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Towards the conservation of the crucian carp in Europe: Prolific hybridization but no evidence for introgression between native and non-native taxa

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Abstract

Hybridization plays a pivotal role in evolution, influencing local adaptation and speciation. However, it can also reduce biodiversity, which is especially damaging when native and non-native species meet. Hybridization can threaten native species via competition (with vigorous hybrids), reproductive resource wastage and gene introgression. The latter, in particular, could result in increased fitness in invasive species, decreased fitness of natives and compromise reintroduction or recovery conservation practices. In this study, we use a combination of RAD sequencing and microsatellites for a range-wide sample set of 1366 fish to evaluate the potential for hybridization and introgression between native crucian carp (*Carassius carassius*) and three non-native taxa (*Carassius auratus auratus*, *Carassius auratus gibelio* and *Cyprinus carpio*) in European water bodies. We found hybridization between native and non-native taxa in 82% of populations with non-natives present, highlighting the potential for substantial ecological impacts from hybrids on crucian carp populations. However, despite such high rates of hybridization, we could find no evidence of introgression between these taxa. The presence of triploid backcrosses in at least two populations suggests that the lack of introgression among these taxa is likely due to meiotic dysfunction in hybrids, leading to the production of polyploid offspring which are unable to reproduce sexually. This result is promising for crucian reintroduction programs, as it implies limited risk to the genetic integrity of source populations. Future research should investigate the reproductive potential of triploid hybrids and the ecological pressures hybrids impose on *C. carassius*.

KEYWORDS
genomics, invasive species, polyploid, postzygotic isolation, RADseq

1 | INTRODUCTION

Hybridization is one of the most impactful processes in evolution, facilitating both local adaptation and the formation of species (Meier et al., 2017). However, hybridization can also lead to the reduction of biodiversity via erosion of reproductive barriers and species collapse (Abbott et al., 2013; Kearns et al., 2018). Due to recent deliberate and non-deliberate translocations, as well as rapid range shifts in response to human-mediated environmental change, species that were once geographically separated are now more likely than ever to come into contact with each other (Seebens et al., 2017; Brondizio et al., 2019). The process of hybridization is therefore likely to become increasingly important in shaping global biodiversity in the future.

In the context of biological invasions, hybridization with non-native species can pose a significant threat to native biodiversity. First, vigorous hybrids can outcompete the native parental species (Arnold & Hodges, 1995; Facon et al., 2005; Hänfling et al., 2005). Second, the wasted reproductive resources that are committed to hybrid offspring can reduce the number of pure native species offspring produced in a given population and generation (Rhymer & Simberloff, 1996). Third, introgression of genomic regions from the non-native to the native genome can result in outbreeding depression and/or loss of locally adapted haplotypes from the native genome (Rhymer & Simberloff, 1996). Finally, introgression can lead to the transfer of beneficial locally adapted genes from the native to the non-native species, thus facilitating further invasion success (Mooney & Cleland, 2001; Muhlfeld et al., 2009; Perry et al., 2002; Rhymer & Simberloff, 1996) and increasing the fitness of invasives in their non-native range. In light of these potential impacts, characterizing hybridization and introgression between native and non-native taxa is an important step in evaluating the threats posed to native taxa and in turn, to their conservation (Mooney & Cleland, 2001; Muhlfeld et al., 2009; Perry et al., 2002; Rhymer & Simberloff, 1996).

One species potentially threatened by hybridization with non-natives is the crucian carp, *Carassius carassius* (L.) (Hänfling et al., 2005). Native to most of central and northern Europe, *C. carassius* is a freshwater cyprinid especially associated with isolated ponds, small lakes and river cut-offs (Holopainen et al., 1997; Olsén & Bonow, 2023). *C. carassius* is well adapted to life in isolated and often anoxic water bodies, having evolved a specialized form of respiration, 'the ethanol cycle', which allows for long-term anaerobic respiration (Blažka, 1958; Fagernes et al., 2017; Olsén & Bonow, 2023). As such, *C. carassius* occupies a highly specialized niche and its presence has been shown to increase pond invertebrate diversity at the landscape scale, making it a valuable species for ecosystem conservation (Harper et al., 2021). Unfortunately, small isolated water bodies such as ponds are among the most vulnerable to environmental degradation (Sayer et al., 2012), and *C. carassius* population declines have been observed in many regions throughout its range due to several human-mediated factors including habitat loss, drought and acidification (Holopainen & Oikari, 1992; Navodaru et al., 2002; Sayer et al., 2011, 2020). Importantly, *C. carassius* are also thought to be threatened by three taxa which are either introduced or invasive across much of the *C. carassius range*, these are the goldfish, *Carassius auratus auratus* (L.); the gibel carp, *Carassius auratus gibelio* (Bloch); and the common carp, *Cyprinus carpio* (L.) (Hänfling et al., 2005; Knytl et al., 2022; Mezhzherin et al., 2012; Papoušek et al., 2008; Sayer et al., 2011; Wouters et al., 2012). Note that the subspecies status of *C. a. auratus* and *C. a. gibelio* is often disputed, with some researchers referring to them as separate species (*C. auratus* and *C. gibelio*). However, this is not supported by phylogenetic evidence (Takada et al., 2010) and the term *C. auratus* is used in the literature somewhat ambiguously to refer to different taxa or different taxonomic levels. We have therefore opted for the more granular subspecies nomenclature here, to minimize the uncertainty around which taxonomic units we refer to. And we will use *C. auratus* subspp. to refer to both *C. a. auratus* and *C. a. gibelio* together when required.

C. a. auratus, *C. a. gibelio* and *C. carpio* are all among the top 25 most important non-native freshwater fish in Europe (Savini et al., 2010; van der Veer & Nentwig, 2015). Their statuses (native, introduced, established or invasive) differ across different European countries, however, due complicated domestication histories, multiple waves of human-mediated introductions and complicated biology, reliable historic information for these taxa is lacking. All three taxa are listed as non-native in the United Kingdom (https://www.nonnativespecies.org/, accessed 16/06/2024). On the European mainland, *C. carpio* is thought to be native to eastern and lower central Europe including the Danube catchment. However, being an important aquaculture species, *C. carpio* is now present across the entirety of Europe as the consequence of extensive introductions over the last thousand years, beginning with those of the Roman Empire (Holčík, 1991). *C. a. auratus* is native to Asia and has also been extensively introduced throughout Europe, mainly through ornamental fish trade, which likely began via introductions to Portugal and France around 400 years ago (Copp et al. 2005; Novák et al., 2020). The status of *C. a. gibelio* in Europe is perhaps the most contentious. Again, likely originating in Asia, it has been suggested that *C. a. gibelio* entered Europe via natural colonization of the Danube catchment in Romania in the early 20th century (Copp et al., 2005). However, its introduction by humans is difficult to rule out. Subsequent artificial introductions are nevertheless clear in northern European countries, Hungary and likely several other European countries including Czechia and Austria throughout the 20th century (Copp et al., 2005). The exact locations and timings of these are unclear and are confounded by the presence of both sexual diploid forms and gynogenetic triploid forms (see below).

These non-native taxa likely impose ecological pressures on *C. carassius* where they are found together. In experimental conditions, the presence of *C. a. auratus* or *C. a. gibelio* has been shown to reduce the growth rates of *C. carassius* (Busst & Britton, 2015; Tapkir et al., 2022). In the wild, *C. carpio* and *C. auratus* subspp. impose potentially detrimental effects on ecosystems and water quality where

they are introduced (Navodaru et al., 2002; Tapkir et al., 2022); however, it is not known whether this directly impacts *C. carassius* (Copp et al., 2010; Tapkir et al., 2022; Tarkan et al., 2009, 2010).

