

Undetected diversity of goldfish (*Carassius* spp.) in North America

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Abstract

The goldfish (*Carassius auratus*) is one of the most readily recognizable fish species in the world. It is found worldwide as a pet, is commonly eaten in many countries, and has been introduced purposefully or inadvertently worldwide. Several of its lesser-known relatives in the genus *Carassius* have also been introduced outside of their native Eurasian range. In North America, *Carassius auratus* was first introduced in the mid-1800s and now widely occurs across the continent. The only other *Carassius* species known to be currently present in North America is *Carassius gibelio*, recently reported from Alberta. In this study, we sequenced cytochrome *b* in *Carassius* specimens from across North America. We confirm the widespread presence of *Carassius auratus*, record *Carassius gibelio* from Saskatchewan as well as Alberta, and report the first known occurrence of *Carassius langsdorfii* in North America, in British Columbia and California. Given their known impacts and high risk, management efforts should be implemented to minimize the spread of *Carassius* species in North America.

Key words: invasive species, introduced species, carp, Cyprinidae, genetic sequencing, mtDNA

Introduction

The goldfish, *Carassius auratus* (Linnaeus, 1758), is one of the most well-known fish species. It is a ubiquitous pet, a food fish in many countries, and has been introduced to every continent except Antarctica (Takada et al. 2010; Rylková et al. 2013; Haynes et al. 2012; Welcomme 1988; Scott and Crossman 1973). A member of the family Cyprinidae, it has several lesser-known relatives: four described species, including the crucian and Prussian carps (*C. carassius* (Linnaeus, 1758) and *C. gibelio* (Bloch, 1782)), and several undescribed forms (Kalous et al. 2012). The genus is native to most of Europe and Asia with the centre of diversity in eastern Asia (Takada et al. 2010). The species in the genus are generally difficult to distinguish morphologically and frequently hybridize where they come into contact; they also hybridize with their close relative, the common carp or koi (*Cyprinus carpio* Linnaeus, 1758) (Taylor and Mahon 1977). Several species in the genus have been widely

introduced around the world and have often had negative ecological effects on the native flora and fauna, including other fish species (Gaygusuz et al. 2007; Lusk et al. 2010), amphibians (Winandy et al. 2015), and vegetation (Richardson et al. 1995). To minimize such impacts, many North American jurisdictions have attempted to prevent the introduction and spread of *Carassius* species beyond their current range; however, *Carassius* continues to be imported to North America, purposely and inadvertently mixed with common carp, as food and ornamental fishes (Mandrak et al. 2013). Preventing further introductions and limiting the impact of the existing North American populations requires an understanding of which *Carassius* species have already been introduced to North America. Although goldfish species are easily identified to the generic level on the basis of morphological characters, it is difficult to morphologically distinguish most species within the genus from each other (Kalous et al. 2013); thus, several goldfish species may have arrived and spread in North America without being detected.

The various species of *Carassius* are widely distributed around the world, although the center of diversity is in eastern Asia (Takada et al. 2010). Wild-type goldfish are native to southern China, particularly the lower Yangtze River basin (Wang et al. 2013). The domesticated form has been introduced into other parts of Asia (Takada et al. 2010; Gao et al. 2012), Europe (Rylková et al. 2013), Australia (Haynes et al. 2012), New Zealand (Smith and McVeagh 2005), Africa (Welcomme 1988), South America (Welcomme 1988) and much of North America, including all 50 states (Nico et al. 2016) and several Canadian provinces (Scott and Crossman 1973). At least two other species in the genus are known to have been substantially introduced outside of their native range. The Prussian carp, *Carassius gibelio*, native from eastern Europe to eastern Asia, has been introduced to other parts of Europe (Kalous et al. 2012; Rylková and Kalous 2013; Rylková et al. 2013) and has recently been reported from North America, in Alberta, Canada (Elgin et al. 2014). The ginbuna, *Carassius langsdorfii* Temminck and Schlegel, 1846, native to Japan, has been reported from several European countries (Kalous et al. 2007; Rylková et al. 2013; Kalous et al. 2013). The genus includes several other species including the crucian carp *Carassius carassius*, which has been reported from North America (Schofield et al. 2017), and the Japanese white crucian carp *Carassius cuvieri* Temminck and Schlegel, 1846, native to Lake Biwa in Japan (Murakami et al. 2001; Yamamoto et al. 2010), along with a number of undescribed lineages from Asia (Takada et al. 2010; Gao et al. 2012; Kalous et al. 2012).

