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ORIGINAL PAPER

Journey into the past: using cryogenically stored samples to reconstruct the invasion history of the quagga mussel (*Dreissena rostriformis*) in German river systems

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Abstract Knowledge about the spatial–temporal dynamics of biological invasions often remains incomplete, because precise information about the invaders' arrival dates is rare. This applies to the quagga mussel, which has become one of the most successful invasive species in Western European freshwaters. We here used cryogenically stored *Dreissena* samples from the German Environmental Specimen Bank to reconstruct the colonization history of the quagga mussel in German river systems. Our retrospective genetic analysis significantly improved upon previous findings of when the quagga mussel arrived in Germany and can be used as chronological landmarks to reconstruct its range expansion. The discovery of *Dreissena rostriformis* in 2004 in the Rhine River near Koblenz presented the first record of this species not only in Germany, but also in Western Europe. Our results show that the quagga mussel had already invaded not only large parts of the Rhine and the Danube, but also the Elbe River. This demonstrates the value of cryobanked biological samples for the retrospective analysis of biological 'pollution' through alien invasive species.

Keywords Colonization · Environmental specimen bank · Genetic monitoring · Microsatellite · Zebra mussel · *Dreissena bugensis*

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Introduction

Emerging biological invasions often remain undiscovered until the invaders become conspicuous due to their numbers or their deleterious effect on the environment or other species (Lockwood et al. 2007). However, invasions frequently result from a long process of transport, introduction, establishment and dispersal (Williamson 1996). Thus, their origins often remain hidden in time and space. Inferring invasion histories is a basic requirement to develop suitable management strategies by identifying vectors, invasion corridors, and speed, as well as for predicting their future dispersal and consequently developing mitigation strategies. Precise knowledge about their arrival dates and locations therefore is essential to invasion analysis. However, this information rarely is available as detections often are made at a late stage of the invasion process (Estoup and Guillemaud 2010; Heiler et al. 2012, 2013).

Estoup and Guillemaud (2010) emphasized that most of our knowledge on introduction routes of invasive species stems from historical and observational data, which often are incomplete, and sometimes may be misleading. Alternatively, invasion

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histories may be inferred from the genetic imprint they leave in the colonizing population. However, such successful reconstruction depends on a variety of factors such as the effective size of the founder population(s), its geographic origin (single or multiple origins), its demographic history, and the mutation rate of the marker system used for reconstruction (Estoup and Guillemaud 2010). We here present the reconstruction of the invasion history of a bivalve species into major river systems of Germany based on standardized biological samples taken over a period of almost 20 years for environmental pollution monitoring under the framework of the German Environmental Specimen Bank.

In recent years the quagga mussel, *Dreissena rostriformis* (Deshayes, 1838), formerly known as either *D. bugensis* or as *D. rostriformis bugensis* (name has been changed per Stepien et al. 2013), has become one of the most actively invading species in European freshwater systems. Native to the Dnieper delta (Son 2007), its range expansion reportedly started in the 1930s, when the species became a major invader in the Volga River (Orlova et al. 2004). In North America, it has been present since at least 1989 (May and Marsden 1992). The earliest records from Western Europe were reported many years later: the species was sampled in 2005 in the Main River, Germany (Imo et al. 2010) and in 2006 in the Dutch Rhine delta (Molloy et al. 2007). Meanwhile, numerous records from several German river and canal systems indicate the rapid dynamics of its current expansion (bij de Vaate 2010; Haybach and Christmann 2009; Heiler et al. 2012, 2013; Imo et al. 2010; Martens et al. 2007; Schöll et al. 2012; van der Velde and Platvoet 2007; van der Velde et al. 2010). The known invaded region includes the entire navigable Rhine River, sections of the Main and Danube rivers, and single records from the Neckar and Elbe rivers, the Rhine-Main-Danube Canal, as well as canals connecting the Rhine and Elbe rivers. Such rapid range expansion usually coincides with high abundances, which often appear to be reached shortly after introduction (e.g. Heiler et al. 2013; Orlova et al. 2004; Zhulidow et al. 2010). Outside its native range, *D. rostriformis* appears to be competitively superior to its likewise invasive relative, the zebra mussel *Dreissena polymorpha* (Pallas, 1771), which populated the Western European river systems several decades earlier. As a new and dominant filter feeder and colonizer of hard substrates, *D. rostriformis* may exert a strong ecological impact on the entire ecosystems in newly populated reaches (e.g. Ricciardi and Whoriskey 2004; Wong et al. 2013; Zhulidov et al. 2010). Despite existing records, much of the Western European colonization history of the quagga mussel remains unverified. This appears due to multiple introductions from various source populations (bij de Vaate and Beisel 2011; Imo et al. 2010; Mayer et al. 2009).

