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The invasive bighead goby *Ponticola kessleri* displays large-scale genetic similarities and small-scale genetic differentiation in relation to shipping patterns

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Abstract

Colonization events, range expansions and species invasions leave genetic signatures in the genomes of invasive organisms and produce intricate special patterns. Predictions have been made as to how those patterns arise, but only very rarely, genetic processes can be monitored in real time during range expansions. In an attempt to change that, we track a very recently established invasive population of a fish species, the bighead goby *Ponticola kessleri*, with high temporal and spatial resolution through 2 years to identify patterns over time. We then compare Swiss and German samples of bighead goby along the river Rhine using microsatellites, mitochondrial D-loop sequences and geometric morphometrics to investigate geographic patterns. We detect weak temporal and strong geographic patterns in the data, which are inconsistent with isolation by distance and indicate long range transport. In search of an explanation for our observations, we analyse the vector properties and travel patterns of commercial vessels on the river Rhine. We present evidence that freshwater cargo ships and tankers are plausible vectors for larvae of invasive goby species. We also present indications that cargo ships and tankers act as differential vectors for this species. In summary, we present genetic data at unique temporal resolution from a vertebrate invasion front and substantiate the paramount role of commercial shipping in freshwater fish translocations.

Keywords: ballast water, invasion genetics, microsatellite, Ponto–Caspian goby, vector

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Introduction

Colonization events, range expansions and biological invasions are predicted to leave certain signatures in the genome of a population (Excoffier *et al.* 2009). On the one hand, these signatures are helpful to track the geographic source regions and the pathways that an expanding species has taken. On the other hand, these signatures can be used to identify genetic challenges arising during range expansions. For example, an invasive population may be founded by just a few individuals that contain only a fraction of the source population's genetic variation (Williamson & Fitter 1996). Also, dispersal from the range margin may be under strong selective forces. For example, dispersal may favour certain traits such as flight capacities, directed movement behaviour, leg length or early flowering (Phillips *et al.* 2006; Hughes *et al.* 2007; Alford *et al.* 2009; Colautti *et al.* 2010). Expanding populations may also be subject to deleterious allele surfing events of

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non-neutral mutations, which can reach unexpectedly high densities at the expanding wave front (Travis *et al.* 2007; Peischl & Excoffier 2015). Such genetic challenges are thought to limit an expanding species' adaptive potential and, thus, its success of establishment in the newly colonized area by reducing genetic diversity, favouring inbreeding, counteracting environmental adaptations or by producing unfavourable allele combinations (Briskie & Mackintosh 2004; Frankham 2005; Dlugosch & Parker 2008; Dlugosch *et al.* 2015).

In some cases, however, species seem to have bypassed those challenges. Invasive species have been found to harbour significant levels of standing variation in the invasive range (Kolbe *et al.* 2004, 2007, 2008; Stepien & Tumeo 2006; Hochkirch & Damerau 2009). In some instances, invasive populations are genetically not diverse or even clonal, but nonetheless highly successful (Dlugosch & Parker 2008; Zhang *et al.* 2010; Carvalho *et al.* 2014; Lobos *et al.* 2014; Pigneur *et al.* 2014; Hagenblad *et al.* 2015; Ray *et al.* 2015). Dependent on the species specific situation, these unexpected observations have been explained by a variety of biological and/or evolutionary genetics mechanisms: large numbers of founding propagules (Simberloff 2009), parallel introduction events from diverse source populations (Durka *et al.* 2005; Henshaw *et al.* 2005; Brown & Stepien 2009; Zalewski *et al.* 2010), clonal reproduction strategies (Chapman *et al.* 2004), introgression events (Choler *et al.* 2004; Suehs *et al.* 2004), fast drift at the wave front of expanding populations (Edmonds *et al.* 2004; Miller 2010), fast adaptation and selection processes in the very early establishment phases (Phillips *et al.* 2006; Kelehear *et al.* 2012) or intraspecific admixture (Kolbe *et al.* 2008).

Most of these assumptions on genetic processes at invasion fronts of wild species, however, are inferential. Genetic data taken at high temporal and spatial resolution from colonizing populations in their early establishment phase are scarce. Observations and genetic investigations on invasive populations usually start many years or generations after the actual introduction event. By that time, secondary introductions and gene flow among introduced populations may have had ample opportunity to obscure early population scale processes (Colautti *et al.* 2005). Also, stratified-dispersal strategies, in which different vector activities blend with natural dispersal in space and time, may yield genetic patterns which no longer contain the genetic signatures of the original introduction events (Bronnenhuber *et al.* 2011). Therefore, many aspects about genetic processes during early invasion stages remain unknown (Bock *et al.* 2014).

Invasive Ponto–Caspian gobies are benthic fish species which present an excellent case study to fill some of these knowledge gaps in vertebrates. As vigorous and adaptive invaders with high reproductive capacities, five species of Ponto–Caspian gobies (*Neogobius melanostomus*, *Ponticola kessleri*, *Neogobius fluviatilis*, *Proterorhinus semilunaris* and *Babka gymnotrachelus*) are presently colonizing European freshwaters, European coasts and the Great Lakes and its tributaries. They are expected to colonize the majority of freshwater and brackish temperate water bodies worldwide (Puntila *et al.* 2013; Snyder *et al.* 2014; Hempel & Thiel 2015). Ponto–Caspian goby invasions have been attributed to shipping traffic (Roche *et al.* 2013). Importantly, however, there are no records of Ponto–Caspian larvae or adults found aboard ships in the scientific literature, and speculations on egg attachment can be traced to a single anecdotal source (Tsepkin *et al.* 1992; Sokolov *et al.* 1994; Moskal'kova 1996; Ahnelt *et al.* 1998). In Europe, several invasion corridors have been proposed (Ricciardi & MacIsaac 2000), but the relative contribution of these pathways to the spread of Ponto–Caspian gobies has not yet been analysed on a molecular level. Importantly, Ponto–Caspian gobies are easy to sample, and it is possible to install extensive passive monitoring schemes to detect very early invasion stages.

We chose one Ponto–Caspian goby species, the bighead goby *Ponticola kessleri*, to evaluate population genetic structure during a vertebrate range expansion in time and in space and to identify processes, such as vector activities, which may have an impact on the genetic structure. We chose this particular species for two reasons. First, very little is known about the phylogeographic and genetic structure of this successful pan-European invader, as the only two existing studies could not detect genetic differentiation between the sampled populations (Ondrackova *et al.* 2012; Cerwenka *et al.* 2014a). Ondrackova *et al.* (2012) compared the genetic diversity of one bighead goby population from the native range with an introduced population from the invasive range using 16 microsatellites. They found those populations to be similar in diversity and attributed this to high propagule pressure during the invasion, which would promote the transfer of a wide spectrum of alleles from the native range to the invasive range. Cerwenka *et al.* (2014a) sampled bighead goby *Ponticola kessleri* and round goby *Neogobius melanostomus* subpopulations at several sites along the Upper Danube for AFLP and mtDNA cytochrome B analysis. They could identify genetic patterns in round, but not in bighead goby and suggested that a genetically impoverished source population or a genetic bottleneck in the bighead goby may be the reason for this low genetic variability. This is, actually, a vivid example on how similar genetic data can be interpreted in very different ways with regard to the unobserved processes that may have caused the observed patterns.

A second reason why the bighead goby is attractive for the study of genetic processes during invasion is that bighead goby usually invades Central European sites before other Ponto–Caspian goby species (Seifert & Hartmann 2000; Paintner & Seifert 2006; Borcherding *et al.* 2011). The bighead goby invaded the Upper Danube River area before the round goby (Seifert & Hartmann 2000; Paintner & Seifert 2006) and developed high population densities, which then decreased after the arrival of the round goby (Cerwenka *et al.* 2014b). In this case, genetic data indicate that the bighead goby may be genetically less diverse and therefore less able to adapt to novel environments than the round goby. The same dynamic pattern of the two species is also visible in the Lower Rhine and, in this location, may be attributable to lower competitive strength on food resources in the bighead goby (S. Gertzen, J. Borcherding, pers. observations). The bighead goby may thus be more revealing with regard to the introduction pathways of Ponto–Caspian gobies in Europe than other invasive goby species because its early establishment is least affected by competitive interactions with sister species.

For this study, bighead goby samplings were initiated at an invasion hotspot, the commercial harbour in the river Rhine at Basel (Switzerland), immediately after fishermen first recorded the species (Kalchhauser *et al.* 2013), and were continued weekly for 2 years. These samples were complemented with samples taken along 16 km of upstream river in the High Rhine in Switzerland, and with samples from the Lower Rhine in Western Germany, taken >600 km downstream. All sampled individuals were genotyped for 15 microsatellites. A subset of individuals, chosen on the basis of microsatellite results, was additionally subjected to mitochondrial haplotype analysis and body morphology quantifications. With the ambition to provide explanations for the observed genetic patterns, information on the use and on the specifications of freshwater ballast water tanks was recovered from locally relevant shipping companies, and the travel patterns and mooring patterns of all ships arriving in Basel in 2012 were analysed.