Several phylogenetic studies have examined the mitochondrial DNA relationships among these taxa (Murakami et al. 2001; Komiyama et al. 2009; Rylková et al. 2010; Yamamoto et al. 2010; Takada et al. 2010; Gao et al. 2012; Kalous et al. 2012; Rylková et al. 2013; Luo et al. 2014). These studies largely support the species relationships within the genus: *Carassius carassius* is sister to all other species; *C. cuvieri* and *C. langsdorfii* form two additional clades, sister to one large remaining clade, which includes subclades representing *C. gibelio*, *C. auratus*, and a number of undescribed Asian lineages (Supplementary material Figure S1). Phylogenetic studies of *Carassius* using nuclear genes are more limited (Luo et al. 2014; Podlesnykh et al. 2015), although one study suggests that domestic goldfish are descendants of an ancient hybridization event between wild *C. auratus* and the common carp, *Cyprinus carpio* (Podlesnykh et al. 2015).

While many of these phylogenetic studies have determined the genetic origins of introduced *Carassius* species in Europe and Asia (e.g. Takada et al. 2010; Kalous et al. 2013; Rylková et al. 2013), no such

studies have been performed elsewhere. The domestic goldfish, *C. auratus*, has been introduced widely throughout North America (Nico et al. 2016; Scott and Crossman 1973), but there has been no assessment of its genetic diversity. Knowledge of the genetic diversity of feral goldfish in North America would be useful in predicting and controlling future spread of the species. Additionally, very little is known about other species of *Carassius* that may be present in North America. *Carassius gibelio* was only very recently discovered in Alberta (Elgin et al. 2014), and *C. carassius* has been reported from Illinois (Schofield et al. 2017); other species of *Carassius* are known to have been introduced into Europe (Rylková et al. 2013; Rylková and Kalous 2013) but have not been searched for in North America. In this study, we obtained goldfish specimens from across North America to determine what genetic diversity exists among different populations, and to determine if other previously undetected species of *Carassius* are present in North America.

Methods

Frozen or ethanol-preserved specimens identified as *Carassius auratus* were obtained from various locations across North America (Figure 1, Supplementary material Table S1). Goldfish, as non-native species, are not frequently targeted for collection by North American museums, nor, when collected, are they usually preserved to allow for later DNA extraction. The samples used in this study were largely new collections sampled and preserved in a variety of ways. The samples received were either entire fish preserved in ethanol or frozen, or only fin clips or samples of muscle tissue preserved in ethanol. Several specimens of *Carassius gibelio* and the undescribed *C. cf. gibelio* from Mongolia were obtained from museums to expand coverage of these taxa (Table S1). Geographical co-ordinates for all collection locations are given in Table S1.

Genomic DNA was extracted by using QIAamp™ tissue-extraction kits (Qiagen Inc.) according to the manufacturer's instructions. The complete mitochondrial cytochrome *b* gene was amplified by performing PCR with the primers HA and LA of Schmidt et al. (1998). PCR was performed in a total volume of 25 µL, containing 5 µL 5x Green GoTaq Flexi Buffer, 1.5 µL 25 mM MgCl₂, 0.5 µL 10 mM dNTPs, 0.03 nmol each of the forward and reverse primers, 0.125 µL GoTaq Flexi DNA polymerase, and approximately 0.5 µg of DNA, under the following thermocycler settings: initial denaturation at 95.0 °C for 1 minute; 30 cycles of 95.0 °C for 30 seconds, 53.0 °C for 1 minute, and 72.0 °C for 2 minutes; and a final extension at 72.0 °C for 10 minutes, with the reaction terminating