There is, however, strong evidence that non-natives threaten *C. carassius* via hybridization. Hybrids are found in almost every studied location where *C. carassius* coexists with *C. a. auratus*. Hänfling et al. (2005) observed that, out of seven ponds in the United Kingdom containing both *C. carassius* and *C. a. auratus*, hybrids occurred in every one. Mezhzherin et al. (2012) found similar results in water bodies of the Dnepr, Donets and Danube watersheds (Ukraine), wherein, nine out of 10 populations containing *C. carassius* alongside *C. a. auratus* also contained hybrids. Hybridization between *C. carassius* and *C. a. gibelio* is less predictable however, owing to the existence of not only diploid lineages of *C. a. gibelio* (able to hybridize) but also several independently arising gynogenetic triploid lineages (which are unable to outcross) (Knytl et al., 2022). For instance, in 12 central European populations where *C. carassius* were found with polyploid (3n, 4n) *C. a. gibelio*, only one population was found to contain hybrids between them (Mezhzherin et al., 2012). Whereas hybridization between *C. a. gibelio* and *C. carassius* has been documented in multiple populations throughout Sweden (Wouters et al., 2012) and Finland (Knytl et al., 2018), though the ploidy distribution in these populations was unknown. Unfortunately, the relative frequency of invasive diploid versus polyploid *C. a. gibelio* populations is hard to assess in the field, as they are morphologically indistinguishable. Thus, the frequency of and potential for hybridization between *C. carassius* and *C. a. gibelio* in many regions is currently unknown. Hybridization between *C. carassius* and *C. carpio* has also been observed, but less often than between *C. carassius* and *C. a. auratus*. Hanfling et al. (2005) found that hybrids occurred in three out of 12 locations containing both *C. carassius* and *C. carpio*, with lower hybridization rates likely reflecting greater evolutionary divergence between these taxa. Finally, hybridization among all combinations of the non-native taxa *C. carpio*, *C. a. auratus* and *C. a. gibelio* is possible, for example, see Hänfling et al. (2005) and Keszte et al. (2021).

Importantly, several independent studies have noted the presence of populations containing predominantly, or exclusively *C. carassius* × non-native hybrids (Hänfling et al., 2005; Mezhzherin et al., 2012; Sayer et al., 2011). This strongly suggests that hybrids can negatively impact and potentially extirpate native *C. carassius* populations. The mechanisms of extirpation in this case remain unknown, but theories include competition with vigorous hybrids (Hänfling et al., 2005; Sayer et al., 2011).

The hybrids observed in the aforementioned studies were largely identified as first generation crosses, but backcrosses have also been observed (Hänfling et al., 2005; Mezhzherin et al., 2012), highlighting the possibility of introgression between *C. carassius* and any of the non-native taxa. Not only could such introgression lead to genetic erosion of reproductive barriers (Abbott et al., 2013; Kearns et al., 2018), outcrossing depression in *C. carassius* (Rhymer & Simberloff, 1996) or adaptive introgression and increased fitness in the non-native taxa (Mooney & Cleland, 2001; Muhlfeld et al., 2009; Perry et al., 2002; Rhymer & Simberloff, 1996), but it could also compromise *C. carassius* populations as sources for reintroduction programmes, which are a staple of *C. carassius* conservation (Sayer et al., 2020). Understanding the extent to which genes can cross species boundaries is therefore crucial to the conservation of *C. carassius* and the native ecosystems in which it resides.

Quantifying the potential for introgression among the taxa in this study is challenging. While backcrosses between *C. carassius* and *C. carassius* × *C. auratus* hybrids have been described in the United Kingdom (Hänfling et al., 2005), and between *C. a. auratus* and *C. carassius* × *C. a. auratus* hybrids in Ukraine (Mezhzherin et al., 2012), classifying individuals into hybrid classes beyond F1 is problematic with traditional methods. Morphological identification, especially among hybrids, is known to be unreliable (Hänfling et al., 2005; Knytl et al., 2018). And while microsatellite markers have been used in the past, the loci used are limited in their ability to distinguish between hybrids of different classes (Anderson, 2008; Boecklen & Howard, 1997; Hänfling et al., 2005). It is therefore possible that backcrosses often go unnoticed using such approaches, and that the potential for introgression among these taxa is high. An additional challenge is to characterize the ploidy of hybrids, and in turn their potential for further backcrossing.

In this study, we aim to inform conservation practices for *C. carassius* in Europe by assessing the potential for introgression between *C. carassius* and three non-native taxa. To that end, we use genetic samples of over 1300 fish from over 70 populations across 14 European countries, which contain either *C. carassius* only or together with one or more non-native taxa. We use microsatellites to assess the levels of hybridization between taxa and use RADseq to search a subset of samples for signs of backcrossing and cryptic introgression.

2 | METHODS

2.1 | Sample collection and DNA extraction

The samples used in this study were collected between 2003 and 2013 by a number of researchers across the various focal countries (see Acknowledgements). There were three main components to our strategy for obtaining samples. First, we collected samples that most closely represented the morphological standard (Kottelat & Freyhof, 2007) for each of the four studied taxa (*C. carassius*, *C. a. auratus*, *C. a. gibelio* and *C. carpio*) to provide baseline data. Second, we obtained samples from populations in which hybrids between *C. carassius* and any of the three invasive taxa had previously been reported (via morphological identification). As it was known that hybridization was occurring in these populations, they were seen to be good candidates for containing backcrossed individuals if they existed. And lastly, we devoted most of the sampling to fish morphologically identified as pure *C. carassius*, to screen for any cryptic hybridization or introgression. These samples covered most of the native range of *C. carassius* (Jeffries et al., 2016), and all locations

are in countries with reports of non-native taxa being present. We note that our sampling of localities and of fish within water bodies was not exhaustive and, in several cases, we preferentially sampled fish which were morphologically identified to be hybrids. Thus, our results cannot be used to infer the absolute frequencies of taxa or hybrids, or co-occurrence rates of native and non-native taxa. They can, however, be used to gain minimum estimates.

This sampling regime resulted in 1366 fish from 72 populations across 14 counties (Figure 1, Table S1), which included individuals morphologically identified as *C. carassius*, *C. a. auratus*, *C. a. gibelio* or *C. carpio* or one of the hybrid combinations. However, sample identification was based primarily on morphological identification, so exact hybrid class, or the existence of cryptic hybrids was not reliably known at the time of sampling.

For all samples, approximately 1 cm^2 of tissue was taken from the lower caudal fin and immediately placed in 95% ethanol for storage at −20°C. Fish collected specifically for this study by DLJ were anaesthetized by a UK Home Office (UKHO) personal licence holder (GHC) using a 1 $mL\,L^{-1}$ anaesthetic bath containing 2-phenoxyethanol. Resulting wounds were treated with adhesive powder (Orahesive) and antibiotic (Cicatrin) (Moore et al., 1990) to prevent infection before releasing the fish. DNA extraction was

