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Short Communication

High prevalence of the parasite *Sphaerothecum destruens* in the invasive topmouth gudgeon *Pseudorasbora parva* in the Netherlands, a potential threat to native freshwater fish

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Abstract

The prevalence of *Sphaerothecum destruens*, a pathogenic parasite, was studied in two wild populations of topmouth gudgeon (*Pseudorasbora parva*), an invasive freshwater fish non-native to the Netherlands. Using genetic markers and sequencing of the 18S rRNA gene, we showed the prevalence of this parasite to be 67 to 74%. Phylogenetic analysis demonstrated a high similarity with known sequences of *S. destruens*. The topmouth gudgeon, which functions as a healthy carrier of the pathogen, is rapidly colonizing the Netherlands, its expansion showing no signs of saturation yet. Both the presence of *S. destruens* and the rapid dispersal of the topmouth gudgeon are considered to constitute a high risk for native freshwater fish.

Key words: *Sphaerothecum destruens;* rosette agent; *Pseudorasbora parva;* topmouth gudgeon; invasive; 18S rRNA

Introduction

Pathogens introduced by invasive species are a risk to native species and a threat to biodiversity (Daszak et al. 2000). The topmouth gudgeon *Pseudorasbora parva* (Temminck & Schlegel, 1846), originating from China and South-East Asia, is a highly successful invasive freshwater fish in Europe, Asia and the Middle East (Bănărescu 1999; Gozlan et al. 2010). Its primary introduction pathway outside its native range is mainly through aquaculture, recreational fishing and ornamental fish trade, whereas the secondary pathway is considered to be natural dispersal (Gozlan et al. 2010). In the 1960s, the topmouth gudgeon was unintentionally imported from China and the former USSR together with herbivorous fish species [*Hypophthalmichthys molitrix* (Valenciennes, 1844), *Hypophthalmichthys nobilis* (Richardson, 1845) and *Ctenopharyngodon idella* (Valenciennes, 1844)], to fish farms in several eastern European countries, such as Romania, Hungary and Lithuania (Barus et al. 1984; Gozlan 2012). In the past forty years, the topmouth gudgeon has invaded five new countries in each decade (Gozlan et al. 2010). In the Netherlands, this invader has been spreading across the country since its initial introduction in 1992. Rivers such as the Meuse function as a dispersal corridor, and high densities of topmouth gudgeon are now found in the adjacent floodplain lakes (Pollux and Korosi 2006). Further expansion of the topmouth gudgeon towards European river basins can be expected (Copp et al. 2009) and

pose a high risk for the spread of pathogens such as *Sphaerothecum destruens*.

The topmouth gudgeon is a healthy carrier of the pathogen *S. destruens* (Gozlan et al. 2005), a rosette-like intracellular eukaryotic parasite belonging to the Ichthyosporea (syn. Mesomycetozoea), within the clade of the Opisthokonta (Arkush et al. 2003). Its life cycle has been described by Arkush et al. (2003) and includes a zooflagellate phase that occurs only in fresh water (Andreou et al. 2009). The spores and zooflagellate stages have a wide temperature tolerance with later onset and longer lifespan of zoospores at lower temperature (Andreou et al. 2009), facilitating the infection of hosts. *S. destruens* causes inflammation, serositis and cell death in the host's organs (Andreou et al. 2011a), increasing the mortality of cyprinid fish such as sunbleak [*Leucaspius delineatus* (Heckel, 1843)], bream [*Abramis brama* (Linnaeus, 1758)], carp [*Cyprinus carpio* (Linnaeus, 1758)], roach [*Rutilus rutilus* (Linnaeus, 1758)], salmon (*Salmo salar* Linnaeus, 1758) and Chinook salmon [*Oncorhynchus tshawytscha* (Walbaum, 1792)] (Andreou et al. 2012; Arkush et al. 1998; Gozlan et al. 2005; Paley et al. 2012). In a cohabitation study with topmouth gudgeon and sunbleak, the latter failed to reproduce and the population dramatically declined over a period of three years (Gozlan et al. 2005). Descriptions of cases of *S. destruens* infected species are mostly based on experimental cohabitation studies, challenge experiments or specimens from hatcheries (Andreou et al. 2012; Paley et al. 2012). Little information exists on the prevalence of *S. destruens* in wild populations of both topmouth gudgeon and native fish species. Andreou et al. (2011a) found an infection rate of 5% in a wild population of sunbleak in the United Kingdom. *S. destruens* has proved to be a true generalist with a broad host spectrum that is likely to expand (Gozlan et al. 2009). As *S. destruens* is slow-growing, causing chronic, steady mortality rather than short-term high mortality, the method used to detect the disease is thought to lead to underestimation of its infection rate (Andreou et al. 2012).

