to fish born in 2004 and later, resulting in a decreased native haplotype frequency in non-adults in the north in 2006. We did not expect the decrease in native

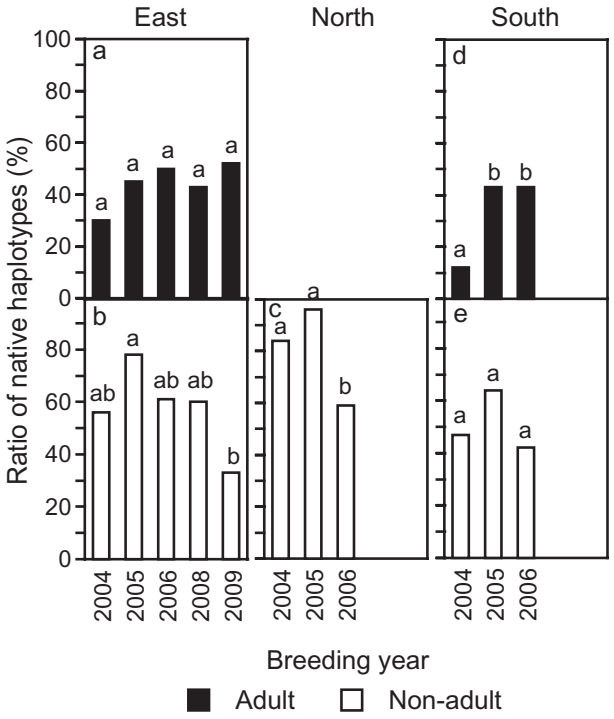

Figure 2 Temporal change in haplotype frequency in adults and non-adults in each of the lake regions (a–e). The ratios of native haplotypes with different superscripts were significantly different after false discovery rate correction ($P < 0.05$) in pairwise exact tests which were conducted separately for non-adults and adults in each lake region.

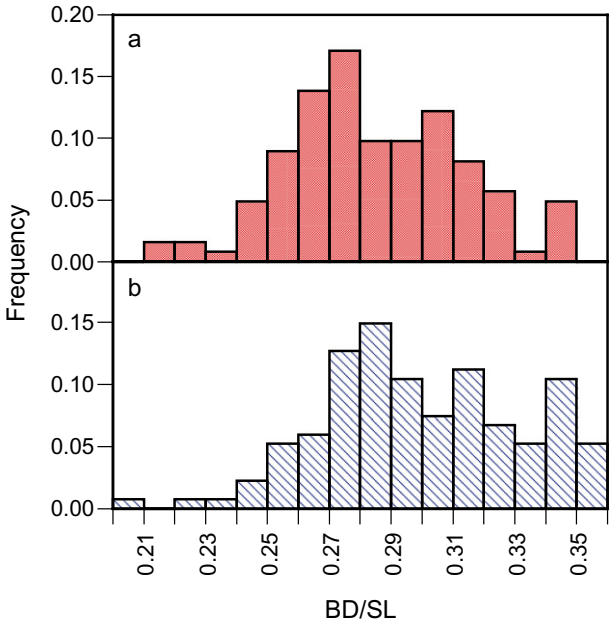

Figure 3 Distributions of body depth/standard length for native (a) and non-native (b) haplotype groups.

haplotype frequency to remain undetected until 2009 in the eastern non-adult subpopulation (Fig. 2b), even though nearly 90% of dead carp in the 2004 CyHV-3 outbreak in this region had native haplotypes (Mabuchi *et al.*, 2010). This delayed decline in native haplotype frequency might be partially explained by the onset of mortality in 2004, that is, a majority of adults in the east might have been able to reproduce before they died. An important implication here is that long-term observations are required when assessing population declines and recoveries following infectious disease outbreaks. A short-term survey can produce misleading predictions of the long-term risks of infectious diseases.

The native haplotype frequency increased in the southern adult subpopulation in 2005 and 2006 compared to 2004 (Fig. 2d). It is likely that the 2004 CyHV-3 outbreak would have reduced the frequency of native haplotypes in adults, and then new adults would have been recruited in 2005 and 2006 from the 2004 non-adult population that retained a high native haplotype frequency. Thus, these increased native haplotype ratios do not indicate recovery of native haplotypes. As fish born after the outbreak, whose haplotype frequency reflects the reduced native haplotype frequency of their parents, are introduced into the adult population, the native haplotype frequency may decline again.