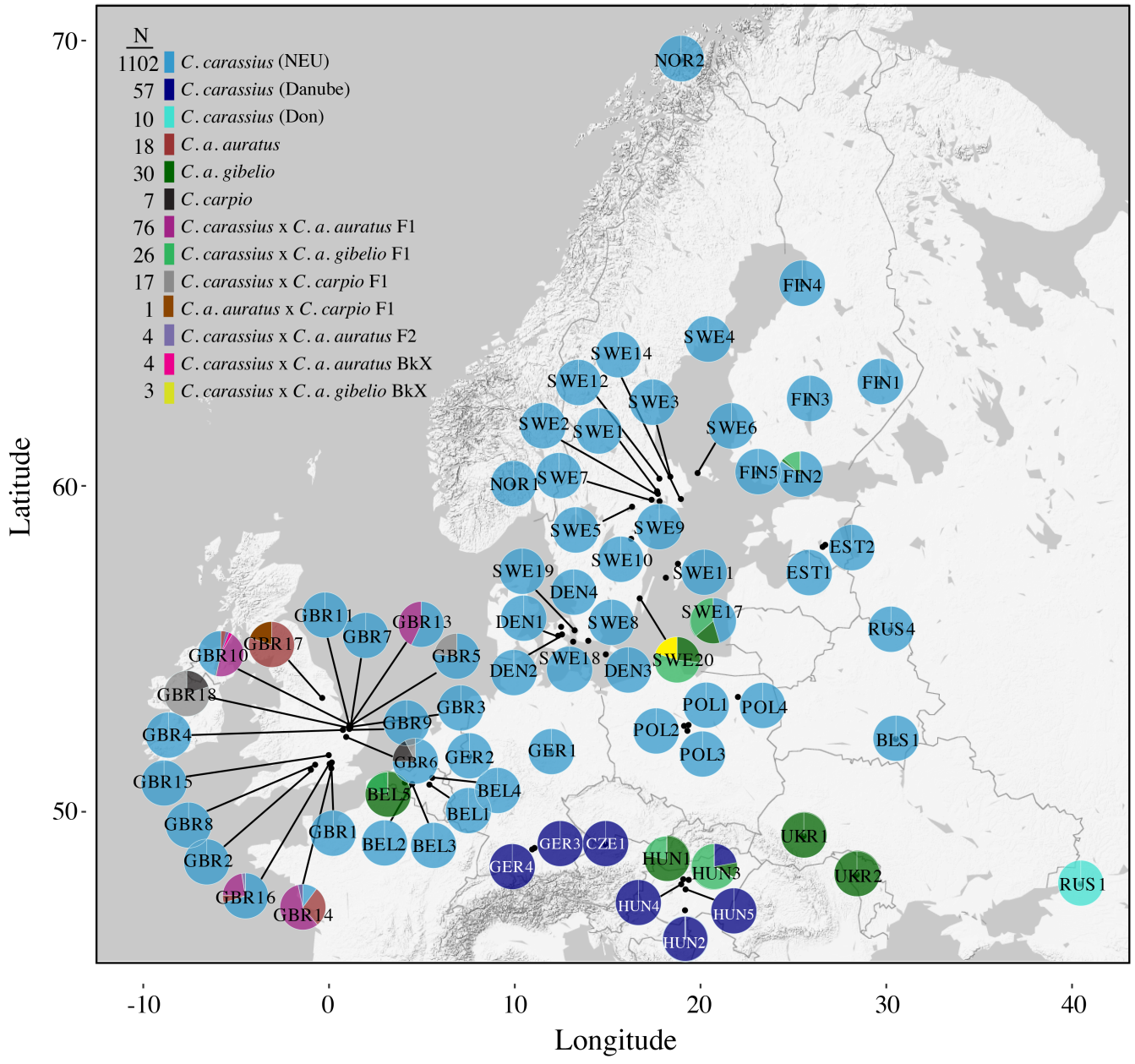

FIGURE 1 Map of sampling locations and taxon composition (inferred with NewHybrids) of samples from each population. Numbers in the key give the total number of samples identified for each taxon and hybrid class identified across the microsatellite and RADseq data sets combined. Numbers per taxa and population can be found in Table S1.

performed from approximately 2–4 mm^2 of tissue using the Qiagen DNeasy extraction kit (Qiagen, Hilden, Germany).

2.2 | Microsatellite amplification and scoring

In order to identify samples as one of the four parental taxa or an early stage hybrid class (F1, F2 or first generation backcross), 1336 of the total 1366 samples were genotyped at six taxonomically diagnostic microsatellite loci (GF1, GF17, GF29 (Zheng et al., 1995), MFW2 (Crooijmans et al., 1997), J7 (Yue & Orban, 2002) and Ca07 (Yue & Orban, 2004)), which were originally developed for use in either *C. auratus* (GF1, GF17, GF29, Ca07), *C. a. gibelio* (J7) or *C. carpio* (MFW2). The diagnostic properties of these loci have been established in previous studies (Hänfling et al., 2005; Maes et al., 2007). GF1, GF29, J7 and MFW2 are diagnostic for all four taxa, whereas GF17 and Ca07 are diagnostic between all three *Carassius* taxa, but do not amplify consistently in *C. carpio*, and were therefore removed from *C. carpio*-specific analyses (Table S2). Loci were optimized for use in a single multiplex PCR reaction, performed using Qiagen multiplex PCR mix in 10 µL volumes, with manufacturer's recommended reagent concentrations, including Q solution and 1 µL of template DNA (see multiplex 1 in (Jeffries et al., 2016)). PCR reactions were run on an Applied Biosciences® Veriti Thermal Cycler and microsatellite fragment lengths were analysed on a Beckman Coulter CEQ 8000 genome analyser using a 400-bp size standard. Microsatellite fragment lengths were analysed and alleles scored using the Beckman Coulter CEQ8000 software.

2.3 | RADseq library preparation and data processing

There were two primary objectives for the RADseq analysis: (i) to leverage the higher density of loci across the genome to validate hybrid classifications based on morphology and microsatellites, and (ii) to search for cryptic introgression in fish previously designated as pure *C. carassius*. To achieve these objectives, RADseq analysis was conducted on a total of 246 fish specimens collected from 32 distinct populations (Table S1). These samples were carefully selected to encompass both putatively pure individuals from each of the four parental taxa, as well as hybrid individuals between them.

Specifically, for the parental taxa baseline data, we sequenced four ornamental *C. a. auratus* (GBR17) which were procured from a pet shop in Hull/ UK, four feral *C. a. auratus* samples (GBR15), identified by microsatellite genotyping in Hänfling et al. (2005), 10 *C. a. gibelio* samples (BEL5, UKR2) again identified as such via previous microsatellite analyses (Maes et al., 2007), and two individuals identified by microsatellite analyses in this study as *C. carpio* (GBR6). For hybrid RADseq samples, we included individuals identified by microsatellite analysis as *C. carassius* × *C. a. auratus* F1 hybrids (6 samples), *C. carassius* × *C. a. gibelio* F1 hybrids (15 samples) and also three samples (SWE20_7, SWE20_8, SWE20_11), which showed high probability of belonging to the F2 hybrid class in the microsatellite analysis. Finally, to test for cryptic introgression not detectable by microsatellites, we included 183 RADseq individuals identified as pure crucian carp on morphological grounds and in the microsatellite analyses (Table S1). Notably, 43 of the latter group originated from populations known to have a history of coexistence with non-native taxa (see 'Non-native taxa present' column in Table S1).

To ensure high-quality RADseq library preparations, DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Assay kit (Invitrogen) and normalized to concentrations greater than 50 ng ml^{-1}. Gel electrophoresis was then used to check that DNA extractions contained high-molecular-weight (i.e. low fragmentation) DNA. Samples were then prepared in 13 RADseq libraries at Edinburgh Genomics (University of Edinburgh, UK), using the enzyme *Sbf*1, according to the protocol in Davey et al. (2012). Libraries were sequenced on five lanes of 2 Illumina HiSeq 2000 flow cells (Edinburgh Genomics). Libraries 1–8 were sequenced using the V3 Illumina chemistry, and libraries 9–13 were sequenced with the V4 chemistry.

RADseq raw data were quality assessed using FastQC (Andrews, 2010) and filtered for PCR duplicates. The remaining reads were then assembled into individual loci and SNPs were called using the de novo STACKS (v2.53) pipeline (Catchen et al., 2013; Rochette et al., 2019). Extensive preliminary tests of the core pipeline parameters (Ustacks: -M, –m; Cstacks: -n) were carried out to identify optimal parameters given the trade-offs between data quality, and the quantity of data that could be kept (Figure S1a). Final locus construction parameters chosen were Ustacks: -M 4, –m 6 and Cstacks: -n 4. We also used the tsv2bam module to incorporate the variable-length paired end sequencing data and assemble contigs for each RAD locus.

The populations module of stacks was then used to filter loci by presence across samples and was run multiple times to produce six SNP data sets, each with SNP and sample combinations best suited to the separate analyses detailed below (Table 1). There are two major difficulties when processing multispecies RADseq data sets and filtering the resulting SNP sets. The first and most common is species-specific locus dropout, often caused by mutations in restriction sites. This is especially problematic for hybrid samples, which will inherit a sequenceable allele from one species, but a null allele from the other. This leads to a large number of hemizygous loci that appear homozygous in sequencing data (see increased number of assembled stacks in hybrids, Figure S1a), and, in turn, this results in substantial biases in resulting SNP calls (Gautier et al., 2012). To overcome this, we required SNP loci to be present in at least 65% of samples from all parental taxa in the sample set (as identified by microsatellite analyses), which ensures that all retained loci are sequenceable in all four taxa and minimizes allele dropout in hybrids. The second major difficulty with multispecies RADseq data sets is the need for generous mismatch allowance when constructing loci. The higher the mismatch allowance, the higher the chance of over merging paralogous loci in de novo locus construction. This

TABLE 1 Metadata for the various data sets and subsets used in the analyses of this study.