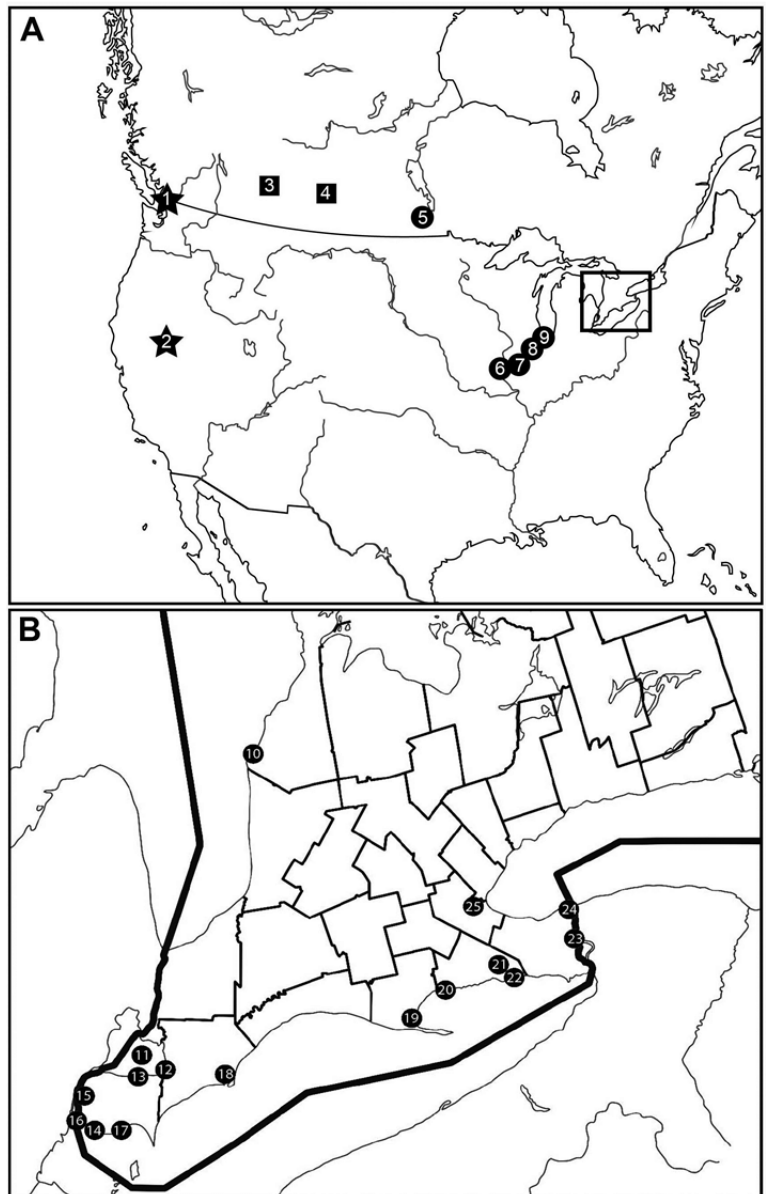


Figure 1. Collection localities of *Carassius* specimens A) outside of Ontario, and B) within Ontario. Species found at each location are indicated as: *Carassius auratus*, circles and stars; *C. gibelio*, squares; *C. langsdorffii* stars. Locations are: 1) Vancouver, British Columbia 2) Lake Tahoe, California 3) Bow River, Alberta 4) South Saskatchewan River, Saskatchewan 5) Winnipeg, Manitoba 6) Mississippi River, Iowa 7) Illinois River, Peoria, Illinois 8) Des Plaines River, Illinois 9) Chicago, Illinois 10) Pine River, Ontario 11) Lake St. Clair, Ontario 12) Thames River, Ontario 13) Ruscom River, Essex County, Ontario 14) Amherstburg, Ontario 15) Detroit River, Ontario 16) Big Creek, Malden Centre, Ontario 17) Cedar Creek, Essex County, Ontario 18) Rondeau Bay, Ontario 19) Long Point Bay, Ontario 20) Nanticoke Creek, Ontario 21) Grand River, Dunnville, Ontario 22) Grand River, Port Maitland, Ontario 23) Niagara River, Niagara Falls, Ontario 24) Niagara River, Niagara-on-the-Lake, Ontario 25) Dundas, Ontario. The rectangular area within Figure 1A shows the location of Figure 1B.

at 4 °C. Amplified DNA was purified using 2 µL of ExoSAP-IT (affymetrix) per 5 µL of PCR product. All purified samples were Sanger sequenced at The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children in Toronto. Sequences were checked for accuracy and assembled in the program Sequencher 4.7 (Gene Codes Corporation). Sequences were aligned manually, and duplicate haplotypes removed, in the program Mesquite 3.02 (Maddison and Maddison 2010).