Here we present (a) the distribution and expansion of topmouth gudgeon in the Netherlands, insofar as it is known, and (b) the first record of *S. destruens* (by molecular detection) in wild populations of topmouth gudgeon in this country. The prevalence of this pathogen provides important information on its potential impact on native freshwater fish in lowland river basins.

Methods

Distribution of topmouth gudgeon in the Netherlands

We used information on the distribution of topmouth gudgeon in the Netherlands from the National Databank on Flora and Fauna (NDFF 2013). These data were gathered from a wide range of surveys, using different equipment and sampling efforts. The validity of these data was scrutinized. All records from the period 1992–2012 were used in the analyses. We used 323 presence records within 156 square kilometre grid cells.

Sampling of topmouth gudgeon populations

Our sampling criterion for selecting sites was opportunistic, in that we sampled water bodies where topmouth gudgeon was expected to occur. Two populations of topmouth gudgeon were sampled, both situated in temporarily isolated water bodies in the floodplain of the river Meuse in the Netherlands: near the Everlose beek (51º24'13"N, 06º08'54"E) and near the Teelebeek (51º43'06"N, 05º55'48"E). The former site was sampled in October 2008, the latter in September 2012. At the Everlose beek site, 20 individuals of topmouth gudgeon were caught by hand netting, while at the Teelebeek site, 38 individuals were caught by seine netting. Densities and occurrence of young of the year (YOY) of other fish species present were noted. After sampling, the collected specimens of topmouth gudgeon were immediately euthanized with a lethal solution of benzocaine (100 mg/L) (Barker et al. 2009), stored in 96% ethanol, put on ice and transported to the laboratory where they were stored at -20°C. After dissection, the kidney, liver, gill and gonad tissue were pooled together for each fish. After immersion in liquid nitrogen, the tissue was ground up using pestle and mortar. The project was approved by the Royal Netherlands Academy of Arts and Sciences ethics committee and was performed under WOD licence No. TRC/VWA/2012/ 4275)

DNA extraction and analysis

Genetic analysis was performed on all 38 topmouth gudgeons caught at the Teelebeek site and on nine of the 20 topmouth gudgeon caught at the Everlose beek site. DNA was extracted from approximately 0.4 g tissue using the Powersoil® DNA isolation kit (MO BIO Laboratories, Inc.