Data set name	N samples	N loci	Analyses	Notes
MICRO_all	1336	6	PCA	
MICRO_CcarNEU_Ccarp	1120	6	NewHybrids	
MICRO_CcarNEU_Caur_subspp	1216	6	NewHybrids	Excluding loci J7 & GF29
MICRO_CcarDAN_Ccarp	91	4	NewHybrids	
MICRO_CcarDAN_Caur_subspp	200	4	NewHybrids	Excluding loci J7 & GF29
SNP_all	246	29,321	PCA, ABBA-BABA	Separate populations, inc. hybrids
SNP_pure_only	214	15,219	ABBA-BABA	Separate populations, excl. hybrids & singles
SNP_pooled	217	29,464	ABBA-BABA	Pure taxa only, pooled.
SNP_Ccar_Caur	214	400	NewHybrids	
SNP_Ccar_Cgib	217	400	NewHybrids	
SNP_Ccar_Ccarp	196	400	NewHybrids	

is especially important in this data set as cyprinids are known to have undergone a whole genome duplication recently (Glasauer & Neuhauss, 2014) and to possess highly repetitive genomes. Thus, to minimize the number of overmerged loci in each SNP data set, samples known to be pure parental taxon individuals based on microsatellite analyses were used to identify and remove loci showing excess coverage (greater than the mean + 1 standard deviation) typical of over merging (Figure S1c). Unfortunately, it was not possible to filter loci based on excess heterozygosity due to the relatively low population sizes. The above filters were used when creating all six of the SNP data sets, which are each described in more detail below.

2.4 | Taxon delimitation and identification of hybrids using microsatellites

Individuals were assigned to parental taxa or hybrid categories using NewHybrids (Anderson, 2008). NewHybrids uses a Bayesian model-based clustering method to calculate posterior probabilities that each sample belongs to either one of two parental taxa, or one of several possible hybrid classes between them. However, as NewHybrids expects only two parental taxa in a given analysis, it was first necessary for us to loosely group samples into taxon-pair data sets. To this end, we performed principle component analyses (PCA) of individual genotypes for the full microsatellite data set *MICRO_all* (1336 samples, Table 1).

It became apparent in using NewHybrids that, while the microsatellite loci used herein are able to reliably discriminate between *C. carassius*, *C. carpio* and the *C. auratus* complex, they were not able to distinguish between the subspecies *C. a. auratus* and *C. a. gibelio*. In addition, the strong population subdivision between *C. carassius* from the Danube river catchment and the rest of Europe (Jeffries et al., 2016; Rylková et al., 2013) also proved confounding in preliminary NewHybrids runs when all *C. carassius* were included together. Specifically, *C. carassius* samples from the Danube and Donets river catchments were found to contain alleles (J7-202, J7-204, GF29-213, GF29-215) previously thought specific to *C. a. auratus* and *C. a. gibelio* (Hänfling et al., 2005; Maes et al., 2007). This resulted in assignment probabilities for Danubian individuals as *C. a. gibelio* or *C. a. gibelio* hybrids. We were able to confirm that many of these individuals were not hybrids based on results from those also present in the RADseq data set. These microsatellite loci were therefore removed from final NewHybrids analyses involving Danubian and Don populations. Lastly, we observed several individuals with microsatellite genotypes consistent with triploidy, for example, three distinct alleles at some loci (discussed further below). As NewHybrids is designed for use on diploid genotypes only, these samples were removed. After accounting for everything described above, the microsatellite data were split into four separate taxon-pair data sets for the NewHybrids analyses: *MICRO_CcarNEU_Ccarp* (*C. carassius* (N. Europe) × *C. carpio*); *MICRO_CcarNEU_Caur*_subspp (*C. carassius* (N. Europe) × *C. auratus* subspp.); *MICRO_CcarDAN_Ccarp* (*C. carassius* (Danube) × *C. carpio*, excluding loci J7 and GF29), *MICRO_CcarDAN_Caur*_subspp (*C. carassius* (Danube) × *C. auratus* subspp., excluding loci J7 and GF29) (see Table 1).

2.5 | Taxon delimitation and identification of hybrids using SNPs

For the SNP-based analyses, we employed the same approach as above for microsatellites, that is, we used PCA to group samples into taxon-pair data sets (including hybrids of unknown class), before running NewHybrids to assign hybrids to specific classes. For the PCA, we created the *SNP_all* (246 samples) data set by first assigning samples to each of the four pure parental taxa or as hybrids of unknown class based on the microsatellite analyses and then filtering the master SNP call set to retain only loci present in at least 65% of all four parental taxa. PCA was then performed in SNPRelate (Zheng et al., 2012, 2017) in R v3.6.3 (R Core Team, 2015).

Dividing samples into taxon pairs for the RADseq data set was much more straightforward. PCA clearly separated parental taxa and hybrids such that we could easily group individuals into three taxon-pair data sets. *SNP_Ccar_Caur*, *SNP_Ccar_Cgib* and *SNP_Ccar_Ccarp*. However, in NewHybrids, the Markov chains can fail to converge if the number of loci greatly exceeds the number of samples in a given data set. As such it was necessary to subset the SNP loci in each data set. We did this (for each taxon-pair data set separately) by first identifying loci with Fst = 1 between groups of samples confidently assigned to each of the two parental taxa in the PCA analyses (Fst calculated using VCFtools v.0.0.14 (Danecek et al., 2011)) and then selecting 400 of these loci that were present in at least 90% of each population. See Table 1 for a summary of all data subsets described above.

NewHybrids was run on each of the above subsets separately. For the RADseq data sets, this was done using the R package implementation parallel newhybrids (Wringe et al., 2017). For each analyses, a total of 1000 sweeps were specified for Markov chains, with a burn-in of 100. For the microsatellite analyses, we computed the posterior probability of assignment of samples to either of the two parental taxa (P1 & P2), or one of four possible hybrid classes: F1, F2 (F1 × F1), backcross 1 (P1 × F1), backcross 2 (P2 × F1). For the RADseq, we extended the analyses to include two additional backcross classes 'backross_2 1' (backcross 1 × P1) and 'backcross_2 2' (backcross 2 × P2).

Where a sample was found to have a posterior assignment probability of greater than zero for more than one genotype class, it was assigned to the class for which the posterior probability was highest. For samples that were represented by both microsatellite loci and RADseq data, if the two data sets disagreed on their assignment, then the result from the RADseq data was used, as a higher number of loci is expected to produce more accurate assignments (Boecklen & Howard, 1997).

2.6 | Testing for past introgression between native and invasive taxa

To test for introgression beyond the early hybrid stages evaluated by Newhybrids we used the ABBA-BABA approach, which examines allele sharing among four lineages of a phylogenetic tree, and tests for a significant departure from the random patterns expected due to incomplete lineage sorting (Durand et al., 2011). We first analysed all populations separately using the *SNP_pure_only* data set. To create this data set, we removed all samples identified as hybrids in Newhybrids analyses, as well as any single samples that represented the only individual of a given taxon in a given population (such instances were found to cause false-positive signal for introgression in preliminary tests). This left 214 samples and resulted in an SNP set of 15,219 SNPs. We then performed a pooled analysis, wherein all hybrids were again removed, and samples were grouped into the six major lineages (*C. carassius* (northern Europe), *C. carassius* (Danube), *C. carassius* (Don), *C. a. auratus*, *C. a. gibelio* and the outgroup *C. carpio*). The resulting data set (*SNP_pooled*) contained 217 samples and 29,464 SNPs.