All available sequences of cytochrome *b* assigned to the genus *Carassius* from Genbank were down-

loaded, assembled and, since the start and end points of many of the sequences varied, aligned with the program ClustalX (Larkin et al. 2007). The sequences newly obtained for this study were then added, and all sequences identical to other sequences were removed, in Mesquite 3.02. The program jModelTest 2.1.3 (Darriba et al. 2012) was used to select a suitable model of evolution for Bayesian phylogenetic analysis. The Akaike Information Criterion was used to select models. A Bayesian likelihood analysis was run in MrBayes 3.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003); the Bayesian

Information Criterion was used to select between an unpartitioned model and one partitioned by codon position. Four separate runs of 10,000,000 generations, each with a burnin of 2,500,000 generations, were performed. The initial temperature was set to 0.1, and the unconstrained exponential branch length prior was set to 100.0; otherwise default settings were used. A maximum likelihood analysis was also run in the program RAxML version 8.2.11 (Stamatakis 2014). The alignment was partitioned by codon, and the GTRCAT model applied to each partition. One thousand rapid bootstrap replicates were performed.

The Bayesian phylogenetic analysis revealed a number of subclades within *Carassius*; the newly collected North American specimens fell within three of these subclades. For these subclades, their phylogenetic structure was further examined by creating unrooted haplotype networks with the program TCS 1.21 (Clement et al. 2000). For these analyses, only the complete (1140 bp) cytochrome *b* sequences downloaded from Genbank were included along with the new sequences.

Results

Cytochrome *b* sequences were successfully obtained for 103 different specimens. These 103 sequences all grouped with the genus *Carassius* in the Bayesian phylogenetic analysis; sequences from a further 24 specimens grouped with *Cyprinus carpio*. Since many of the specimens were delivered to us only as fin clips or tissue samples, it was not possible to determine if these were misidentified *Cyprinus carpio*, hybrids, or otherwise pure goldfish with introgressed *Cyprinus carpio* mtDNA. However, since the majority of hybrids between *Cyprinus* and *Carassius* are known to have *Carassius* mtDNA (Haynes et al. 2012, Šimková et al. 2015), we believe that these specimens were most likely common carp that were misidentified in the field and exclude these specimens from further analyses. All mitochondrial sequences obtained from *Carassius* specimens were deposited in GenBank under accession numbers MG281847–MG281953.

A Bayesian phylogenetic analysis partitioned by codon position was favoured by the Bayesian Information Criterion. The models selected by jModelTest 2.1.3 were SYM+I+G for the first position, HKY+I for the second position, and GTR+I+G for the third position. The Bayesian phylogenetic analysis reconstructed a number of strongly supported clades, including *Carassius carassius*, *C. cuvieri*, three subclades within a *C. langsdorffii* clade, *C. gibelio*, *C. cf. gibelio* from western Mongolia (Kalous et al. 2012), four subclades within *C. auratus*, and several

undescribed Asian clades sister to *C. auratus* and *C. gibelio* (Figure S1). The maximum likelihood analysis also recovered these clades; support values did not differ notably between the two analyses, and in no case were conflicting clades significantly supported in either analysis.

The 103 new *Carassius* sequences obtained in this study comprised four different haplotypes. In the phylogenetic analyses, two of these haplotypes fell within one of the subclades of *Carassius auratus*; all sequences from domestic goldfish belong to this clade (Figure 2). One of these haplotypes, *auratus1*, was found in 86 of the newly sequenced specimens (Figure 2). This haplotype is also found in 10 different Genbank sequences; it is a widespread haplotype found throughout Europe and Asia and in most domestic goldfish specimens (Figure 2). In the newly sequenced North American specimens, this haplotype was found in specimens from Ontario, British Columbia, Manitoba, California, Illinois, and Iowa. The second haplotype belonging to this group, *auratus2*, was found only in two specimens collected in Long Point Bay (Location 19 in Figure 1), on the north shore of Lake Erie in Ontario. This haplotype differs from *auratus1* only by a single silent third-position mutation and was not previously recorded in Genbank.