We considered the river Rhine, which is the second largest river in Central Europe and heavily impacted by shipping traffic, a uniform introduction route, and commercial shipping a homogeneous vector, and thus expected the samples from the invasion front in Switzerland to be genetically homogeneous. We also expected to identify time-dependent patterns from the genetic markers in the Swiss harbour population, based on the idea that ships would continuously supply new propagules and thus add new alleles to the recently established population. We figured that the samples from Western Germany would likely differ genetically from the Swiss samples, based on the notion that geographically separated populations are usually genetically more distant than geographically close populations ('isolation by distance'). We further expected that maternally inherited mitochondrial haplotypes and nuclear markers would give comparable results, because to date, there is no record of sex-specific invasion behaviour in Ponto–Caspian gobies.

Body morphology was expected to be independent of either genetic markers. In fish, and also in several goby species, body morphology is an ecologically relevant phenotypic trait that reflects how an individual interacts with its environment (Smith & Skulason 1996; Hirsch *et al.* 2013). Ecological theory predicts that invasive species should be highly plastic because this would enable a faster adaptation to new environmental conditions such as novel food sources (Agrawal 2001; Davidson *et al.* 2011). However, morphology is of course not completely independent from genetic features. Aspects of morphology may be encoded by loci that are linked to microsatellites, and may therefore differ between genotypes, as has been seen for the lateral plate phenotype in sticklebacks (Colosimo *et al.* 2004). Finally, we expected that patterns in vector behaviour and genetic patterns at the invasion front would match and thus confirm previous notions on the vector activities responsible for the introduction of the species.

Materials and methods

Sampling and geography

Bighead gobies were sampled at several sites in the Lower Rhine in Western Germany, where the bighead goby established before 2006, and at several sites in the High Rhine in Switzerland, where the bighead goby established just before 2012 (Kalchhauser *et al.* 2013; Lower Rhine: km 660 to 1.033 of the river Rhine; High Rhine: km 0 to 165 of the river Rhine). Sampling sites were named from 1 to 16 and are indicated in Fig. 1. Sites 1–5 are situated in the commercial harbour of Basel, Switzerland, and were probed biweekly between 2012 and 2014. Sites 6–11 are situated within 16 km upstream from the commercial harbour in the High Rhine in Switzerland, and were probed at varying time points between 2012 and 2013. Sites 12–16 are situated in Western Germany in the Lower Rhine and surrounding channels and were probed at varying time points between 2013 and 2014. Site coordinates as well as geographic distances between sites are indicated in Table 1. Catch methods included minnow traps, spawning traps (Hirsch *et al.* 2015), angling and electro fishing in accordance with national legal requirements. All fish were

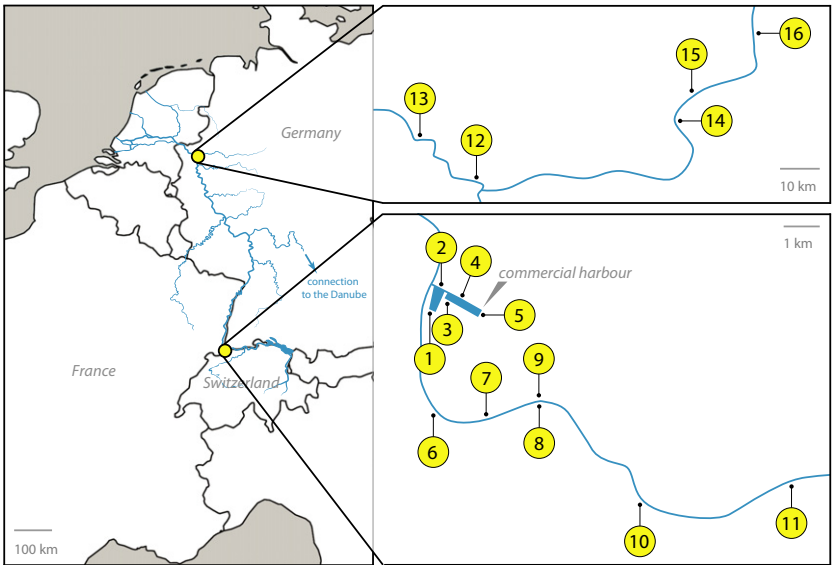

Fig. 1 Map of sampling sites. Sampling sites are situated in Switzerland in the river Rhine (1–11) and in Western Germany (12–16) in the river Rhine and surrounding channels. Swiss sampling sites are spread along 16 km and are separated from German sites, which are spread across 100 km, by 640 km. Site coordinates and distances between individual sites are given in Table 1. At sites 1–11, bighead gobies were first reported between 2012 (site 6) and 2014 (site 11). At sites 12–16, bighead gobies were first reported in 2006. The town Basel (lower yellow dot in the left panel) is situated at 47° 33′28.1″N 7°35′17.2″E.

Table 1 Sampling site coordinates

Sampling site	Latitude	Longitude	Region	Sampling site name	*n*
1	47°34′58.8″N	7°35′18.3″E	Switzerland	Commercial harbour, site A	74
2	47°35′20.2″N	7°35′32.7″E	Switzerland	Commercial harbour, site B	73
3	47°35′14.9″N	7°35′36.4″E	Switzerland	Commercial harbour, site C	73
4	47°35′07.4″N	7°35′55.0″E	Switzerland	Commercial harbour, site D	74
5	47°35′05.0″N	7°36′06.1″E	Switzerland	Commercial harbour, site E	74
6	47°33′25.9″N	7°35′32.8″E	Switzerland	Münstergalgen	1
7	47°33′29.9″N	7°36′39.0″E	Switzerland	Galgen 30	6
8	47°33′45.5″N	7°37′47.2″E	Switzerland	Galgen 8	4
9	47°33′33.2″N	7°38′10.9″E	Switzerland	Birsfelden	32
10	47°31′59.2″N	7°40′25.8″E	Switzerland	Schweizerhalle	1
11	47°32′27.8″N	7°43′16.5″E	Switzerland	Kaiseraugst	5
12	51°45′50.4″N	6°20′13.2″E	Germany	Rhein km 842	2
13	51°39′28.9″N	6°35′38.1″E	Germany	Rhein Wesel	5
14	51°47′17.5″N	7°23′39.6″E	Germany	DEK Abfahrt Lüdinghausen	32
15	51°50′42.5″N	7°28′08.0″E	Germany	DEK Abfahrt Senden	1
16	51°59′05.5″N	7°39′37.8″E	Germany	DEK Schleuse Münster	17

n, number of individuals sampled at the respective site.

frozen at −20 °C after catch, later thawed on ice and weighed, measured, sexed and photographed in a standardized manner. All details on the samples, such as sampling time points, catch methods, weight, length and sex of each individual are indicated in Table S1 (Supporting information).

Microsatellite analysis

We tested 46 published goby microsatellites (Dufour *et al.* 2007; Vyskocilova *et al.* 2007; Feldheim *et al.* 2009; Ruggeri *et al.* 2012) for amplification from bighead goby DNA in single amplicon PCRs at annealing temperatures between 54 and 64 °C with FastStart Taq DNA Polymerase from Roche [amplification protocol: 4′ 94 °C; 30″ 94 °C, 30″ 54–64 °C, 1′ 72 °C (35 cycles); 7′ 72 °C; 4°C ∞]. For reactions which failed, alternative oligos were designed, renamed (f.ex., Nme3.1 fw is the redesigned Nme3 fw), and amplification was retested. In total, 36 of 46 microsatellites could be amplified, cloned and sequenced for bighead goby using this procedure.

All 46 microsatellites were also tested and, if they could be amplified successfully, cloned in round goby to serve as a resource for similar studies in this species. Microsatellite sequences from both species are compiled

in Appendix S1 (Supporting information). All oligos used in this study are listed in Table S2 (Supporting information).

Multiplex sets were compiled and amplified with the Qiagen Multiplex PCR Kit (amplification protocol: 15′ 95 °C; 30″ 94 °C, 90″ 56 °C, 1′ 72 °C (35 cycles); 30′ 60 °C; 4 °C ∞) according to the manufacturers' instructions. A total of 200 bighead goby individuals from sites 1–5 were initially genotyped to identify polymorphic, reliably amplifying microsatellites among the 36 cloned microsatellites. A total of 20 microsatellites were polymorphic, and 15 amplified reliably in multiplex PCR. The oligo sets that worked reliably were: Set 1: Ame10, NG92, NG150, NG195, NG70, Nme3.1; Set 2: NG71, NG111, NG135, NG184; and Set 3: NG132, NG167, NG236, NG28, Nme6. Oligos were fluorescently labelled with the dyes TAMRA, ROX, 6-FAM and JOE as indicated in Table S2 (Supporting information). An overview of the microsatellite selection process is presented in Table S3 (Supporting information).