ABBA BABA tests were performed using Dsuite (v0.4) (Malinsky et al., 2020). The function *Dtrios* was first used to perform ABBA BABA tests on all possible combinations of population/lineage. The *Fbranch* function was then used to calculate f_d, which allows for the interpretation of *D* statistics (calculated from independent ABBA BABA tests of multiple population combinations) while accounting for phylogenetic correlation between populations.

To run *Fbranch*, it was necessary to supply a phylogenetic tree. To produce these trees, we exported all polymorphic sites from the respective SNP data set as a concatenated FASTA sequence for each separate population/lineage. These sequences were aligned using Clustal Omega (Madeira et al., 2019) and the alignments were used to construct maximum-likelihood phylogenetic trees in RAxML (Kozlov et al., 2019). For these trees, we used an unpartitioned model, the GTR substitution matrix, a Gamma among-site rate heterogeneity model and consensus trees were each optimized based on 100 trees.

2.7 | Inferring the ploidy of samples

The genus *Carassius* and particularly the *C. auratus* complex contain polyploid lineages (Takada et al., 2010). While the origin of many of these is unknown, some are known to have arisen from hybridization events (Kuhl et al., 2022; Murakami et al., 2001). As polyploidy can have important implications for fertility and offspring fitness, we assessed both the microsatellite and RADseq data sets for signs of polyploidy in hybrids. For the microsatellite data, this was done by eye from individual chromatograms. Specifically, we searched for three or more allele peaks at a given locus, or for distinct peak ratio differences between two alleles. Multiple 'stutter' peaks and peak height variation are often seen for individual alleles. Thus, we only designated individuals as polyploid if two or more loci showed such signals, and if these signals were distinct from the characteristic behaviour of the loci or alleles in question across other samples.

For the RADseq data, we tested for polyploidy using allele read depth ratios. In a diploid organism, sequence reads at an SNP locus will, on average, be distributed among the two alleles (A and B) at a ratio of 1:1. However, in triploids (and higher odd-numbered ploidies), allele copy numbers will be uneven (e.g. AAB or ABB) and, when using an SNP caller that assumes diploidy, as here in Stacks, read depth ratios will reflect this (e.g. 0.66:0.33 for AAB). For tetraploids, and higher even-numbered ploidies, both uneven and even allele numbers are possible (e.g. AAAB, AABB), however, given that most alleles are rare in a population, uneven allele combinations are much more likely to occur, and thus, when considering many loci, uneven read depth proportions should occur often (McKinney et al., 2017). To assess the read depth proportions, we used the *SNP_all* data set (29,321 SNPs), containing all pure and hybrid samples. For each sample, we calculated read depth proportions at all heterozygous loci as: (N_reads(A)/N_reads(A) + N_reads(B)). The allele assigned as allele A was initially randomly chosen for each locus. As read depth ratios are highly sensitive to the noise inherent in coverage data, we ignored loci with less than 10 reads total. Additionally, to remove the confounding effect

of repeats, we ignored loci with read depth greater than 60 (roughly twice the mean coverage of all samples). Once all read depth proportions were calculated, we fitted a kernel density estimation (KDE) model to the distribution of allele depth proportions in each sample, allowing identification of single or multiple modes. If diploid, a single mode is expected at an allele depth ratio of approximately 0.5. If triploid, two modes are expected, one at 0.33 and the other at 0.66. Due to differing levels of diversity among the populations sampled, and the high heterozygosity of hybrids, the numbers of heterozygous loci were highly variable between samples. We employed this approach only for samples with more than 40 heterozygous loci, as KDE models were found to be unreliable below this point.

The above approach identified several samples likely to be triploid. For these samples, we tested which genome was in two copies by identifying the taxon of origin of each allele. To do this, we filtered loci in each sample for those with fixed allelic differences between the relevant parental taxa, we then repeated the allele depth calculation above while always assigning allele A as the *C. carassius* allele.

3 | RESULTS

3.1 | Taxon delimitation and identification of hybrids

3.1.1 | PCA

In the microsatellite data set, all loci displayed diagnostic alleles or allele ranges between *C. carassius* and at least one of the invasive taxa; however, diagnostic power between *C. a. auratus* and *C. a. gibelio* was limited (Tables S2 and S3). The initial PCA for the whole microsatellite data set (*MICRO_all*) was effective at discriminating between northern European *C. carassius* and all three invasive taxa (Figure 2a, Figure S2). PC1 clearly separated *C. carassius* and the *C. auratus* complex and PC2 separated the *Carassius* genus and *C. carpio* (see PCA figures for variances explained). Principal components 3 and 4 both captured some of the variation between the lineages of *C. carassius* and between *C. a. auratus* and *C. a. gibelio* (Figure S2a); however, no component in the PCA completely discriminated between *C. a. auratus* and *C. a. gibelio*. Based on these results, the microsatellite data were split into four subsets for NewHybrids, separately comparing the main *C. carassius* lineages against non-native taxa. Due to the lack of discriminatory power between *C. a. auratus* and *C. a. gibelio*, these samples were included together in the NewHybrids analyses (see Section 2 for further details).

In contrast, the PCA of the full SNP RADseq data set (*SNP_all*: 246 samples, 29,321 loci), representing all taxa in the present study, was effective at discriminating between all parental taxa and hybrids (Figure 2b, Figure S3), allowing us to split samples into three taxon pair data sets in the NewHybrids analyses.

3.2 | Hybrid classification

Of the 1336 fish samples included in the microsatellite taxon-pair subsets (*MICRO_CcarNEU_Ccarp*, *MICRO_CcarNEU_Caur_subspp*, *MICRO_CcarDAN_Ccarp*, *MICRO_CcarDAN_Caur_subspp*),

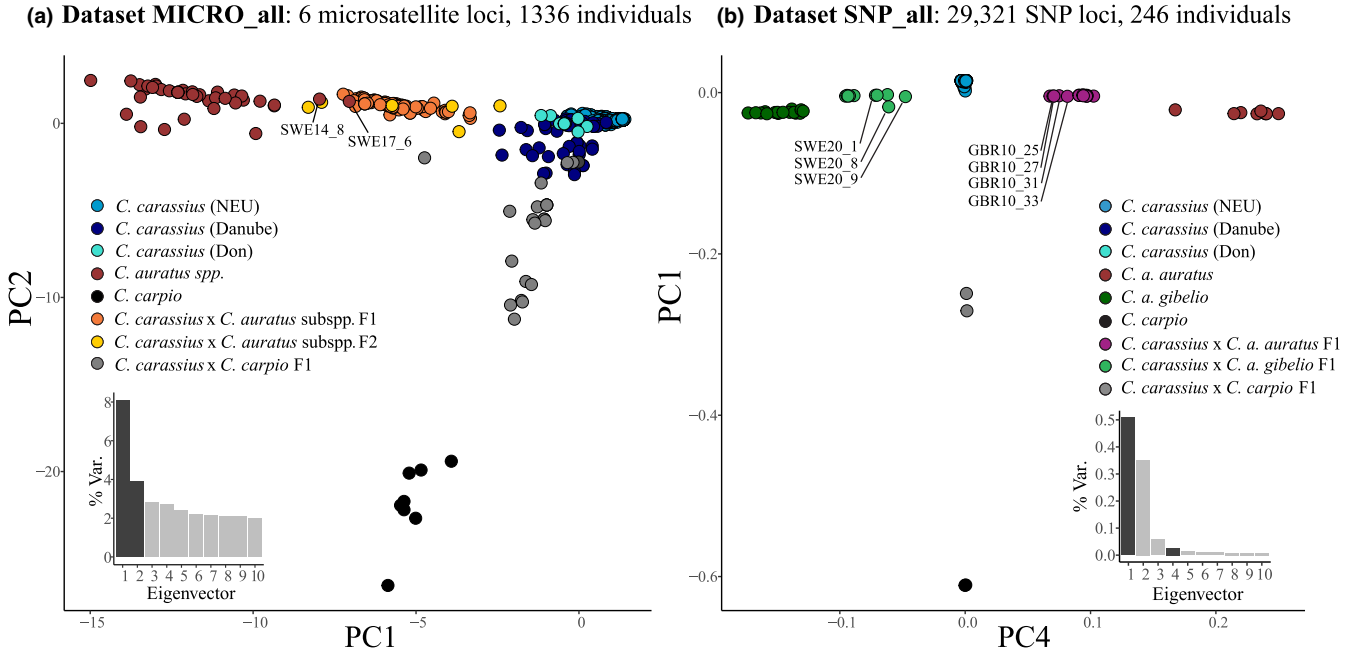

FIGURE 2 Principal component analyses of all samples in this study based on (a) microsatellite loci (data set: *MICRO_all*) or (b) RADseq (data set: *SNP_all*). Principal components shown were chosen to best display interspecies variation. Additional principal component comparisons can be found in Figure S2. Samples are coloured according to the NewHybrids assignments to parental taxa or hybrid class. Labelled samples are those discussed in the text.