All nine specimens from Alberta and Saskatchewan bore a haplotype belonging to the *Carassius gibelio* clade. This haplotype, *gibelio1*, is a widespread haplotype that is also found in six Genbank sequences from eastern Europe and Asia (Figure 3A).

Two specimens from British Columbia and four from California bore a haplotype that grouped with one of the *Carassius langsdorffii* subclades (Figure 3B). This haplotype, *langsdorffii1*, was previously unknown and not represented in Genbank. The clade to which it belongs appears to be native to Honshu, Japan (Takada et al. 2010); other haplotypes belonging to this clade have been reported from Greece, Germany, and the Czech Republic (Kalous et al. 2013). Haplotype *langsdorffii1* differs from the nearest Genbank sequence (DQ868879) by seven mutational steps; intermediate haplotypes are missing or were not sampled (Figure 3B).

Discussion

Eurasian *Carassius* have been extensively sampled genetically (Murakami et al. 2001; Komiyama et al. 2009; Rylková et al. 2010; Yamamoto et al. 2010; Takada et al. 2010; Gao et al. 2012; Kalous et al. 2012; Rylková et al. 2013; Luo et al. 2014), but this has not been the case for *Carassius* in North America (but see Elgin et al. 2014). This study represents the

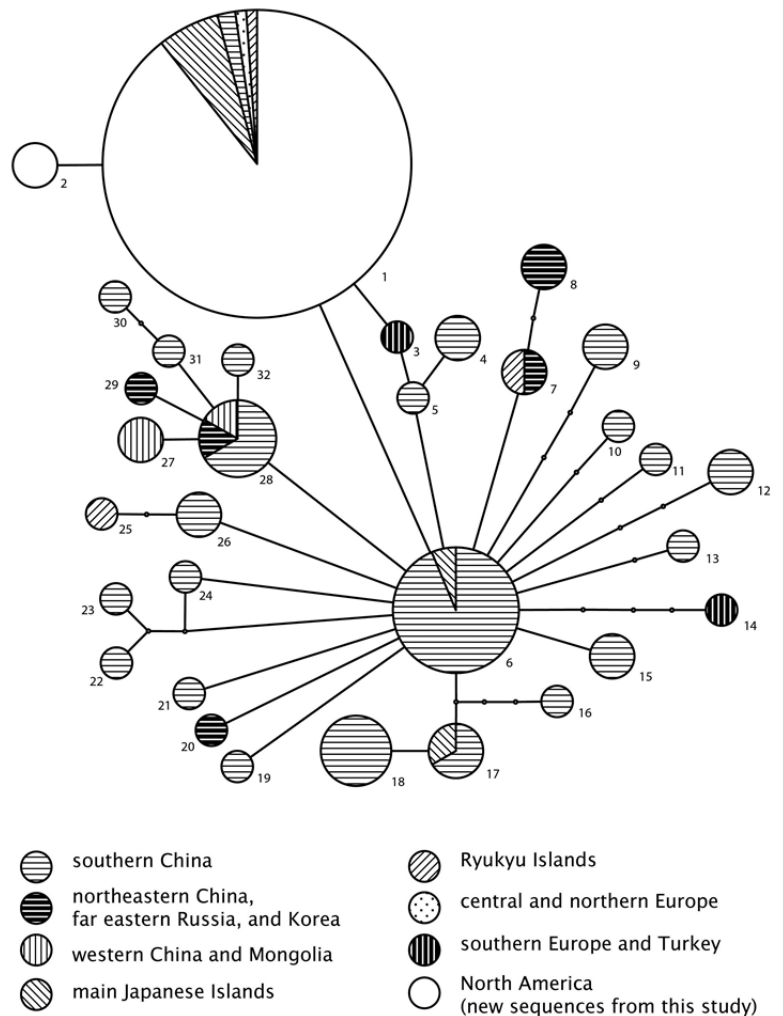


Figure 2. Cytochrome *b* haplotype network for *Carassius auratus* clade D. Different patterns in each circle refer to the different parts of the world in which fish bearing those haplotypes are found. The size of each circle is proportional to the number of individuals sharing that haplotype. Each line connecting haplotypes represents a single point mutation. Dots along the lines connecting haplotypes represent hypothetical haplotypes which were not sampled. The numbers next to each haplotype refer to Tables S1 and S2, where complete information on the sequences and localities represented by each haplotype is given.