Between May 2012 and April 2014, more than 1000 bighead gobies were caught in the harbour at sites 1–5. To reduce the size of the harbour sample, while preserving the ability to identify genetic changes over time, we decided to use the first and the last fish caught at each site in the commercial harbour for microsatellite analysis. For the '2012' group, we chose the first 37 individuals caught at each site, starting May 2012. For the '2014' group, we chose the last 37 individuals caught at each site, up to April 2014. An overview of the samples chosen is given in Fig. S1 (Supporting information). From all sites outside the commercial harbour, all available individuals entered microsatellite analysis. In total, 474 individuals were genotyped: 368 from the Swiss harbour (sites 1–5), 49 from the High Rhine (sites 6–11) and 57 from the Lower Rhine and surrounding channels (sites 12–16).

DNA was isolated from muscle samples using the DNeasy Blood and Tissue 96 well Kit from Qiagen. Microsatellites were amplified from 1 microlitre of eluate in PCR plates using the oligo sets indicated above and the Qiagen multiplex PCR Kit. Amplified samples were spiked with GeneScan – 500 LIZ Size Standard from Applied Biosystems and analysed on an ABI sequencer. Microsatellite traces were scored using Peak Scanner 2. Fragment lengths were rounded in Excel after manual inspection of the length value distributions of each microsatellite. Population structure was determined for all samples including females, males, juveniles and nonsexable individuals together (n = 474), as well as for females (n = 239) and males (n = 214) separately, and for harbour samples (sites 1–5, n = 368) and all nonharbour samples (sites 6–16) separately, using Structure 2.3.4 (Pritchard *et al.* 2000), under the admixture model with 10^5 burnings and 10^6 iterations. Structure Harvester 0.6.8 (Earl & vonHoldt 2012) was used to implement the Evanno method (Evanno *et al.* 2005) to find the most probable number of genetic clusters K. Runs were performed to K = 8 (all samples), K = 5 (males and females), K = 5 (harbour samples only) and K = 7 (all samples except harbour samples). Based on a first Bayesian cluster analysis including the complete data set, three groups were determined. These groups largely correspond to the different localities of catchment and were accordingly named Swiss harbour SH, Swiss Rhine SR and German Rhine GR (see results part for details). Based on this finding, all loci were checked group-wise for genotyping errors such as large allele dropout and stuttering and the presence of null alleles using the software Microchecker (van Oosterhout *et al.* 2004). Indications for stuttering were found in two loci (Ame10 and NG150), null alleles were indicated for three loci (Ame 10, NG150 and NG111) in one cluster and for NG071 in another group (Table S4, Supporting information). However, none of the loci showed a consistent pattern of genotyping errors occurring in more than one group and exclusion of the four loci did not alter the results for the pairwise F_{ST} comparisons. Therefore, all 15 loci were used in further analysis.

Possible deviations from the Hardy–Weinberg equilibrium (HWE) were calculated by comparing the number of observed and expected heterozygotes and tests for locus by locus linkage disequilibrium using Arlequin 3.5 (Excoffier & Lischer 2010). Arlequin 3.5 was also used to calculate pairwise F_{ST} comparisons between all localities sampled and between the three groups found by the Structure program. To look for a pattern of isolation by distance, a Mantel test (10 000 permutations) was conducted in Arlequin 3.5 correlating pairwise F_{ST} comparisons between sampling locations with geographic distance (km). To adjust for strongly unequal sample sizes, the disproportionally large harbour sample was reduced using only a random subset from harbour locations (sampling site 1 from 2012, sampling site 2 from 2012, sampling site 4 from 2014 and sampling site 5 from 2014, n = 148). Allelic counts, richness and prevalence were calculated using the hierfstat package in R [Version 2.13.1; R Core Team (2014)]. Data not presented in the results part are summarized in Table S4 (Supporting information).

Analysis of mitochondrial haplotypes

To complement the nuclear microsatellite data, we established and analysed mitochondrial markers. The mitochondrial D-loop contains the replication origin and regulatory sequences and is considered the most variable region in the mitochondrial genome. Therefore,

the D-loop is considered a suitable mitochondrial sequence to discriminate populations that are suspected to be closely related. It has been previously used to infer on-site evolutionary divergence in North American invasive goby populations (Dillon & Stepien 2001). We first identified polymorphic nucleotides by sequencing the entire mitochondrial D-loop (Kalchhauser *et al.* 2014; D-loop: nucleotide 15961–16890 and 0–527 from GenBank accession no. KM583832, 2029 bp in total) of a subsample of 37 individuals chosen randomly from the sample set to represent all major sampling sites (choice of individuals indicated in Table S1, Supporting information). We identified four deviations from the published mitochondrial genome in 14 of 37 individuals [nt 239 A->G (1×), nt 16038 G->A (5×), nt 16249 G->A (10×) and nt16393 G->A (1×)]. We then developed a PCR-based SNP-genotyping assay for the two more frequent polymorphisms, nt 16038 G/A and nt 16249 G/A. In this assay, the 3′ nucleotide of the forward primer of the PCR assay binds to the polymorphic site, which results in differential amplification behaviours of the two alleles and differential band patterns of the PCR products after separation on an agarose gel. Oligos SL_F16024_pmA and SL_R16367 were used at an annealing temperature of 49 °C to genotype nt 16038 G/A. SL_F16231_pmG and SL_R16503 were used at an annealing temperature of 60 °C to genotype nt 16249 G/A. Illustra PuReTaq Ready-To-Go PCR Beads were used to amplify the fragments according to the manufacturers' instructions. The assay was then performed on 147 additional individuals that were carefully chosen to represent individuals from all microsatellite clusters and from all major sampling sites. Chosen individuals are indicated in Table S1 (Supporting information). Individuals were genotyped and assigned to one of the four D-loop haplotypes, GG, GA, AG or AA. We then tested whether populations and clusters as defined from microsatellite analysis would differ in mitochondrial haplotype proportions with the prop.test function from the stats package in R [Version 2.13.1; R Core Team (2014)].

Analysis of phenotypic differentiation

Geometric morphometrics are an established way to assess body shape differences independently of body size. For geometric morphometrics, each individual was photographed with its fins spread and fixed to the surface of a polystyrene bed. We chose 22 landmarks on the left side of each specimen following general guidelines for placement of landmarks (Zelditch *et al.* 2012). We also digitized five semilandmarks to account for shape differences in regions of the fish body that do not naturally contain landmarks, such as fin insertions (see Fig. S2, Supporting information). To quantify morphological variation in body shape among individuals, we performed multivariate geometric shape analysis. After digitizing the landmarks using TPSDIG (all pictures clicked by one person), we analysed each landmark's relative position and hence overall variation in body shape using TPSRW [Thin-Plate Spline Relative Warp (Rohlf & Marcus 1993), all TPS-software and information available for download at http://life.bio.sunysb.edu/morph/index.html]. TPSRW allowed calculation of the partial warp and uniform scores that denote the differences in body shape among the individuals. To account for differences in size among specimens, the geometric morphometrics analysis includes a scaling procedure. During this scaling procedure both partial warps and uniform scores are scaled to centroid size as part of a generalized procrustes analysis (GPA; please refer to Rohlf & Slice (1990) for details of the method). We then analysed the partial warps and uniform scores using a multivariate discriminant function analysis (DFA using Statistica version 11) based on the classification of individuals into genetic clusters. Following a significant DFA, we calculated a canonical variance analysis (CVA). The CVA combined all partial warp and uniform scores for each individual into a single score that maximally discriminates between the previously chosen classifications. The CVA scores were used solely for visualization of the differences in morphology because they represent single values for an individual that are easy to use in software designed to visualize shape differences. For visualization of the body shape differences between classifications, we manually connected the landmarks of two extreme (5× the observed range of scores) individuals that lie on opposite ends of the morphology spectrum. Body shape depictions were created using the software TPSREGR that regresses the variation in body shape with independent variables such as CVA scores.

Analysis of vector plausibility

Many species invasions depend on a vector, which picks up individuals in the native range, transports them across a distance, which they would not be able to cover on their own, and releases them alive at a location where the species is not native. A transport vehicle can be considered a plausible vector for a certain species when it has properties that allow the pickup and release of individuals of this species, and when the species displays features that promote pick-up by the vehicle, such as attachment organs or a small life stage. To investigate whether commercial freshwater vessels were a plausible vector for Ponto–Caspian gobies, we gathered information on vessel properties from shipping

companies operating in Basel. We contacted all shipping companies listed by the Ports of Switzerland per March 2014 (http://www.port-of-switzerland.ch/) and asked for an opportunity to interview a representative with expertise in ship construction. A total of 11 out of 42 officially listed companies could be reached and were willing to get involved. Phone conversations with company representatives were conducted in a flexible, situation-dependent manner, but followed guideline questions. Guideline questions focused on (i) whether the company's vessels would use ballast water, in which situations, and how much, (ii) what kind of filters were used to prevent particulate material from entering ballast water tanks and (iii) whether the interviewee could imagine any other transport opportunities for small sticky items such as eggs (Hirsch *et al.* 2015), small floating items such as larvae (Hensler & Jude 2007; Janac *et al.* 2013), or items of the size of an adult goby on board the company's vessels. To investigate whether freshly hatched goby juveniles could be taken up through ballast water filters with the obtained specifications, we collected clutches from the wild, hatched larvae in the laboratory (Hirsch *et al.* 2015) and measured their size.