NewHybrids identified 1169 as *C. carassius*, 18 as *C. a. auratus*, 30 as *C. a. gibelio*, 7 as *C. carpio*, 102 as *C. carassius* × *C. auratus* subspp. *F1*, 8 as *C. carassius* × *C. auratus* subspp. F2, 17 as *C. carassius* × *C. carpio F1* (Figure 1, Figure S4, Table S1). As there were no populations with both *C. a. auratus* and *C. a. gibelio* detected, we were able to assign samples identified in the microsatellite analyses as *C. carassius* × *C. auratus* subspp. hybrids to taxon-specific hybrid classes based on their coexistence with the parental taxa. Thus, we found 76 as *C. carassius* × *C. a. auratus* F1s, 26 as *C. carassius* × *C. a. gibelio* F1s, 4 *C. carassius* × *C. a. gibelio* F2s and 4 as *C. carassius* × *C. a. auratus* F2s (Figure 1, Table S1).

In eight of the above samples (GBR5_56, GBR14_27, GBR14_34, GBR14_38, GBR16_21, GBR18_2, SWE20_7, SWE20_14, SWE20_15), hybrid class assignments were ambiguous, whereby more than one genotype class had high assignment probabilities (Figure S4). In all of these samples, the ambiguity existed between hybrid classes F1, F2 and Backcross. These NewHybrids results generally agreed well with the clustering of samples in the PCA (see colours in Figure 2a). However, there were some exceptions, for example, several individuals which were identified as F2 or backcrosses in the NewHybrids analysis, clustered close to samples identified as being F1 hybrids in the PCA (Figure 2a). Also, two individuals, SWE17_6 and GBR14_8 (labelled on Figure 2a), were identified as pure *C. a. gibelio* and *C. a. auratus*, respectively, but clustered close to F1 hybrids, though they were on the periphery of this group (Figure 2a).

NewHybrids analyses of the RADseq taxon-pair subsets (*SNP_Ccar_Caur*, *SNP_Ccar_Cgib*, *SNP_Ccar_Ccarp*) identified 190 *C. carassius*, 9 *C. a. auratus*, 16 *C. a. gibelio*, 2 *C. carpio*, 16 *C. carassius* × *C. a. auratus*, 10 *C. carassius* × *C. a. gibelio* and 2 *C. carassius* × *C. carpio* (Figure S5). Sample assignments in NewHybrids were unambiguous, with all samples having posterior assignment probabilities of >0.99 to a single taxon or hybrid class (Figure S5). We combined these results with those from the microsatellite analyses results to generate final assignments of each sample to taxon-specific hybrid classes. For the 217 samples that were genotyped at both microsatellite loci and using RADseq, the assignment of individuals to parental taxa or hybrid class was identical in both data sets except for three individuals, SWED20_1, SWED20_8 and SWED20_9, which were identified as F2 *C. carassius* × *C. a. gibelio* hybrids by microsatellite analysis, but as F1s in the RADseq analysis. We investigated this disparity below.

Based on the final assignments of all individuals from NewHybrids analyses of both microsatellite and RADseq data, of the 18 populations putatively identified as containing *C. carassius* and non-native taxa, 14 were found to contain hybrids (Table S1). Therefore, discounting FIN5, which contained only triploid *C. a. gibelio* (which are likely unable to sexually reproduce with *C. carassius*), hybridization was observed in 82% of populations where *C. carassius* coexisted with sexual non-native taxa (Table S1).

3.3 | Testing for cryptic introgression between native and invasive taxa

Despite the high prevalence of hybridization where *Carassius* taxa are in sympatry, ABBA-BABA tests did not reveal any evidence for cryptic introgression between any taxon pairs beyond early generation hybrids in either the population-separated (*SNP_pure_only*) or the pooled (*SNP_pure_pooled*) data sets (Figure 3). The only evidence of gene flow identified using the ABBA-BABA approach was within taxa (Figure 3), including weak signal for gene flow between the northern European and Danubian watersheds in the population-specific analysis.

3.4 | Polyploidy in *Carassius*

Of all of the microsatellite genotypes collected (*MICRO_all*), only four *C. a. auratus* and 12 *C. a. gibelio* samples showed evidence of triploidy, and only one *C. a. auratus* sample showed evidence of tetraploidy. However, in all cases, most loci possessed only one or two different alleles, and evidence for polyploidy was often based on the genotypes of only one or two loci out of a possible 12. This is not surprising given that the loci used were generally chosen to be diagnostic between taxa and not variable within *C. a. auratus* or *C. a. gibelio*. We also found that the confounding effects of peak stuttering and variable peak heights made ploidy estimation unreliable across the microsatellite data set. It is therefore possible that we are underestimating ploidy in many of the samples genotyped using microsatellites. Nevertheless, the individuals mentioned above were removed from further analyses, which assume diploidy.

For the RADseq data (*SNP_all*), ploidy estimation was far more reliable. Fifteen individuals showed clearly bi-modal patterns of allele depth ratios, with modes close to 0.33 and 0.66, consistent with triploidy. Among these were all seven pure *C. a. gibelio* in the RADseq data set, four *C. carassius* × *a. auratus* hybrids from population GBR10 and four *C. carassius* × *C. a. gibelio* hybrids (one from population BEL5, and three from population SWE20 Figure S6).

Several additional samples (e.g. GBR4_6, GBR4_11) showed some signs of polyploidy in the RADseq data (i.e. multiple allele depth ratio modes inferred); however, they had low numbers of heterozygous loci, had modes that did not correspond to expected ratios of 0.33 or 0.66 and were not identified as hybrids in any of the above analyses. Thus, while it is possible that they could be autopolyploids with a ploidy of 4n or above, we cannot confidently conclude this based on the data at hand.

For seven of the eight triploid hybrids, we were able to assign alleles to their respective taxon of origin, allowing us to infer the combination of subgenomes within each hybrid (the exception being BEL5_6, for which we could not confidently assign alleles to taxa for enough loci). In every case, triploid hybrids contained two *C.*