widest genetic sampling of North American *Carassius* to date. It adds a third species of *Carassius*, *C. langsdorffii*, new to the North American ichthyofauna (Page et al. 2013), and expands the known range of another species, the recently identified *C. gibelio*. At the same time, it demonstrates very limited genetic diversity in all three *Carassius* species now known to inhabit North America.

The majority of specimens sequenced in this study were *Carassius auratus*, the goldfish species previously known to be present in North America. All but two of the 88 specimens sequenced bore the same cytochrome *b* haplotype. This is not surprising, as it is the most common cytochrome *b* haplotype found in domestic goldfish (Wang et al. 2013). While other cytochrome *b* haplotypes are known in domestic goldfish, these are mostly found in ornamental varieties unlikely to survive for long in

the wild (Wang et al. 2013). The second most common haplotype was found only in two specimens from Long Point Bay on Lake Erie and has not been previously reported in Genbank; therefore, it appears to be a *de novo* mutation in Long Point Bay.

All specimens from Alberta and Saskatchewan shared the same haplotype, which belongs to *Carassius gibelio*. *Carassius gibelio* was first collected in Alberta in 2006, but has only recently been identified there (Elgin et al. 2014) and has not been reported elsewhere in North America. The specimens sequenced from Saskatchewan represent the first record of *C. gibelio* in that province. These specimens might represent a separate introduction to Saskatchewan, but are more likely the result of downstream spread from the introduced populations in Alberta. In the Czech Republic, introduced populations of *C. gibelio* have been shown to undergo long-distance

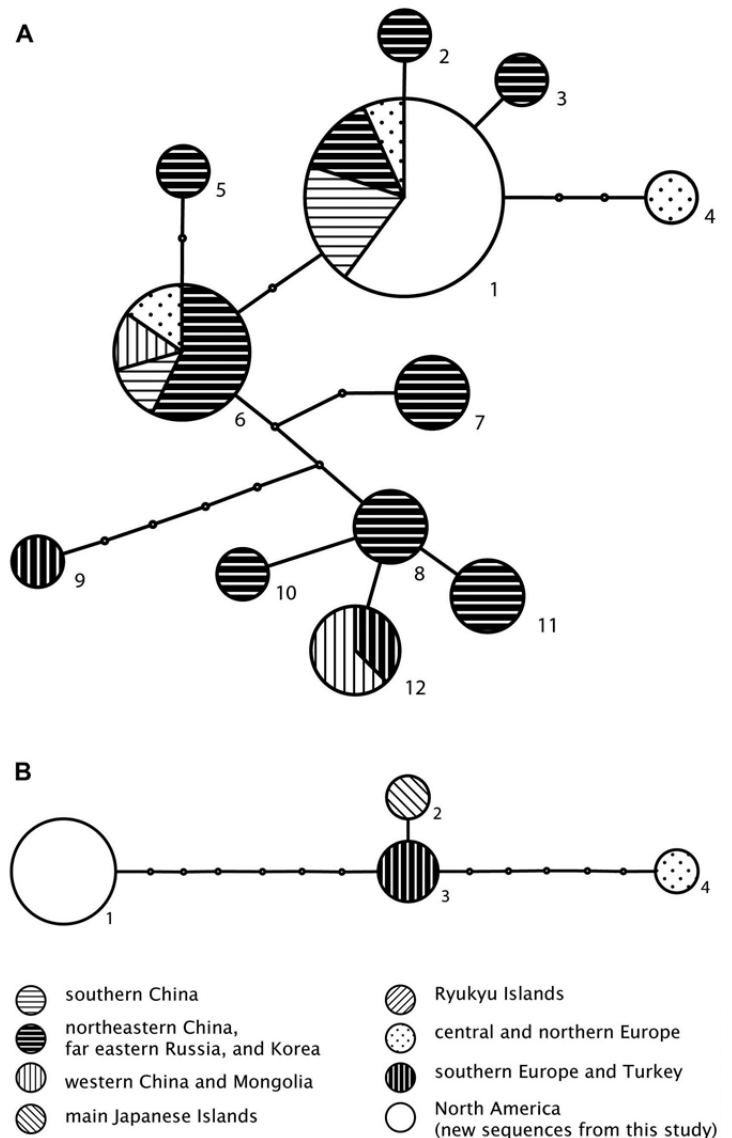


Figure 3. Cytochrome *b* haplotype networks for A) *Carassius gibelio*; B) *Carassius langsdorfii* clade B. The explanation of the figure is the same as for Figure 2.