Analysis of commercial vessels' mooring patterns in Switzerland

Patterns of genetic differentiation among members of a very recently introduced population are an indication for differential introduction pathways. In search of an explanation for the genetic structure observed among Swiss samples (sites 1–11), we analysed port call data provided by the Ports of Switzerland. These data contain information on the accurate mooring position(s), as well as exact arrival and departure times, of all vessels that use infrastructure of the Ports of Switzerland. We chose to analyse data from the year 2012, when the bighead goby was first recorded in Switzerland (Kalchhauser *et al.* 2013). As we were interested in incoming voyages with long distance vector potential, we excluded local ferry services and local small-scale cargo shipping among local ports from the data set. We did this by filtering for newly incoming cargo ships and tankers that had been absent from Basel for at least 20 days before in R [Version 2.13.1; R Core Team (2014)] and Excel. A total of 4419 arrivals passed this filter and were grouped by mooring site and ship type.

Analysis of commercial vessels' travel patterns

If the localities of certain vector types, such as cargo ships and tankers in our case, overlap with the localities of certain genotypes, differential properties of these vector types may represent the underlying cause for the observed genetic pattern. We tested whether cargo ships and tankers arriving in Switzerland displayed such differences with respect to their travel patterns. Travel data of all vessels arriving in Basel in 2012 were procured from the FleetMon database (https://www.fleetmon.com/en/). These data contain information on all stops (location and time) which a vessel travelling towards Switzerland in 2012 had made in a 14-day interval before arrival. Unreasonable and faulty data were excluded from the set by removing those voyages that contained stop records outside of Europe as well as those voyages that exceeded 40 km/h. For the remaining 4469 voyages, the locations of source and stopover ports were plotted with the packages 'maps', 'mapdata', 'mapproj' and 'gpclib' of the software R [Version 2.13.1; R Core Team (2014)].

Results

Genetic processes in the Swiss harbour population

We first examined samples from the commercial harbour (sites 1–5) in detail. From 2012 to 2014, the bighead goby population displayed a short phase of gradual growth, followed by exponential population growth and a population peak in 2013. This was followed by a retrogression phase, in which catch per unit effort decreased substantially. The retrogression phase coincided with the arrival and establishment of the round goby in the harbour (Fig. 2A). Structure analysis (Fig. 2B) suggested the presence of two major genetic clusters in the commercial harbour. Increasing the most probable number of clusters K did not reveal any further substructure (Fig. S3, Supporting information). Admixed individuals could be identified, as only 51% of all individuals showed $q < 90\%$ for either cluster. Both the Structure plot as well as pairwise population F_{ST} values close to zero (Table 2) indicated a high degree of homogeneity among samples. However, we found evidence for subtle temporal changes. At site 5, where population growth and crash were most pronounced (Fig. S4, Supporting information), both Structure and F_{ST} value indicated that the '2012' and the '2014' samples differed to some degree. Also, when inspecting loci individually, we found that two loci experienced allele frequency changes. The 192 nt allele of NG167 was present in the beginning but disappeared towards 2014, while the 165 nt allele of Ame10 was absent in 2012 and appeared towards the end of the sampling period (Fig. 2C).

Microsatellite population structure

A first Structure run including all individuals from all samples indicated a most probable number of $K = 2$

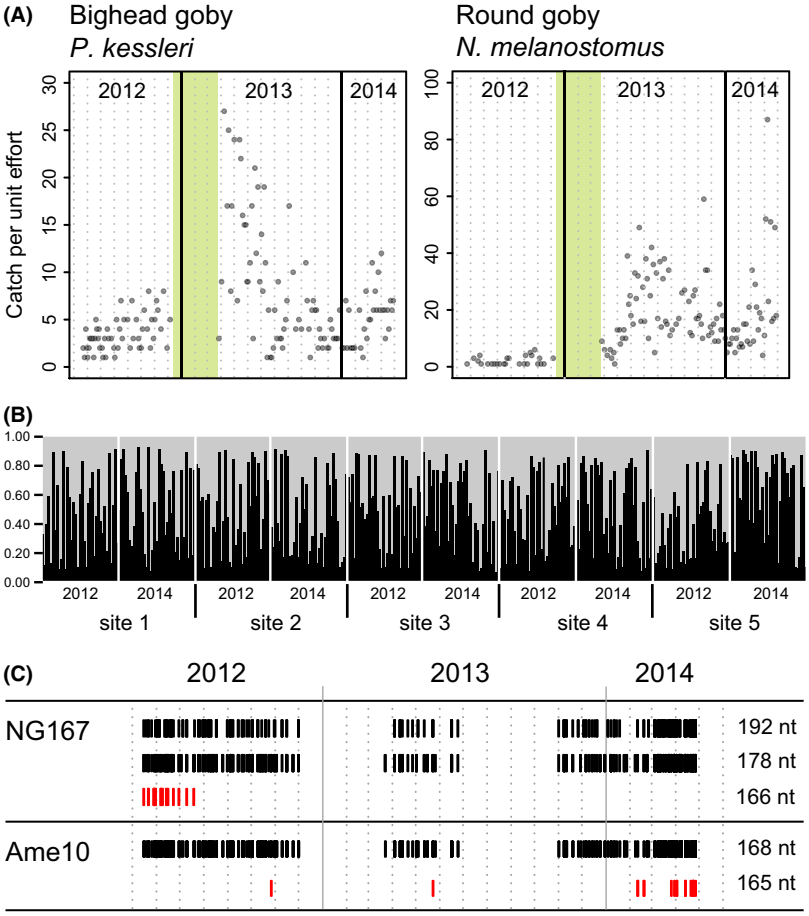

Fig. 2 Genetic processes in the harbour population. (A) Catch data from sites 1–5 in the commercial harbour from 2012 until 2014. Each dot indicates how many individuals of bighead goby or round goby were cumulatively caught in the commercial harbour on the respective field day. Vertical dotted lines indicate the first day of a new month. Vertical straight lines indicate the first day of a new year. No sampling took place from December 2012 to March 2013 (green bar). (B) Structure plot of '2012' and '2014' bighead goby samples from the indicated sites in the commercial harbour. Each vertical line represents one individual. The grey and black colour, respectively, indicates the degree of affiliation of the individual with the respective genetic cluster. (C) Allele occurrence of the microsatellites NG167 and Ame10 between 2012 and 2014. Each vertical bar represents an individual carrying the respective allele. Bars of alleles that experience frequency changes over time are drawn in red. The 166 nt allele of NG167 disappears in 2012, the 165 nt allele of Ame10 increases in incidence towards 2014.

genetic clusters (Fig. 3A, Fig. S5, Supporting information). Close inspection of Structure plots revealed two main groups or populations. The first group included individuals from the commercial harbour (sites 1–5) and the two most upstream locations in Switzerland, sites 10 and 11. These are hereafter referred to as 'population SH'. The second group included sites 6 to 9 in the Swiss Rhine, and the German sites 12 to 16, which are situated more than 600 km downstream (Fig. 1). An additional peak in the Delta K distribution at $K = 7$ however indicated further substructure in the entire data set (Fig. S5, Supporting information). Based on these findings, we confirmed substructuring in the second group, between gobies from the Swiss Rhine (hereafter called 'population SR') and the German sites (hereafter called 'population GR'). While gobies from population SR showed no further structure, additional substructuring was indicated for the German sites (Fig. S3, Supporting information).

We used the population structuring as indicated by the Structure runs to calculate the classical genetic diversity indices and pairwise F_{ST} comparisons population-wise. For eight of the 15 loci, we found deviations from the HWE for population SH, while only one locus deviated from HWE for population SR and none for population GR. The tests for deviations from linkage disequilibrium were significant in four of 315 comparisons at $P < 0.001$. However, because the significant linkage tests involved different pairs of loci in different populations, we concluded that they were more likely effects of type I errors than physical linkage between loci. The population substructure as indicated in the Structure runs was supported by significant pairwise F_{ST} comparisons (Table 2). Mantel tests were weakly significant (correlation coefficient: $r = 0.37$, $P = 0.025$) when all samples were included, and hardly significant when the dominating harbour sample was reduced in size (correlation coefficient: $r = 0.304$, $P = 0.049$).