The German Environmental Specimen Bank (ESB) (Federal Environment Agency 2008) has been using the zebra mussel *D. polymorpha* as an indicator of water pollution for approximately 20 years. In order to achieve the mandatory sample size required for long-term cryogenic storage by the ESB, a great number of zebra mussels were collected per sample site and time period. During recent years, some known quagga mussel populations have been confirmed during routine zebra mussel sampling, and additional populations have been discovered at further ESB sampling sites. Due to their similar shell morphology and the intraspecific variability of shell characteristics of both *Dreissena* species, as well as its unknown occurrence in Western Europe for many years, it is reasonable to assume that small numbers of *D. rostriformis* also may have been included among previous samples with *D. polymorpha*.

We used microsatellites to test if quagga mussels may have been already included in old zebra mussel samples and thus may indicate a much earlier colonization of the respective rivers systems than previously thought. This retrospective genetic analysis is based on the cryogenically stored ESB samples and aims at an improved reconstruction of the colonization history of quagga mussels in German river systems. Use of cryobanked biological samples in systematic monitoring of an invasion process represents a powerful approach for historical reconstructions. Retrospective analyses were complemented by morphological analyses of individual mussels for the validation of first records of *D. rostriformis* at ESB sampling sites in current sampling years. We also calculated its relative abundances compared to the zebra mussel to estimate how far the displacement of *D. polymorpha* has advanced.

Materials and methods

Sampling

Zebra mussels were sampled annually at 14 sites including the Rivers Elbe (E1–E5), Rhine (R1–R4),

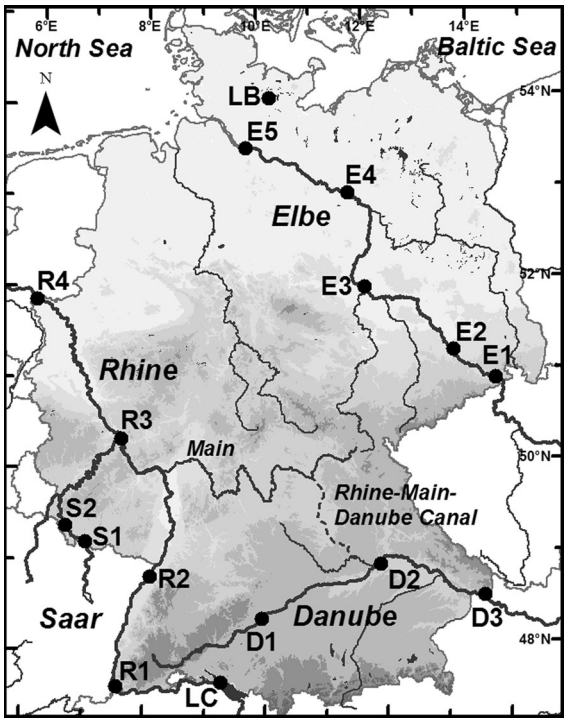

Fig. 1 Zebra mussel sampling sites of the German ESB (*E* Elbe, *R* Rhine, *S* Saar, *D* Danube, *LB* Lake Belau, *LC* Lake Constance (used for colonization of plate stacks)

Danube (D1–D3) and Saar (S1–S2); one sample from the Lake Belau (LB) was taken biennially (Federal Environment Agency 2008; Fig. 1). In order to ensure comparability of the samples, sampling was based on standard operating procedures (Wagner et al. 2003).

Zebra mussel samples were collected on stacks of polyethylene plates, exposed in Lake Constance (Fig. 1) at the beginning of the zebra mussel spawning season (Wagner et al. 2003). In autumn, when they were densely populated with young mussels, the plate stacks were removed and transported to the sampling sites of the ESB (Fig. 1), where they were exposed for 1 year. A single plate stack remained in Lake Constance as control. If stack exposure was unsuccessful, effort was put into gathering naturally occurring zebra mussels from river banks beneath the low water line. In order to achieve the required 2,000 g soft tissue of zebra mussel, 5,000–7,000 fresh mussels with a shell-length range of c. 15–25 mm had to be collected at each sampling site. The soft-tissues were pooled and homogenated per sampling site and subsequently divided into c. 200 aliquots for long-term cryogenic storage.