migration, with individual fish moving up to 85 km downstream during the spawning season (Slavík and Bartoš 2004). As the river systems in which *C. gibelio* have been found in Canada, the Bow and Red Deer, flow into the South Saskatchewan River, which drains through Saskatchewan and Manitoba into Lake Winnipeg, the possibility exists for further spread of *C. gibelio* eastward into Manitoba, and subsequently southward into the United States through the Red River of the North. The native range of *C. gibelio* includes large areas of northern Asia with a similar climate to much of the Canadian prairies and north-central United States (Takada et al. 2010; Gao et al. 2012; Elgin et al. 2014), therefore, the potential exists for *C. gibelio* to survive

throughout much of northern North America in areas too cold for survival of *C. auratus*, which is native largely to subtropical and warm-temperate regions of southern China (Takada et al. 2010; Gao et al. 2012).

Specimens representing a third species, previously unknown in North America, were collected in western North America. Two specimens from British Columbia, from a pond on the University of British Columbia campus, and four specimens from Lake Tahoe in California, shared the same *Carassius langsdorfii* haplotype. Specimens from several countries in Europe have recently been determined to be *C. langsdorfii*, mostly belonging to the same subclade as the haplotype found in this study (Kalous et al. 2007; Rylková et al. 2013; Kalous et al. 2013). Other

than these introductions and its native range of Japan, *C. langsdorfii* has not been reported elsewhere in the world. Our specimens from British Columbia and California represent the first records of *Carassius langsdorfii* in North America. Interestingly, our *C. langsdorfii* haplotype does not match any of the sequences available in Genbank for this species, in contrast to the most common haplotypes found in North American *C. auratus* and *C. gibelio*, which are also common globally. The nearest sequence differs from it by seven mutations, which is a considerable difference. *Carassius langsdorfii* in Europe is believed to have arrived as unintentional introductions in shipments of *Cyprinus carpio* for the aquaculture trade, or in the aquarium trade with ornamental koi. A similar origin is possible for the North American specimens. However, the distinct haplotype of the North American specimens suggests that the source is different to that of the European introductions, one that has not been sampled in Japan. Discovering the original source of this haplotype would help in determining how it was introduced and how it might spread. Another possibility is that these specimens represent *Carassius auratus* with introgressed *C. langsdorfii* mtDNA. Hybridization and backcrossing among *Carassius* species is common (Mezhzherin et al. 2012; Wouters et al. 2012), and it is possible that such hybridization has occurred in places where both species occur, such as Japan or Europe. There are no clear morphological characters that distinguish *C. langsdorfii* from *C. auratus* (Kalous et al. 2013), so any hybrids would likely be indistinguishable morphologically from *C. auratus*. The difficulty of detecting individuals of *C. langsdorfii* without genetic sampling means that there may be additional populations that have been misidentified in North America. It is still unclear to what extent *C. langsdorfii* is spreading in Europe, if at all, but it is notable that many of the European specimens have been collected in areas where *C. auratus* cannot survive over winter (Rylková et al. 2013). This suggests that *C. langsdorfii* in North America may also be able to persist in areas where *C. auratus* cannot.