Mitochondrial haplotypes and phenotypic differentiation

In a next step, we tested whether mitochondrial markers and body shape would mirror the microsatellite population structure. One-hundred fifteen individuals from population SH, 36 individuals from population SR

Table 2 Pairwise F_{ST} values between sampling sites. Sites with $n = 1$ were excluded from analysis. Top and bottom panel are identical except for the colour coding. F_{ST} values are indicated below, *P*-values above the diagonal. Green colour indicates *P*-values below 0.05. (A) F_{ST} values are heat-map colour coded from blue (lowest values) to red (highes values). (B) F_{ST} values are coloured yellow if they were significant after sequential Bonferroni correction

n	site	1 (2012)	1 (2014)	2 (2012)	2 (2014)	3 (2012)	3 (2014)	4 (2012)	4 (2014)	5 (2012)	5 (2014)	7	8	9	11	12	13	14	16
(A)																			
37	1 (2012)		0.41511	0.75596	0.75992	0.42026	0.27364	0.14385	0.16622	0.05613	0.49906	0.00792	0.00000	0.00000	0.04435	0.05445	0.07544	0.00000	0.00000
37	1 (2014)	0.00049		0.37046	0.21641	0.12840	0.67746	0.01455	0.19137	0.00000	0.53480	0.00139	0.00000	0.00000	0.05594	0.05168	0.12019	0.00000	0.00000
36	2 (2012)	−0.00360	0.00109		0.75220	0.65904	0.37303	0.54341	0.17434	0.01841	0.72191	0.00545	0.00010	0.00000	0.07455	0.09761	0.28769	0.00000	0.00000
37	2 (2014)	−0.00363	0.00338	−0.00351		0.63291	0.58578	0.56767	0.56153	0.14999	0.54331	0.01337	0.00010	0.00000	0.10445	0.13593	0.31225	0.00000	0.00000
36	3 (2012)	−0.00048	0.00469	−0.00303	−0.00279		0.35175	0.95921	0.44392	0.22136	0.61350	0.00495	0.00000	0.00000	0.02604	0.05079	0.11316	0.00000	0.00000
37	3 (2014)	0.00209	−0.00269	0.00078	−0.00181	0.00018		0.24116	0.96753	0.02267	0.52510	0.00238	0.00000	0.00000	0.08534	0.10702	0.20919	0.00000	0.00000
37	4 (2012)	0.00430	0.01203	−0.00152	−0.00167	−0.00792	0.00233		0.47906	0.13306	0.09336	0.00139	0.00000	0.00000	0.02792	0.10167	0.26552	0.00000	0.00000
37	4 (2014)	0.00495	0.00461	0.00507	−0.00110	−0.00034	−0.00763	−0.00061		0.21523	0.48659	0.00317	0.00000	0.00000	0.13692	0.10484	0.27512	0.00000	0.00000
37	5 (2012)	0.00793	0.02470	0.01246	0.00464	0.00243	0.01046	0.00470	0.00385		0.00673	0.00079	0.00000	0.00000	0.01762	0.09266	0.08039	0.00000	0.00000
37	5 (2014)	−0.00072	−0.00105	−0.00343	−0.00132	−0.00268	−0.00127	0.00644	−0.00031	0.01654		0.00327	0.00010	0.00000	0.22552	0.04168	0.08118	0.00000	0.00000
6	7	0.05286	0.07148	0.05727	0.04922	0.05220	0.06372	0.06230	0.07211	0.07080	0.06896		0.57945	0.92030	0.03218	0.72488	0.14484	0.00000	0.00129
4	8	0.13733	0.14847	0.12859	0.11254	0.12842	0.13333	0.14233	0.14833	0.15398	0.13848	−0.00445		0.29482	0.04970	0.06514	0.00762	0.00000	0.00020
32	9	0.05972	0.06979	0.06768	0.05484	0.05955	0.06569	0.07293	0.06901	0.06949	0.07073	−0.02172	0.01333		0.00050	0.30749	0.03703	0.00000	0.00000
5	11	0.03871	0.03775	0.03551	0.02820	0.04214	0.03119	0.04597	0.02828	0.05321	0.01685	0.15883	0.16219	0.12899		0.17860	0.19543	0.00000	0.00129
2	12	0.07617	0.08089	0.06262	0.04801	0.06130	0.05538	0.05224	0.06591	0.05589	0.09377	0.02055	0.05986	0.03166	0.17501		0.61994	0.01287	0.17434
5	13	0.02626	0.02193	0.00840	0.00617	0.01694	0.01192	0.00525	0.01190	0.02658	0.02958	0.04101	0.08779	0.04968	0.04631	−0.04070		0.00040	0.20374
32	14	0.12861	0.11317	0.13236	0.10842	0.10880	0.11257	0.12619	0.12453	0.11704	0.13485	0.14422	0.17393	0.13020	0.15977	0.11129	0.08819		0.00000
17	16	0.08020	0.09691	0.08527	0.07837	0.09727	0.09786	0.08631	0.10612	0.09055	0.11880	0.09854	0.16478	0.09735	0.14343	0.04869	0.01476	0.13154	
(B)																			
37	1 (2012)		0.41511	0.75596	0.75992	0.42026	0.27364	0.14385	0.16622	0.05613	0.49906	0.00792	0.00000	0.00000	0.04435	0.05445	0.07544	0.00000	0.00000
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37	3 (2014)	0.00209	−0.00269	0.00078	−0.00181	0.00018		0.24116	0.96753	0.02267	0.52510	0.00238	0.00000	0.00000	0.08534	0.10702	0.20919	0.00000	0.00000
37	4 (2012)	0.00430	0.01203	−0.00152	−0.00167	−0.00792	0.00233		0.47906	0.13306	0.09336	0.00139	0.00000	0.00000	0.02792	0.10167	0.26552	0.00000	0.00000
37	4 (2014)	0.00495	0.00461	0.00507	−0.00110	−0 00034	−0.00763	−0.00061		0.21523	0.48659	0.00317	0.00000	0.00000	0.13692	0.10484	0.27512	0.00000	0.00000
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37	5 (2014)	−0.00072	−0.00105	−0.00343	−0.00132	−0.00268	−0.00127	0.00644	−0.00031	0.01654		0.00327	0.00010	0.00000	0.22552	0.04168	0.08118	0.00000	0.00000
6	7	0.05286	0.07148	0.05727	0.04922	0.05220	0.06372	0.06230	0.07211	0.07080	0.06896		0.57945	0.92030	0.03218	0.72488	0.14484	0.00000	0.00129
4	8	0.13733	0.14847	0.12859	0.11254	0.12842	0.13333	0.14233	0.14833	0.15398	0.13848	−0.00445		0.29482	0.04970	0.06514	0.00762	0.00000	0.00020
32	9	0.05972	0.06979	0.06768	0.05484	0.05955	0.06569	0.07293	0.06901	0.06949	0.07073	−0.02172	0.01333		0.00050	0.30749	0.03703	0.00000	0.00000
5	11	0.03871	0.03775	0.03551	0.02820	0.04214	0.03119	0.04597	0.02828	0.05321	0.01685	0.15883	0.16219	0.12899		0.17860	0.19543	0.00000	0.00129
2	12	0.07617	0.08089	0.06262	0.04801	0.06130	0.05538	0.05224	0.06591	0.05589	0.09377	0.02055	0.05986	0.03166	0.17501		0.61994	0.01287	0.17434
5	13	0.02626	0.02193	0.00840	0.00617	0.01694	0.01192	0.00525	0.01190	0.02658	0.02958	0.04101	0.08779	0.04968	0.04631	−0.04070		0.00040	0.20374
32	14	0.12861	0.11317	0.13236	0.10842	0.10880	0.11257	0.12619	0.12453	0.11704	0.13485	0.14422	0.17393	0.13020	0.15977	0.11129	0.08819		0.00000
17	16	0.08020	0.09691	0.08527	0.07837	0.09727	0.09786	0.08631	0.10612	0.09055	0.11880	0.09854	0.16478	0.09735	0.14343	0.04869	0.01476	0.13154	

and 32 individuals from population GR (individuals indicated in Table S1, Supporting information) that could unambiguously be assigned to a certain microsatellite genotype were genotyped for their D-loop haplotype. We could distinguish four D-loop haplotypes GG, GA, AG and AA, which we found to occur in a site-specific manner. GG occurred preferentially in the commercial harbour, GA occurred preferentially in the Swiss Rhine and AA occurred preferentially in the German Rhine. This site-dependent distribution was statistically significant, also when we restricted our analysis to individuals associated with the same microsatellite cluster (Fig. 3B, Table 3). The same was true for body shape. Individuals from the German Rhine and from the Swiss Rhine differed significantly in body shape, even when only individuals affiliated with the same microsatellite genotype were analysed. At the same time, we could not identify a significant difference in body shape between individuals caught in the same location, the commercial harbour, but affiliated with different genetic clusters (Fig. 3C, Table 4).