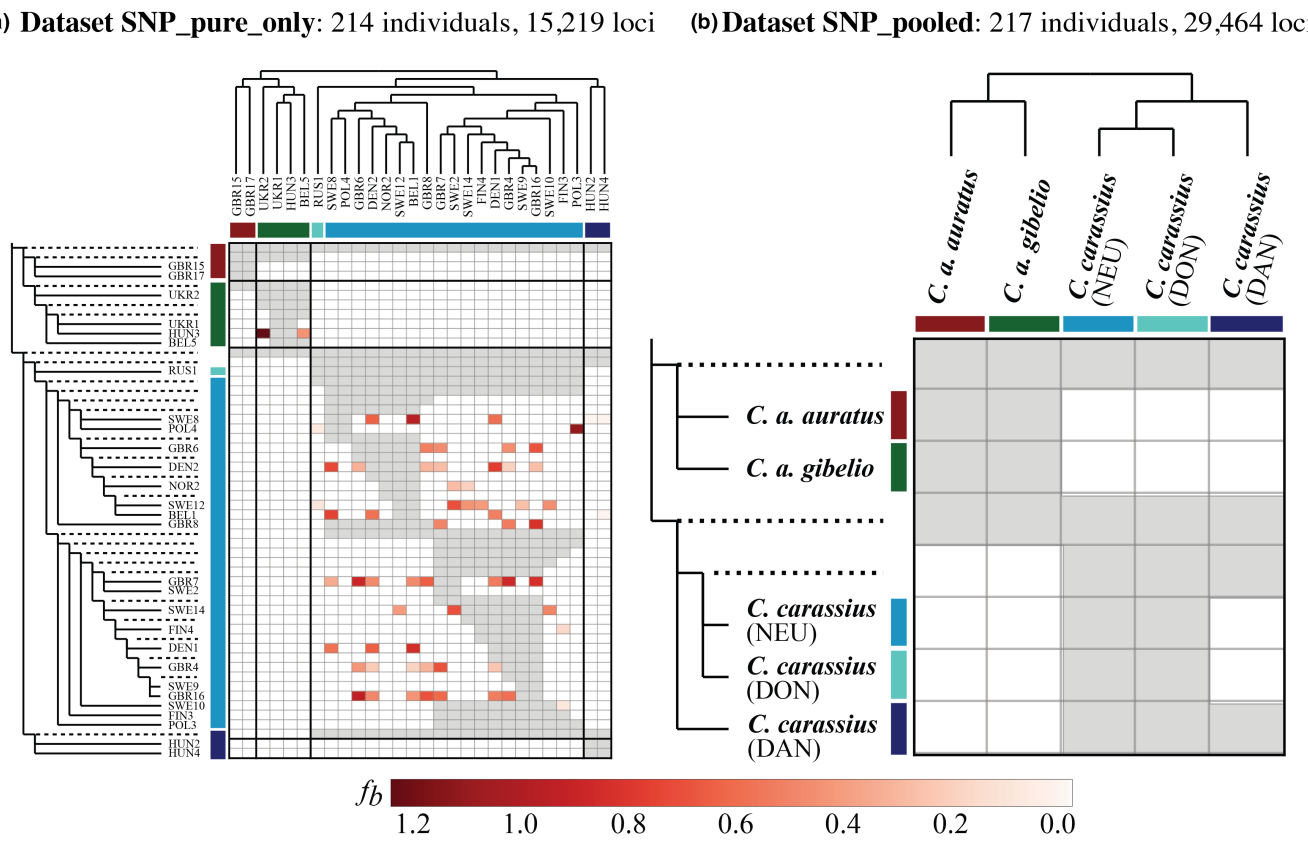

FIGURE 3 Results from the ABBA-BABA analyses with (a) all populations separated (data set *SNP_pure_only*, N samples: 214, N loci: 15,219) and (b) pooled by taxon and *C. carassius* lineage (data set: *SNP_pooled*, N samples 217, N loci 29,464). Populations in (a) corresponding to pools in (b) are shown with the coloured bars next to inferred phylogenies. *f-branch* value for each pair of populations is coloured according to the red scale shown, with higher *f-branch* values indicating higher rates of gene flow. Population or pool comparisons which do not conform to the phylogenetic relationship required to calculate *f-branch* are greyed out.

carassius genomes and one *C. a. gibelio* genome (Figure 4). This result also validates the finding that these individuals are triploid, as this is the only explanation for the alleles of one taxon only being consistently higher coverage.

We note that, while we did not deem it reliable to infer triploid hybrids with our microsatellite loci alone, four GBR10 hybrids identified as triploid in the RADseq analysis did in fact display triploid-like genotypes at one microsatellite locus GF29, all sharing the genotype 221/223/195. Alleles 221 and 223 are specific to *C. carassius*, while allele 195 is specific to *C. a. auratus* and *C. a. gibelio*. Thus, while we did not analyse these data using NewHybrids (which deals only with diploid genotypes), this locus did in fact present signal for triploid hybrids. In contrast, we did not detect triploidy in the SWE20 triploid hybrids with the microsatellites, though they were assigned to abnormal hybrid classes in the NewHybrids analysis (F2 instead of F1 in this case). If such a result is typical of triploid hybrids when only diploid genotypes are called, then there is also some evidence for triploid hybrids in several other populations from the microsatellite results, including populations GBR14, GBR16 and additional samples from GBR10 and SWE20 (Figure S4).

4 | DISCUSSION

4.1 | Prevalent hybridization but no evidence for introgression between *C. carassius* and non-native taxa

Hybridization with non-native taxa has the potential to severely impact native species and ecosystems, especially those that are already threatened by other factors. Here, we have shown that hybridization between the native and non-native taxa is extremely prevalent in this system, occurring in 82% of sampled locations where *C. carassius* and non-native taxa were found together. This is a minimum estimate, as our sampling of each population was not exhaustive, and hybrids may have been present but unsampled in the three remaining populations. This result corroborates the findings of previous studies (Hänfling et al., 2005; 2007; Mezhzherin et al., 2012; Smartt, 2007), adding to the consensus that hybridization between *C. carassius* and closely related non-natives is almost certain to occur where they are found together.

Despite the prevalence of hybridization in this system, microsatellite and RADseq screening of over 70 populations across the *C.*

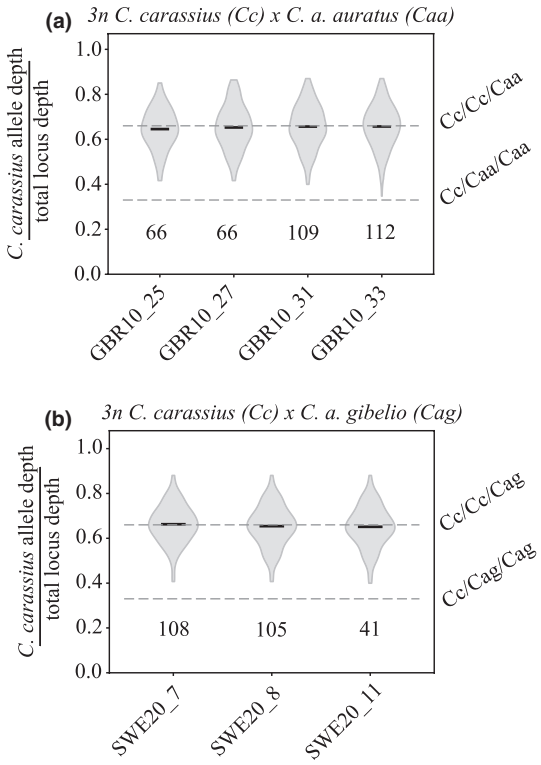

FIGURE 4 Polarized allele depth balance in seven triploid hybrids between *C. carassius* and (a) *C. a. auratus* and (b) *C. a. gibelio*. Expected allele balance ratios for the possible genome compositions of hybrids are given by dashed lines. Black lines within violins show the mean allele depth balance per sample. Numbers below violins show the number of loci used for each sample.

carassius range failed to identify any cryptic introgression between *C. carassius* and non-native taxa. These samples included 18 water bodies known to have non-natives present, and 54 with no known non-natives present. However, all water bodies in the sampled geographic regions were known to have non-native taxa present. A similarly low frequency of backcrossing was found by Hänfling et al. (2005), who identified only four individuals as backcrosses between *C. carassius* and *C. a. auratus* in the United Kingdom.

It is possible, however, that introgression does occur but that the approaches used in this study were unable to detect it. For example, introgression can, in some cases, occur at the scale of a few genes (for example see The Heliconius Genome Consortium, 2012). The genome of *C. carassius* is thought to be approximately 2.14 GB (Vinogradov, 1998); therefore, the ~30,000 SNP loci used to search for introgression here constitutes approximately one marker every ~71 kb (under the assumption that these markers are evenly spread throughout the genome). It is possible that small genomic regions have introgressed between these taxa but are not represented in our data set. However, if such small introgressed regions existed, it is likely that they resulted from old introgression events, as the size of introgressed linkage groups is eroded over time by recombination (Twyford and Ennos, 2011). The amount of time required to reduce a linkage group to a size undetectable in the present study would be dependent on the size of the original introgressed region, and any adaptive forces acting upon it, both of which are unknown. However, if such ancient introgression existed, there would have been more time for these regions to have segregated throughout each taxa, making them more likely to be detected with a limited sample number. Thus, while we cannot rule out that we have missed introgression in unsampled locations or genome regions, our results suggest that it is at least rare. Further analyses, using whole genome sequencing, are required to rule out introgression between these taxa more confidently.