Further descriptions of the ESB, including latitude and longitude for all sampling sites, are provided under www.umweltprobenbank.de.

Morphological screening for quagga mussels

To confirm that quagga mussels at ESB sampling sites were not introduced by the ESB procedures (e.g. via contaminated plate stacks), we tested for presence of the species in Lake Constance. A total of 1,000 mussels from the control samples of 2011 and 2012 were examined morphologically. Additionally, a composite sample consisting of 200 individuals from each of the 2 years was genetically analyzed for quagga mussel DNA (see below).

The first in situ discoveries of quagga mussels were made on plate stacks and river banks during field sampling in autumn 2009. Since then, we have checked all samples morphologically for quagga mussel occurrence. The relative abundance of both species was determined during ESB sampling operations. For this purpose, 300 mussels were gathered at suitable low water levels from river banks of the Rhine in 2011 and from the Elbe and Danube in 2012. Mussels were collected haphazardly, sorted by size and subsequently identified. Identification of quagga mussels during sampling and sample preparation was based on external morphological shell features following May and Marsden (1992), Pathy and Mackie (1993), and Martens et al. (2007). The transition from the lateral to the ventral side, which is acutely angled in the zebra mussel but rounded in the quagga mussel, served as key diagnostic character (Ram et al. 2012).

Genetic analyses

Due to the high variety of shell characteristics, species identification via microsatellite analysis was conducted for all suspected initial recordings of the quagga mussel in the morphological screening. The retrospective analysis commenced with samples from 2010 in reverse chronological order until no quagga mussel DNA was detected for a minimum of two consecutive years. The time series, especially for the Rhine sampling sites R1 and R2, was sometimes incomplete due to a lack of sample material.

Table 1 Arrival years of *D. rostriformis* directly at (results from present study) and in the vicinity (cited references) of ESB sampling sites, considering all available record data

Sampling site	Previous records	Present study
LB	No records	No records
LC	No records	No records
R1	2011 [1]	2011
R2	2009 [2]	2011
R3	2009 [2]	2004
R4	2008 [3]	2005
S1	No records	No records
S2	No records	No records
E1	No records	No records
E2	No records	No records
E3	No records	2010
E4	No records	2007
E5	No records	2010
D1	No records	No records
D2	2009 [2]	2008
D3	No records	2010

[1] Schöll et al. (2012), [2] Heiler et al. (2012), [3] Haybach and Christmann (2009)

DNA was extracted with the Qiagen Inc. DNEasy blood and tissue kit according to the manufacturer's protocol. We tested twenty microsatellite loci for species specificity and reliable amplification using pure tissue of *D. polymorpha* and *D. rostriformis*. We finally chose the following loci, which either amplified in only one species or had species-specific alleles: Dbug4, Dbug5 (Wilson et al. 1999), DpolB9 (Astanei et al. 2005; Naish and Boulding 2001), Dpol6 (Thomas et al. 2011), and Dbu92 (Feldheim et al. 2011). PCR was performed in a Multigene Gradient Thermal Cycler (Labnet) with the Qiagen Type-it Microsatellite PCR kit and PCR conditions as recommended by the manufacturer. Dbug4 and Dbug5 were amplified in a multiplex PCR with an annealing temperature of 60 °C, Dpol6 and DpolB9 at 57 °C and Dbu92 in a singleplex reaction at 50 °C. In order to obtain information upon the sensitivity of this method for *D. rostriformis* detection, we tested this method on artificial DNA mixes of both species at concentrations of 1:10,000, 1:1,000, 1:500, 1:100, 1:50, 1:10, 1:1, 1:0 and 0:1 (*D. rostriformis* : *D. polymorpha*). This test revealed that *D. rostriformis* could be reliably detected up to concentrations of 1:1,000 (data not shown). We then applied this method to the pooled ESB samples using a multiple-tube approach.