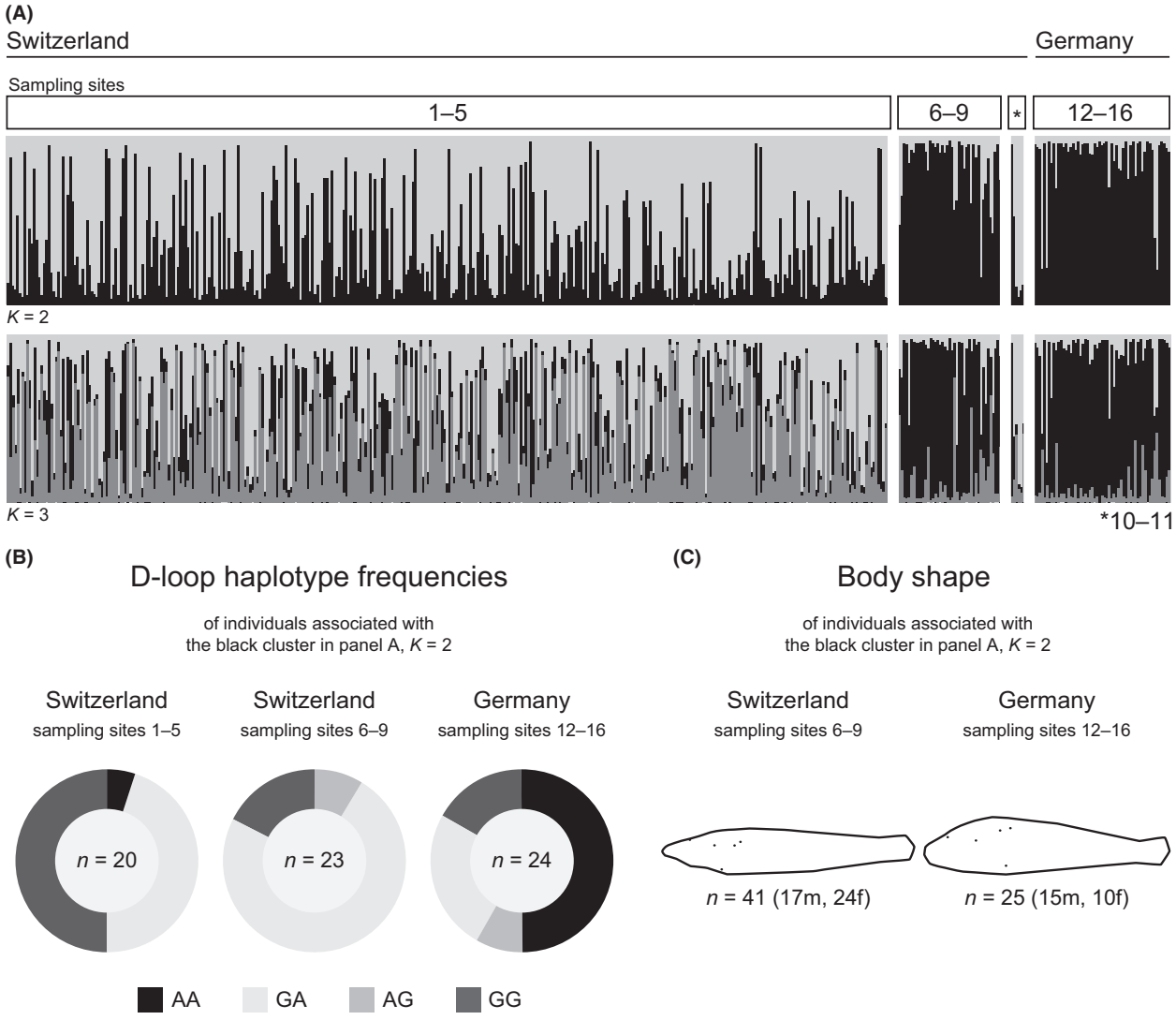

Fig. 3 Comparison of microsatellite structure, D-loop haplotypes and body shape. (A) Structure plot of all samples. Each individual is represented by a vertical line. The grey and black colour, respectively, indicates the degree of affiliation with the respective genetic cluster. Sampling sites and geographical region of origin of individuals are indicated above the plot. (B) Mitochondrial haplotype frequencies. The indicated numbers (*n*) of selected individuals from the indicated sites that were affiliated with the black cluster in panel A, *K* = 2 were genotyped for their D-loop haplotypes. (C) Body shape. The indicated numbers (*n*) of selected individuals from the indicated sites that were affiliated with the black cluster in panel A, *K* = 2 were analysed for their body shape using geometric morphometrics. m, males. f, females. Body outlines represent the connected landmarks of two extreme (5× the observed range of scores) individuals that lie on opposite ends of the morphology spectrum.

Table 3 D-loop haplotype affiliations. (A) Numbers of individuals affiliated with the respective haplotype are given for all genotyped individuals (top) and for genotyped individuals affiliated with the black cluster in Fig. 3A, $K = 2$ (bottom). (B) Pairwise comparisons of haplotype proportions between indicated groups of individuals. *P*-values below 0.05 indicate that haplotype distributions are significantly different between the groups compared

(A) Haplotype counts				(B) Pairwise comparisons of haplotype proportions		
Country	Switzerland		Germany			
Pop	SH	SR	GR			
Sampling site	1–5	6–9	12–16	First data set	Second data set	*P*-value
Data set: all individuals	$n = 184$			Pop SH	Pop SR	0.00002791*
Haplotype AA	1	0	15	Pop SH	Pop GR	0.00000000000182*
Haplotype AG	4	2	3	Pop SR	Pop GR	0.000006397*
Haplotype GA	45	30	9	POP SH, black cluster only	Pop SR, black cluster only	0.04895*
Haplotype GG	65	4	6	Pop SH, black cluster only	Pop GR, black cluster only	0.002604*
Data set: black cluster ($K = 2$)	$n = 67$			Pop SR, black cluster only	Pop GR, black cluster only	0.0006286*
Haplotype AA	1	0	12	Pop SH	Pop SH, black cluster only	0.4018
Haplotype AG	0	2	2	Pop SR	Pop SR, black cluster only	0.6809
Haplotype GA	9	17	6	Pop GR	Pop GR, black cluster only	0.99
Haplotype GG	10	4	4			

*$P < 0.05$.

Evidence for commercial vessels as vectors for the bighead goby

Structure runs suggested that fish from the Swiss Rhine may be more similar to fish from Germany than to fish from the commercial harbour. Such a pattern would imply some kind of connection between the geographically widely separated populations SR and GR. When we interviewed ship inspectors, captains, executive directors and fleet inspectors on the vector potential of their company's vessels, eight of eleven interviewees confirmed the use of ballast water. They indicated that all commercial ship types travelling on the Rhine towards Switzerland – tankers, cargo vessels and passenger boats – use large amounts of ballast water to stabilize empty vessels and to pass below bridges. The mesh sizes of ballast water tank filters were specified as ranging from 3 to 8 mm. Ponto–Caspian goby larvae that were hatched from eggs (Hirsch *et al*. 2015) for comparison with mesh sizes were found to be approximately 2 × 2 × 7 mm in size (Fig. 4A). In addition to ballast water tanks, company representatives pointed at the ships' cooling systems as a potential hideaway for adult individuals. They indicated that these so-called sea chests were continually flushed with fresh river water through entry slits that were 3–8 cm wide. Adult bighead gobies in Switzerland reach about 3–4 cm in head width and no more than 3 cm body height, and can be much smaller than that (own observation, data not shown).

Structure runs and pairwise FST comparisons revealed genetic structuring among Swiss sampling sites, in particular between the harbour population SH and the adjacent river population SR. The genetic differences observed between population SH and population SR may be caused by differential introductions. When analysing mooring patterns of cargo ships and tankers in Switzerland, we found that these two ship types use available anchoring sites in a nonuniform manner. The major mooring sites for cargo ships overlap with sites associated with population SH (sites 1–5 and sites 10 and 11). Tanker mooring sites on the other hand overlap with sites associated with population SR (sites 6–9) (Fig. 4B).

When analysing travel patterns of vessels heading towards Switzerland to test whether these ship types may potentially pick up propagules from different source populations, we found that cargo ships and tankers used different harbours before their arrival. Tankers almost exclusively called at harbours along the river Rhine, while cargo ships also used ports along the Danube and Rhine–Main–Danube channel, as well as ports in Northern Germany, before they arrived in Switzerland (Fig. 4C).

Discussion

In this study, we have for the first time identified genetic structuring among invasive populations of the bighead goby *Ponticola kessleri*. The observed structure carries a number of signatures of a recent and ongoing range expansion. Also, the genetic structure is informative with respect to introduction routes. Observed genetic patterns relate well to vessel anchoring and vessel travel patterns. In accordance with these findings, we describe that ballast water use and ballast water tank specifications in the freshwater environment are permissive for the introduction of invasive gobies by commercial vessels.

Table 4 Geometric morphometrics

Group 1							Group 2						Results					
		n	m	f	j	NA		*n*	m	f	j	Eigenvalue	Canon R	Wilk's Lambda	Chi-Square	df	*P*-value
All individuals	Male	**216**	216	0	0	0	Female	**240**	0	240	0	1.683008	0.792013	0.372716	418.4619	50	**<0.001**
harbour population	Grey cluster (*K* = 2)	**35**	13	18	3	1	Black cluster (*K* = 2)	**168**	85	75	8	0.406310	0.537512	0.711081	60.01064	50	**0.1570**
Black cluster (*K* = 2)	German Rhine	**41**	17	24	0	0	Swiss Rhine	**25**	15	10	0	2976.009	0.999832	0.000336	159.9735	40	**<0.001**

The body shapes of indicated groups with the indicated affiliation were compared. *n*, total number of individuals entering analysis from the indicated group. m, f, j, NA, number of male, female, juvenile and not sexed individuals, respectively. *P*-values below 0.05 indicate that the body shapes of the compared groups are significantly different.