Hybridization, a common phenomenon in cyprinids, is known to occur among several *Carassius* species (Hänfling et al. 2005) and also between *Carassius* species and the common carp (Taylor and Mahon 1977; Pullan and Smith 1987). Many hybrids between *Cyprinus carpio* and *Carassius* species appear to be sterile, or at least have reduced fertility (Smith and McVeagh 2005; Kucinski et al. 2015), but this is not universal. The existence of backcrosses indicates that some hybrids must be fertile (Haynes et al. 2012). Hybrids of different *Carassius* species are frequently

fertile and, in some parts of Great Britain, hybrids between *C. carassius* and *C. auratus* outnumber pure individuals of either species (Hänfling et al. 2005). In North America, extensive hybridization between introduced bighead carp (*Hypophthalmichthys nobilis* (Richardson, 1845)) and silver carp (*H. molitrix* (Valenciennes, 1844)) in the Mississippi River is leading to the creation of a hybrid swarm (Lamer et al. 2010). While such hybrid swarms may show decreased fitness compared to the parental species, due to reduced fertility or other effects (Kucinski et al. 2015), increases in fitness are also possible, a concern for control of introduced populations. Šimková et al. (2015) demonstrated that hybrids between *Cyprinus carpio* and *Carassius gibelio* had lower overall parasite loads than either parental species, even though they hosted parasites of both parental species and, thus, carried a greater diversity of parasites. This might be due to the inheritance of complementary immune mechanisms from each parental species, resulting in a more effective defense against parasites (Šimková et al. 2015). Hybridization between species, if backcrossing occurs, could also lead to the transfer of advantageous alleles between species. In Australia, hybridization between *Carassius auratus* and *Cyprinus carpio* is a concern because backcrossing could lead to the transfer of resistance to the koi herpes virus from goldfish to common carp, which is susceptible to the virus. This would complicate efforts to control common carp populations in Australia by using the virus (Haynes et al. 2012).

Carassius species have a number of detrimental effects on native species that are undesirable from conservation and fisheries management perspectives. The goldfish feeds on plants and detritus, often resulting in the uprooting of submerged vegetation and an increase in turbidity (Richardson et al. 1995). The goldfish has been shown to have negative effects on amphibian populations in ponds, reducing their reproductive success (Winandy et al. 2015). In Europe and Asia, competition between introduced *Carassius* populations and native fish species has been shown to cause decreases in native fish populations (Gaygusuz et al. 2007; Lusk et al. 2010).

Several *Carassius* species are regulated in Canada and the United States. The ecological risk of *C. carassius* was assessed as high in the United States (USFWS 2014) and Canada (Mandrak et al. 2013). In the United States, it is considered an injurious species under the *Lacey Act*, and its importation and interstate transport is prohibited. Although it is high risk throughout Canada, federal AIS regulations only prohibit its importation and possession in Manitoba. The ecological risk of *C. gibelio* was assessed as

high in the United States (USFWS 2012) and it is listed under the *Lacey Act*, but it has not been assessed or regulated in Canada. The ecological risk of *C. langsdorfii* has not been assessed by, nor is it regulated in, either country. The occurrence of *C. gibelio* and *C. langsdorfii* in the wild may reduce their chances of being regulated in both countries, thereby increasing the probability of their spread and consequent ecological impacts. Therefore, given their known impacts and high risk, and despite being present in the wild, management efforts (e.g. implementation of legislation and regulation) should be undertaken to minimize the spread of *Carassius* species in North America.

Carassius auratus has been present in North America since at least the 19th century (Scott and Crossman 1973) and it appears to be a stable element of the North American ichthyofauna (Page et al. 2013); however, unless *C. gibelio* and *C. langsdorfii* have been present for a long time but misidentified, they appear to be newly introduced. It is still unclear to what extent they will spread in North America, and what effect they will have on native North American fish species and freshwater ecosystems. Ecological studies on North American populations of these *Carassius* species, as well as on the impacts of introduced *Carassius* elsewhere, are necessary to determine their potential ecological impacts. The difficulty of morphologically distinguishing *C. gibelio* and *C. langsdorfii* from *C. auratus* indicates that much more extensive genetic sampling should also be pursued to determine the full extent of the current range of these species in North America.

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Supplementary material

The following supplementary material is available for this article:

Figure S1. Phylogenetic tree for all *Carassius* cytochrome *b* sequences.

Table S1. Geographic co-ordinates of collection localities.

Table S2. Geographic origins of haplotypes in parsimony networks.

This material is available as part of online article from:

http://www.aquaticinvasions.net/2018/Supplements/AI_2018_Halas_etal_Figure_S1.pdf

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