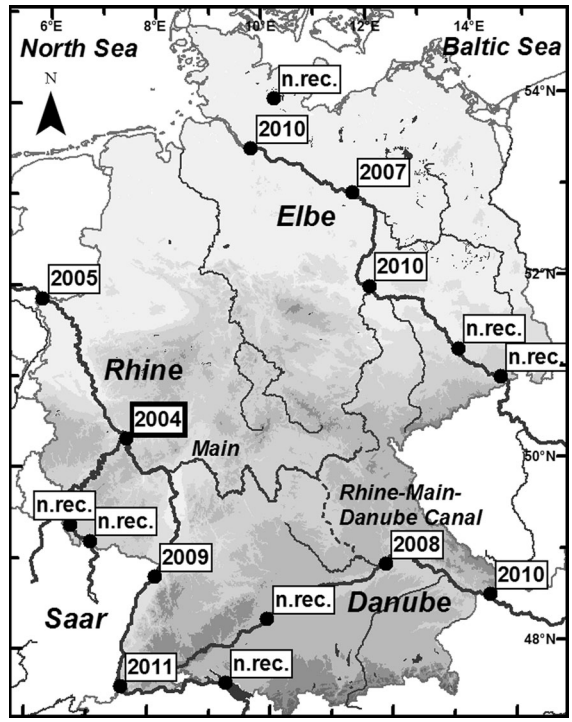

Fig. 2 Earliest dates of quagga mussel verification at zebra mussel sampling sites of the German ESB. The thick frame represents the first record not only in Germany, but also in Western Europe (*n.rec.* no records)

Results

The morphological screening revealed records of *D. rostriformis* from the Rivers Rhine (R1, R2, R3, R4), Elbe (E4, E5) and Danube (D2, D3). Retrospective genetic analysis showed that *D. rostriformis* has been present in the samples since at least 2004 (R3). We found no evidence for occurrence of *D. rostriformis* in the lakes and in the river Saar. The first record for the Elbe (E4) stems from 2007, for the Danube (D2) from 2008. It should be noted that no continuous time series was available for sites R1 and R2. Therefore, it was not possible to date the arrival of the quagga mussel at these sampling sites accurately. However, some of our results predate the existing colonization estimates significantly (Table 1, Fig. 2; additional data are given in Online Resource 1).

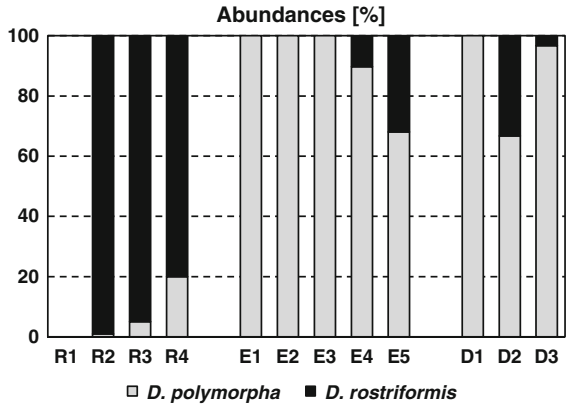

Fig. 3 Relative abundances (%) of *D. polymorpha* and *D. rostriformis* at Rhine (R; 2011), Elbe (E; 2012), and Danube (D; 2012) river banks (evidence of *D. rostriformis* at R1 and E3 was from just single individuals from plate stacks in 2011 and therefore not included)

The relative abundance of the quagga mussel compared to zebra mussel was highest in the Rhine, which also seems to have been colonized earlier by quagga mussels compared to the other rivers (Fig. 3). In the Danube and Elbe zebra mussels were still more abundant than quagga mussels, with the highest relative abundance of the latter close to the Rhine-Main-Danube Canal (D2) and close to the coast (E5), respectively.

Discussion

We applied genotyping of cryogenically stored samples for inferring the colonization history of an invasive species, the quagga mussel. The improved information for the arrival of adult individuals can be used as chronological landmarks to reconstruct the range expansion of the species. The ESB samples provided evidence that the quagga mussel had reached the Rhine in 2004 at the latest and expanded along its entire navigable length. The first occurrence in the Danube was detected in the 2008 samples at D2, followed by D3 in 2010. These results together with the relative abundance data support the hypothesis of invasion via the Rhine-Main-Danube Canal, as suggested by Heiler et al. (2012, 2013) and Schöll et al. (2012), rather than directly from its native range through the Danube itself. Remarkably, our results also showed that the range of the quagga mussel now encompasses a rather large part of the Elbe. It can be expected that, following Rhine and Danube, the Elbe will soon also be populated along its entire navigable length. The currently northernmost record of the quagga mussel in Western Europe stems from downstream Hamburg port (E5).