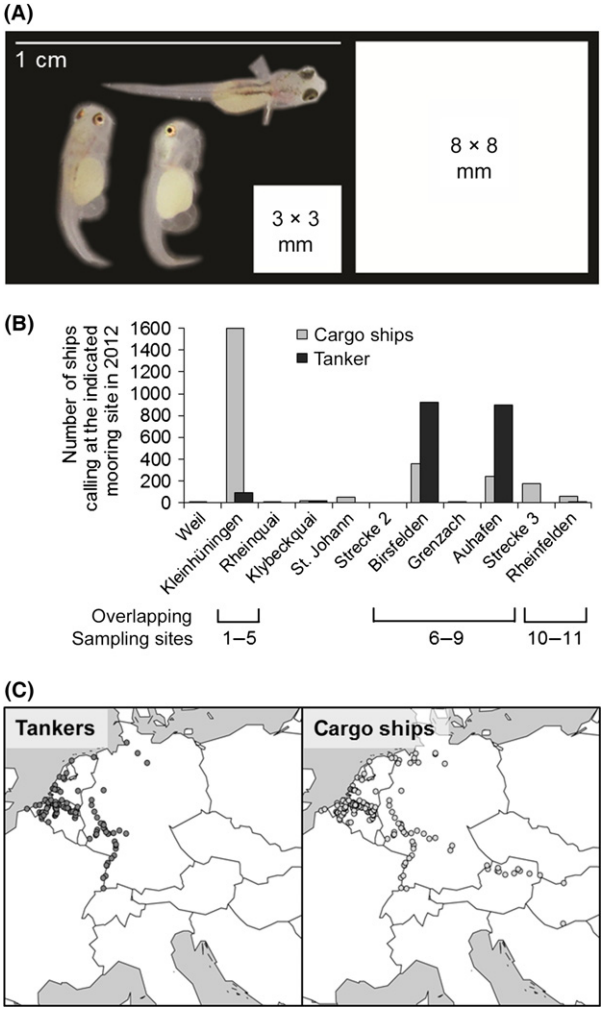

Fig. 4 Evidence for commercial shipping as vector for non-native gobies. (A) Freshly hatched goby larvae displayed at scale next to drawings of ballast water tank filter pores. (B) Bar plot of the numbers of cargo ships and tankers calling at the indicated mooring sites in Switzerland. Goby sampling sites closest to these mooring sites are indicated below the plot. Sites 1–5, 10 and 11 affiliate with one genetic cluster, sites 6–9 affiliate with a different genetic cluster (Fig. 3A). (C) Source harbours of cargo ships and tankers arriving in Basel, Switzerland. Each dot on the map indicates a harbour where a ship of the indicated type that arrived in Basel in 2012 had stopped within 14 days prior to arrival.

Invasive bighead goby populations are genetically differentiated

Previous studies (Ondrackova *et al.* 2012; Cerwenka *et al.* 2014b) could not observe any structuring on small or large scales for invasive bighead goby populations. This may reflect a low overall genetic diversity in this species, at least in the invasive range. In contrast to these reports, we detected genetic structuring among bighead goby populations and could even resolve

temporal turnover in a densely sampled harbour population. This difference between studies may be attributable to the marker types and sampling schemes used. Microsatellites and D-loop sequences as used in this study are fast evolving sequences and therefore may yield higher resolution than AFLP markers and Cytochrome B sequences as used by Cerwenka *et al.* (2014a). Also, our data suggest that the sampling scheme may have an impact on the ability to detect structuring. If we would have sampled less sites (e.g. only sites 6–9, or only sites 1–5, 10 and 11), we would not have been able to detect genetic differences between sites and would have come to similar conclusions as Ondrackova *et al.* (2012) and Cerwenka *et al.* (2014b).

Genetic signatures of an ongoing invasion

Our catch data suggest that the goby population in the commercial harbour has arrived recently and was going through an establishment period during our sampling interval. The relative dynamics of round and bighead goby catches, with the bighead goby being present in low numbers from the beginning of monitoring and with the round goby arriving during the monitoring, represents a typical pattern for the establishment period of Ponto–Caspian gobies in Central Europe. In the Upper Danube River, the bighead goby invaded shortly before the round goby (Seifert & Hartmann 2000; Paintner & Seifert 2006) and decreased in abundance after the arrival of the round goby. A similar pattern was also observed in the Lower Rhine (Borcherding *et al.* 2011; J. Borcherding, S. Gertzen, P. Jurajda, pers. communication). Also, fishermen at the Rhine at Basel did not register non-native gobies before 2012, although they were informed about their expected arrival (Dönni 2002).

Our genetic results support the idea that the bighead goby arrived in Switzerland very recently. We identify deviations from HWE specifically in the harbour population, indicating that this population is either substructured or subjected to selection and/or drift processes. As we could exclude geographic, temporal or sex-dependent substructuring, we propose that the population is currently subject to genetic processes. Indeed, we could detect turnover of individual microsatellite alleles in the commercial harbour. At site 5, genetic turnover may also be ongoing at the population level, as suggested by Structure analysis. This turnover may be linked to the population growth and crash that was suggested by catch data from this site. In summary, these observations validate our approach to monitor genetic processes at an invasion front in real time. We look forward to similar experiments on the round goby, which started invading the harbour during our sampling timeline and thus also represents an excellent case of an ongoing invasion.

Genetic evidence for vector activities of cargo ships and tankers

Swiss populations were introduced recently, are closely spaced, and are likely linked through larval drift. Based on our understanding of commercial shipping as a single and homogeneous vector, we expected samples from Switzerland to be genetically homogeneous. Based on the fact that German populations established in 2004, and invasive gobies arrived in Switzerland not before 2011, we expected German and Swiss samples to differ pronouncedly. Yet, the invasion front in Switzerland is genetically fragmented, and German and a subset of Swiss samples do seem to bear some kind of similarity. As Swiss populations are young, the genetic differences among them cannot be attributed to evolutionary divergence.

Our results suggest that shipping travel behaviour may underlie the observed pattern. While round goby translocations to and within the Great Lakes have been attributed to shipping traffic (LaRue *et al.* 2011), the potential of commercial river shipping to transport fish propagules is to date unresolved. Here, we demonstrate that commercial freshwater vessels may present excellent vectors for upstream transport of invasive goby species. Freshwater vessels do indeed use ballast water on their way to Switzerland, and larvae are without any doubt small enough for uptake in freshwater ballast tanks. Ponto–Caspian goby larvae are present in the water column in massive amounts (Hensler & Jude 2007; Janac *et al.* 2013) and drift downstream in rivers from March/April to August (Janac *et al.* 2013). During this time, they can easily be taken up with ballast water by commercial vessels in harbours or during shipping, and released with the ballast water upon discharge.

In addition, our observations and our data suggest that eggs may also serve as propagules. Ponto–Caspian gobies readily accept any kind of narrow cave-like structure, such as PVC tubes, as shelter and spawning substrate, both in the laboratory and in the field (Hirsch *et al.* 2015). Adult invasive gobies may thus seek shelter in the cooling systems of commercial vessels while these are anchored in a harbour. The openings of those systems are large enough to accommodate Ponto–Caspian gobies. While it is unlikely that individual adults would be able to hold on to the cooling systems during transport, goby eggs are highly resistant to dragging forces and other stressors (Hirsch *et al.* 2015). Eggs deposited in the cooling system or on any other crevice of the ship may be transported with the ship to the next destination and would eventually hatch.

Our genetic data suggest that individuals may have been introduced to Switzerland from different source populations. Shipping data support this interpretation. The mooring sites of cargo ships and tankers, two ship types that we find to come to Basel from different source regions, mirror the geographic occurrence of populations. Also, we observe four unique alleles in the harbour population, but none in the Swiss Rhine population and only one in the German Rhine population (Table S4, Supporting information), indicating that the harbour population receives input from somewhere else – putatively, from populations in the Danube. It seems like bighead goby population genetics may accurately mirror the introduction pathways. This could be further investigated in the future using mtDNA haplotype data, as these were found to differ between all three investigated populations in this study. Source regions were not sampled in this study, and it is important to note in this context that very little is known about the genetic structure of Ponto–Caspian goby species in the native area. An elaborate phylogeographic analysis investigating mtDNA haplotypes of Ponto–Caspian gobies in their native range, combined with common shipping travel routes, would eventually provide detailed information about the source of goby introductions for all sites investigated. Comparing these patterns for different invasive goby species would be highly interesting.

We are not aware of any alternative variable such as habitat structure or water temperature that would covary with the genetic patterns observed. Also, we can exclude that bait-bucket transfers play a major role in setting up the observed structure. Bait-bucket transfers have been shown to be relevant in Northern America (Drake *et al.* 2014). However, fishermen in Switzerland were not yet routinely catching invasive gobies when sample collections started. Also, the use of live bait is generally forbidden in the area. In such a situation, the propagule pressure exerted by bait-bucket transfers would be, if present at all, minuscule.

Mitochondrial markers and body shape

As there is no evidence for bottlenecks during Ponto–Caspian goby invasions (Stepien & Tumeo 2006), and propagule numbers are therefore assumed to be high, we expected that maternally inherited mitochondrial haplotypes would yield similar patterns as nuclear markers. Body morphology was expected to be independent of either genetic marker, as fish are known to be morphologically plastic, particularly during development (Langerhans & Reznick 2010).