4.2 | Barriers to introgression between native and non-native taxa

The prevalence of hybridization in this system prompts the question, why has there been no (or very low levels of) introgression between taxa beyond the early hybrid stage? Answering this question is vital for planning conservation and recovery strategies for *C. carassius*. Our observations of not only diploid hybrids but also triploid hybrids across multiple populations and between *C. carassius* and both *C. auratus* subsp. may offer an explanation. Triploid hybrids often result from hybridization via genome addition, wherein one parent (usually a hybrid) contributes an unreduced (diploid) gamete, while the other contributes a standard reduced gamete. Unreduced gametes are likely the product of dysfunctional meiosis in hybrids as a result of the divergence between their genomes. This phenomenon has been documented in many vertebrates, and especially teleosts (Neaves & Baumann, 2011). For instance, it has been observed that diploid female F1 hybrids of *Cobitis taenia* and *Cobitis elongatoides* produce unreduced oocytes. When these females backcross with diploid males of either taxon, it results in the production of triploid backcross offspring (Choleva et al., 2012; Janko et al., 2007). Similarly, in Poeciliid fish, *Poecilia mexicana limantouri* x *P. latipinna* F1 hybrids produce diploid oocytes that in turn lead to triploid offspring when fertilized (Lampert et al., 2007). We therefore propose that a similar situation exists in the hybrids between *C. carassius* and taxa of the *C. auratus* complex. The most likely scenario is that the triploid hybrids found in this study are in fact backcrosses that arose when diploid F1s produced unreduced gametes and backcrossed with a pure individual of one parental taxon. In the present case, as all confirmed triploid hybrids had subgenome composition Cc/Cc/Cag (Cc = *C. carassius*, Cag = *C. a. gibelio*), the most likely origin of such hybrids was via mating between F1 hybrids which produced diploid (Cc/Cag) gametes and pure *C. carassius* contributing haploid gametes (Cc). The same process likely also produces the suspected triploid hybrids with *C. a. auratus*.

Beyond the results of this study, this hypothesized mechanism is supported by two additional lines of evidence. First, triploid lineages are well documented within the *C. auratus* complex (Takada et al., 2010), and several of these have been shown to be of hybrid origin (Kuhl et al., 2022; Murakami et al., 2001). Importantly, if

they are not completely sterile, these triploid lineages are known to reproduce via gynogenesis, that is, clonal reproduction in females induced by the sperm of a conspecific or closely related taxa, but without incorporation of DNA from the paternal gamete (Knytl et al., 2022). Second, controlled crossing experiments (D. K. Lamatsch, *unpublished*) have shown that, while backcrossing F1 hybrids between *C. carassius* and diploid *C. a. gibelio* with *C. carassius* produced viable offspring, these offspring showed a high incidence of triploidy and propagation of crossing lines beyond this point was not possible.

The above evidence and the results of the present study suggest that meiotic dysfunction in hybrids may be responsible for partially, if not completely prohibiting gene flow between *C. carassius* and both *C. a. auratus* and *C. a. gibelio*. This would explain the lack of introgression observed in our genomic analyses of the many natural populations herein. However, confirming this hypothesis requires further work and will face several challenges. First, additional crossing experiments of multiple hybrid classes and different ploidy combinations are required to fully understand the mechanisms at work and the strength of the reproductive barrier between these taxa. Second, much more detailed data are required from natural populations, as it is currently impossible to ascertain, based on the published data available, the relative frequencies of each hybrid and ploidy class in nature. Indeed, reliably identifying triploid backcrosses is not possible with morphological data alone (Knytl et al., 2018), or even specific amplified genetic markers (e.g. microsatellites). Future work in this system must therefore prioritize not only the identification of hybrids but also their specific hybrid class, ploidy and reproductive potential, in order to fully exclude the possibility of gene flow between *C. carassius* and non-native taxa.

4.3 | The impact of hybridization on *C. carassius*

Despite the assumed low risk of introgression from non-natives, observations of populations composed entirely of hybrids between *C. carassius* and taxa of the *C. auratus* complex suggest that hybrids can negatively impact *C. carassius* in other important ways (Hänfling et al., 2005; 2007; Mezhzherin et al., 2012). However, previous studies have failed to find any obvious negative ecological impacts of non-natives on natural *C. carassius* populations (Copp et al., 2010); thus, it remains unknown how extirpation of *C. carassius* occurs. Based on the results found here and elsewhere (Smartt, 2007), hybridization could pose a substantial threat to *C. carassius*, even in the absence of introgression. If a population consists of, for example, *C. carassius*, *C. a. auratus* and their hybrids, then six types of matings can occur: Cc × Cc, Caa × Caa, Cc x Caa, Cc × (Cc/Caa), Caa × (Cc/Caa) and (Cc/Caa × Cc/Caa). Only one of these combinations (Cc × Cc) would maintain the crucian population in the long term, one benefits the non-native taxa (Caa × Caa) and the remaining four all will result in some type of hybrid. Thus, without strong pre-zygotic barriers to reproduction, the laws of probability predict that populations would inevitably shift to a composition of mostly hybrids. This situation is even more likely if triploid hybrids can reproduce gynogenetically, which releases them for the need for conspecific mates. However, ecological studies of direct pressure from hybrids as well as mating preferences are needed before we can truly evaluate the threat that hybrids pose to *C. carassius*. Furthermore, we are also lacking reliable estimates of how often *C. carassius* co-occurs with the non-native taxa throughout Europe. This knowledge is essential to gauge the scale of the threat faced from these taxa. However, such knowledge may soon be accessible given the rise of eDNA sampling techniques.

5 | CONCLUSIONS

The results of the present study confirm the high hybridization rates between *C. carassius* and the three non-native taxa studied here, with hybridization occurring in almost all populations where they were sympatric. However, the lack of introgression suggests little risk of genetic contamination of the *C. carassius* genome. This is reassuring news for ongoing *C. carassius* reintroduction and conservation programmes, as it increases the usability of existing *C. carassius* populations for reintroduction programmes. Additionally, facilitating the recovery of *C. carassius* populations by removing non-natives and hybrids (e.g. via Fyke or Seine netting) also becomes a viable conservation strategy in small water bodies when we can be confident that the remaining fish are indeed pure *C. carassius*. However, additional sampling and detailed monitoring of the frequency of diploid/triploid hybrids would be invaluable for confirming such assumptions. The genomic resources produced here would allow for time and cost-efficient assays for this purpose, for example, via quantitative PCR amplification of taxon-specific alleles (easily designed from the RADseq markers) or flow cytometry (Lamatsch et al., 2000). However, it is also important to further elucidate the ecological impacts imposed on *C. carassius* by the non-native taxa themselves and especially via their hybrids.

AUTHOR CONTRIBUTIONS

DLJ and BH conceived the project, with input from LLH. DLJ, BH, KHO, CDS and GC collected samples. DKL provided samples and advice throughout the project. DLJ performed all wet lab work and analyses. DLJ and BH led the writing of the manuscript with input from all other authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence data and associated metadata can be found on the Sequence Read Archive using the accession number SRP063043. Individual SNP and microsatellite genotypes and all scripts can be found on GitHub here: https://github.com/DanJeffries/Carassius_hyrbidisation_paper.

BENEFIT SHARING STATEMENT

All researchers who contributed genetic samples from across Europe are included either as co-authors on this manuscript, or in the acknowledgements section according to their individual contributions and requests. All researchers involved, and those in the wider freshwater ecology community in Europe will benefit from the data generated described herein, which is freely available as described above.

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SUPPORTING INFORMATION

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