The morphological analysis revealed indiscrete phenotypic distributions between the two haplotype groups (Fig. 3), implying possible hybridization and introgression. A high proportion (81%) of the native haplotype individuals was morphologically native, whereas less than half of the non-native haplotype individuals were morphologically non-native, which suggest that introgression might be greater in the direction from the native strain to the non-native strains. Although the degree of introgression in the Lake Biwa population is not known, the latest study reported that native haplotype individuals captured from the northern coast in 2004, which were thought to have been born before the CyHV-3 outbreak, showed a high genetic purity with little introgression of exotic genes based on polymorphisms in seven nuclear DNA loci (Mabuchi *et al.*, 2012). This result was consistent with the very high frequency of native haplotypes estimated for the northern subpopulation before the CyHV-3 outbreak, suggesting that the native strain was well-conserved in this region until the outbreak. Further studies are required to elucidate the extent of introgression by applying a newly developed method for detecting hybridization between the native and non-native strains (Mabuchi *et al.*, 2012).

We expect the CyHV-3 outbreak to have two consequences for the common carp population. First, CyHV-3 would be spread successively by surviving adults, as common carp that survived CyHV-3 infection would become carriers, transmitting viruses during mating (Uchii *et al.*, 2011). In general, the existence of reservoirs of pathogens enhances their survival rate independent of susceptible host abundance and thus may increase the risk of extinction of the host populations (De Castro & Bolker, 2005). The

more resistant non-native strains might act as a reservoir by virtue of their stronger resistance to CyHV-3, thereby competitively eliminating the more susceptible native strain by spreading the viruses through sharing spawning grounds. Additionally, shared infectious pathogens appear to accelerate the replacement of less resistant native species by exotic species even at a low prevalence of infection, as theoretically demonstrated in the decline of red squirrels caused by exotic grey squirrels in the UK (Tompkins *et al*., 2003). Therefore, we would need to continue long-term monitoring of the population dynamics of the native and non-native strains even after the CyHV-3 infection becomes less apparent.

Second, as native common carp populations decrease in size, increased introgression of exotic genes to the population may occur. A theoretical study showed that shared diseases help exotic species having stronger disease resistance to expand their ranges by reducing the population size of competing native species (Bell *et al*., 2009). The CyHV-3 disease might have allowed the non-native strains to invade the habitats once occupied by the native strain such as the northern coast, enhancing the introgression risk especially in the 'pure' native population. Hybridization of the native and non-native strains was reported for 9% of the eggs spawned in a breeding habitat in the northern coast in 2006 (Kume, 2007), suggesting that an increased introgression of exotic genes is likely. Considering that Lake Biwa is one of the largest genetic sources of the native strain, and that both CyHV-3 and non-native Eurasian strains have already invaded many lakes and rivers in Japan (Mabuchi *et al*., 2008; Minamoto *et al*., 2012), Japanese native common carp may be facing their greatest ever threat of extinction.

We suggest two possible countermeasures for conserving Japanese native common carp in Lake Biwa. One is the complete cessation of stocking of domesticated strains because the stocking not only increases the population size of the non-native strains but might also multiply the risk of recurrent CyHV-3 outbreaks by increasing the density of susceptible hosts and the risk of introduction of other pathogens derived from domestic environments. The other would be to identify the breeding habitats of the native strain that evaded the invasion of the non-native strains, by investigating the hybridization degree of spawned eggs, and then to conserve these habitats. The northern and western coastal areas, which have deep waters near shore, may contain good candidate habitats because the native strain likely prefers deep habitats (Hurukawa, 1966; Mabuchi *et al*., 2010).

In conclusion, introduction of exotic species involves not only the introduction of new infectious diseases but also interactions with these diseases. When exotic species have a stronger resistance to emerging pathogens, they may replace native species even after the apparent disease outbreak has diminished. The concurrent introduction of exotic species and accompanying infectious diseases is becoming more common and it was very difficult to prevent these diseases from damaging the native populations in most reported cases (e.g. Schmitz & Nudds, 1994; Tompkins *et al*., 2003; Edgerton *et al*., 2004). Our study emphasizes the importance of conservation strategies that prevent the unexpected introduction of infectious diseases and closely related exotic species that often share these diseases.

Acknowledgments

We thank K. Mabuchi, M. Nishida and their group for providing the data in Mabuchi *et al*. (2010). This research was supported by the Research Institute for Humanity and Nature (project number: C-06) and a Grant-in-Aid for JSPS Fellows to K.U. (10J09347).

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Supporting information

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

Appendix S1. Occurrence of native and non-native haplotypes of common carp after the CyHV-3 outbreak by breeding year, size class, lake region and sampling site. Ratios of native haplotypes were calculated when the numbers within each group were > 10. Data of the years 2004 and 2005 were cited from Mabuchi *et al*. (2010).