We found that individuals associated with the same microsatellite cluster would differ in both mitochondrial haplotype and in body shape when they came from different sites. While this is an interesting observation, it is important to note that these analyses were all based on the result of the Structure run at $K = 2$. At $K > 3$, the cluster uniting population SR and population GR splits up. Differences in morphology and in mitochondrial markers may therefore not be surprising when comparing population SR and GR individuals.

For both markers, we also found that individuals associated with different microsatellite clusters would be similar if they came from the same site. In the case of morphology, these results add to recent studies on phenotypic differentiation in goby species, which assume that morphological differences among subpopulations arise from plasticity rather than rapid genetic adaptation (Simonovic *et al.* 2001; Polacik *et al.* 2012; Cerwenka *et al.* 2014b). However, as microsatellites are generally considered to be neutral genetic markers because they rarely occur in coding regions (Li *et al.* 2002), we may have missed an association between body shape and genotype due to our choice of markers.

For mitochondrial haplotypes, the observation indicates that maternally inherited mitochondrial genotypes and nuclear genotypes have the potential to yield divergent patterns for this species. Similar diverging patterns of nuclear and mitochondrial markers have been observed, for example in brown and polar bears (Hailer *et al.* 2012; Bidon *et al.* 2014) and have been attributed to male-biased introgression due to migratory males (Bidon *et al.* 2014). In the case of invasive gobies, a sex-specific bottleneck would provide an explanation for diverging patterns. Invasive goby males and females do indeed differ in traits that might be relevant to dispersal. Sexual dimorphism in size is common in gobiids and might lead to different swimming and range expansion performance between larger male individuals and smaller female individuals. Also, behaviour can differ fundamentally between sexes. In the round goby, males were found to be more active and more prone to explore novel environments than females. Consequently, in the field males move larger distances than females (Marentette *et al.* 2011). Conversely, recent research in the Danube suggested that migrating adult females (and not males) were mainly driving a range expansion (Brandner *et al.* 2013). However, sexual dimorphisms themselves can change as an invasive population expands its range. For example, size differences between males and females increased as a population of the round goby expanded its range (Brandner *et al.* 2013). In general, sex-biased dispersal is well described in mammals and birds, and mounting evidence from studies with fish suggests that differences between the sexes can create complex range expansion dynamics. Alternatively, a sex-specific bottleneck may be independent from differential behaviour of males

and females and arise simply through the reduced population size of males/females in relation to the entire population. Together, our observations advertise caution when inferring population structures from one marker type only. Even when not expected, sex-specific processes may be at work, and may affect the results. In this context, it is important to note that the observed microsatellite-based population structure was sex-insensitive. The population structure was equally supported by males and females (Fig. S6, Supporting information). Our data also indicate that invasive Ponto–Caspian gobies may be well suited as models for research addressing the differential contribution of sexes to a range expansion (Prugnolle & de Meeus 2002).

Implications for biological invasions and invasion genetics

Our results provide important insights into population genetics of recently invasive species and propose relevant conclusions on how to study invasive populations.

First, our data support the notion that isolation by distance applies only weakly to invasive gobies, and very likely, to most invasive species in general. In fact, our observation of very low levels of isolation by distance may be attributable to the unequal numbers of fish sampled at individual sites. In our data set, 75% of all samples originate from within <2 km. Indeed, Mantel tests become less significant when we reduce the harbour data set by arbitrarily removing a fraction of individuals from the analysis. Isolation by distance thus actually may not apply to our data set. Our results therefore support the idea that invasive organisms experience a distorted distance landscape in which vector activity complements, or possibly even replaces, geographic separation as distance measure (LaRue *et al.* 2011; Darling *et al.* 2012; Ghabooli *et al.* 2013a,b; Schrey *et al.* 2014).

In addition, our observations indicate that human actions may promote spatial differentiation of invasive species through cryptic diversity in vector behaviour. Our data indicate that hidden variations among closely spaced sampling sites, such as the slightly shifted travel patterns of cargo and tank ships in our case, may be sufficient to generate a signature in population genetics. Consequently, our observations suggest that, if one wants to investigate population structures and invasion pathways with restricted resources, sampling more sites less intensely may be better than sampling few sites more intensely. Temporally widely stretched and geographically very restricted sampling schemes, such as those used by Stepien & Tumeo (2006) or Brown & Stepien (2009) for the round goby, sometimes cannot be avoided, but are problematic because such schemes do neither take temporal turnover into account nor do they subsample potentially fragmented source populations.

Invasive populations are expected to lose diversity and fitness at the range margin during expansion processes (Peischl & Excoffier 2015). Frequently, however, invasive populations are highly successful. Importantly, current models of expanding populations are linear and do not deal with multiple sources (Peischl *et al.* 2015). Successful invasions, however, are often associated with multiple introductions and subsequent mixing (Bock *et al.* 2014). Our data provide further evidence that invasive populations integrate input from diverse sources. Linear expansion models, although highly relevant for cancerous tissue expansion processes or for slow post-glacial species expansion processes, may not be able to properly recapitulate genetic processes during species invasions.

Our data indicate that the genetic clusters of bighead goby have started to interbreed where they meet. The harbour population contains a low fraction of admixed individuals. Accordingly, interbreeding has either not been going on for very long or 'true type' individuals keep arriving and maintain a relevant proportion of nonadmixed individuals. Depending on the mechanism at work, genetic structuring will disappear in the future through continuous interbreeding in combination with larval drift and local migration or will be maintained by continued input of true type individuals. In this context, it is interesting to note that subpopulations in Western Germany, which established around 2006 (Borcherding *et al.* 2011), show some degree of substructuring. Whether they started off that way, or whether this substructure resulted from selection and adaptation processes since 2006, is unclear. Future research will show whether the Swiss subpopulations will be able to maintain the existing genetic differences, homogenize or establish novel genetic differences.

Finally, we propose that vector-induced genetic fragmentation of the invasion front may explain the lag phase frequently observed during species invasions. It has been proposed previously that introduction sites may serve as melting pots when different genotypes from different sources are introduced to the same site (Brown & Stepien 2009). We do observe such a phenomenon in the harbour population. Additionally, our data suggest that subtle differences in vector behaviour may result in a geographically–genetically structured invasion front. In such a scenario, geographically separated and genetically discrete subpopulations may have to go through a phase of natural migration and small-scale translocations before they would be able to mix. In such a model, a lag phase preceding exponential population growth may not just represent the time needed for reshuffling of alleles between two genotypes

introduced at the same spot to yield novel beneficial allele combinations, but may rather represent the time needed for different genotypes to get to the same spot (and mix afterwards).

Implications for ballast water management

Ballast water in the marine sector contains a diverse community of organisms and taxa (Gollasch *et al.* 2002). As freshwater vessels take smaller volumes of ballast water compared to marine vessels, their potential to transport reasonable amounts of organisms is not fully acknowledged, and freshwater ballast water is thus not managed. Our data substantiate the relevance of freshwater vessels for the translocation of non-native vertebrate species. With this study, we hope to contribute to the establishment of freshwater ballast water management procedures such as proposed by Briski *et al.* (2015) and also hope to provide decision makers in the freshwater sector with the evidence they need to promote ballast water hygiene measures.

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I.A.K. designed the project. I.A.K., P.E.H., J.B.G., A.N., S.W., S.L. and S.G. collected data. I.A.K. and J.B.G. analysed genetic data. I.A.K. analysed shipping data. P.E.H. analysed morphometric data. I.A.K., P.E.H., J.B.G., A.N., J.B. and P.H. wrote the study.

Data accessibility

All data are accessible in the Supplementary Material provided with this article.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sample information including sampling date, site, catch method, weight, length, sex, microsatellite genotype, D-loop haplotype and cluster affiliation for $K = 2$.

Table S2 Primer sequences for primers used in this study.

Table S3 Microsatellite set establishment process indicating which of the published goby microsatellites passed which analysis stage.

Table S4. Information on stutter, allele dropout, null alleles, HWE, linkage disequilibrium, population F_{ST}, Mantel tests, allele frequencies, allelic richness, F_{IS} and genetic diversity.

Appendix S1 Microsatellite sequences for bighead goby and round goby as determined in this study.

Fig. S1 Weight vs. Catch Date plots, separately drawn for sites 1–5 from the commercial harbour, are illustrating which 37 first and last individuals caught at each site entered analysis.

Fig. S2 Landmarks used for Geometric Morphometrics.

Fig. S3 Structure plots for $K = 2$ to $K = 5$–8, drawn separately for all samples, the harbour samples, and all samples minus the harbour samples.

Fig. S4 Catch data for bighead goby split by sites 1–5 from 2012 to 2014. Solid lines indicate the beginning of a year, and dotted lines indicate the beginning of a month.

Fig. S5 Structure Harvester plot indicating the most probable K for all samples.

Fig. S6 Structure plots for $K = 2$–5 for all samples, all male samples and all female samples.