The discovery of *D. rostriformis* in 2004 at R3 represented the first record of this species in Western Europe. However, it has to be considered that all dates referred to the first record of adult mussels. Thus, it cannot be excluded that the arrival may have taken place earlier. Current arrival estimates of the quagga mussel commonly were based on shell length of the initially detected individuals at a given site based upon growth data of zebra or quagga mussels in other river systems (e.g. bij de Vaate and Beisel 2011; Imo et al. 2010; van der Velde and Platvoet 2007). The present findings indicated that the range expansion of the quagga mussel not only included larvae and juveniles, but also considerable numbers of adults (Mayer et al. 2009). We found numerous quagga mussels with shell lengths >20 mm (up to 30 mm) just 4 weeks after placement of the plate stacks at R3, suggesting that colonization took place during the adult stage. 1 year after exposure, their relative abundance accounted already for 55 %. This documents that adult quagga mussels must be able to rapidly colonize hard substrate. We therefore recommend adopting the initial discovery date of quagga mussels either as juveniles or adults as the real arrival date rather than using estimations based on shell lengths.

The relative abundance of both species confirms that the quagga mussel was able to rapidly displace zebra mussels. Heiler et al. (2012, 2013) described a time dependency of this displacement and calculated a common rate of displacement for Western Europe that can be used during the initial growth phase to estimate the time since introduction of a given quagga mussel population (Heiler et al. 2013). As the quagga mussel has colonized R3 at least six and R4 at least 5 years before our abundance study started, it is reasonable to assume that the saturation phase of displacement has been reached. According to Heiler et al. (2012), our relative abundance data cannot be used to estimate the arrival date of quagga mussel and the period of time for zebra mussel displacement at these sampling sites. Concerning R2, where *D. rostriformis* was initially detected in 2009, the proposed common rate of displacement per year as suggested by Heiler et al.

(2012, 2013) was confirmed. However, our data also showed that the displacement process seems to have proceeded significantly slower in the Danube and Elbe Rivers. The rapid displacement in the River Rhine might have been promoted by receding nutrient availability for zebra mussels during the dispersal period of the quagga mussel (Haybach and Christmann 2009). The competitive advantage of quagga mussels over zebra mussels may be explained by their higher growth rate and better energy supply of the soft-tissue at equal food availability (Zhulidov et al. 2010) as well as their considerably higher filtration rate (Baldwin et al. 2002; Diggins 2001).

Our analyses significantly improved some of the previous reported findings of quagga mussel occurrence at the studied sampling sites. The main reason for the later observation of *D. rostriformis* during the morphological screenings compared to our genetic analysis may be that the search was restricted to shallow water in most cases, whereas it is known that the quagga mussel has a competitive advantage towards the zebra mussel particularly in deeper water (e.g. Orlova et al. 2005; Ram et al. 2012; Zhulidov et al. 2010). Therefore, it is possible that initial colonization mainly takes place in deeper water. Its discovery in the initial colonization phase thus frequently is rather difficult and may happen mostly by chance. This hypothesis is supported by Popa and Popa (2006), who discovered *D. rostriformis* "in an abandoned fishing net on the bottom of the Danube River" and Molloy et al. (2007), who found quagga mussels during sampling in the Rhine with a trawl net at a depth of five to seven meters. The ESB plate stacks are exposed at about 0.5–1 m above the river bed and seemed to provide a suitable substrate for the quagga mussel during its initial colonization phase. Furthermore, our findings from the Elbe suggest that screening for introduction events should focus around harbors. Martens et al. (2007) showed that the species already had reached a high abundance in harbors, while just a short section of the main canal downstream of the respective harbors was populated. Similarly, all of our initial recordings at the Elbe were made in harbor basins, which is further evidence of the importance of inland navigation as a crucial dispersal vector for the quagga mussel.

Analyzing the cryogenic composite samples of *Dreissena* from the German ESB enabled us to significantly refine the current picture of its spatial and temporal invasion history. Although initially designed for the retrospective documentation of the spread of chemical pollutants in freshwater ecosystems, the ESB samples have proven to be invaluable to reconstruct the history of an alien mussel species and thus may contribute to a better understanding of the invasion process, which has the potential to effectively alter Western European freshwater ecosystems. This documents the high sensitivity of molecular methods, helping to reconstruct invasion processes.

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