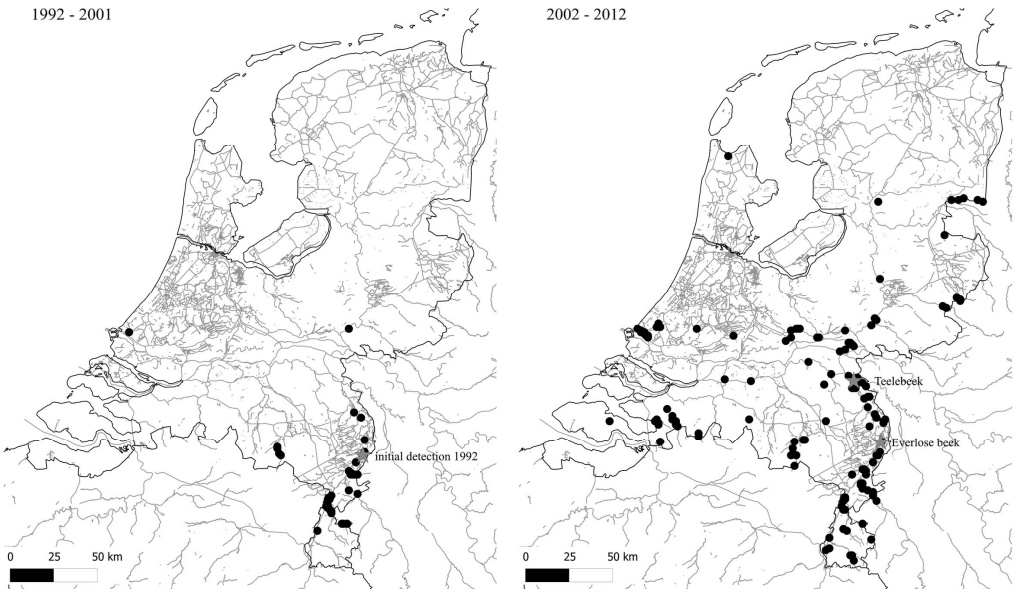

Figure 1. Expansion of topmouth gudgeon (*Pseudorasbora parva*) in the Netherlands (square kilometre grid cells) (data source: NDFF 2013). Initial introduction in the Meuse basin (1992) (location indicated by a star in the left-hand map) was followed by both upstream and downstream dispersal within the Meuse basin and colonization of other basins in the southeast of the country. Locations with *Sphaerothecum destruens* infected populations (Teelebeek, Everlose beek) are indicated in the right-hand map (stars).

Sanbio, Uden NL) according to the manufacturer's protocol. DNA was dissolved in DEPC-treated water and stored at -20 °C. The quality of the isolated DNA was checked by subjecting aliquots of the samples to agarose gel (1%) electrophoresis. All DNA samples were used as a template in a PCR using a specific combination of primers targeting the 18S rRNA gene of *S. destruens*. These specific primer sets were 5'-AAT CGT ATG ACA TTT TGT CGA C-3' (F1) and 5'-GAA GTC ACA GGC GAT TCG G-3'(R1) in combination with 5'-ACA GGG CTT TTT AAG TCT TGT-3' (F2) and 5'-ATG GAG TCA TAG AAT TAA CAT CC-3' (R2) (Gozlan et al. 2005). Thermal cycling conditions for the PCR were: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and elongation at 72°C for 1.5 min. A final elongation step at 72°C for 10 min was performed. After agarose (1%) electrophoresis to check if the PCR resulted in a unique band of the expected size, PCR products were purified using a GeneJET PCR Purification Kit (Thermo Scientific, Landsmeer, NL). The PCR fragments were cloned in *E. coli* XL1Blue cells using the pGEM-T easy cloning kit (Promega, Leiden, NL) according to the manufacturer's protocol. Plasmids isolated from selected colonies were sequenced at the sequencing facility at the Department of Human Genetics, Radboud University Medical Center, using M13 forward and reverse primers. Sequences were checked with Chromas Lite (Technelysium Pty Ltd.) and analysed by BLASTn searches (http://www.ncbi.nlm.nih.gov) against the GenBank nucleotide database. Alignment (ClustalW) and phylogenetic analysis (neighbour joining) was performed using MEGA 5.1 (Tamura et al. 2011). The 18S rRNA gene sequences from the closely related Dermocystida *Rhinosporidium cygnus, Dermocystidium salmonis* and *Amphybiocystidium ranae* were used as an out-group. Accession numbers for these species are AF399715, U21337 and AY692319, respectively. The 18S rRNA gene sequences of the clones depicted in Figure 3 have been deposited in GenBank under accession numbers KC896010 to KC896020.

Results

Distribution of topmouth gudgeon in the Netherlands

Topmouth gudgeon was first detected in the Aalsbeek brook near its confluence with the river Meuse in 1992 (Figure 1). After a two-year lag

Table 1. Densities (number of individuals per m^2) and presence of young of the year (YOY) of topmouth gudgeon (*Pseudorasbora parva*) and sympatric species.

	Everlose beek		Teelebeek	
Species	ind/m^2	YOY present	ind/m^2	YOY present
Cyprinus carpio	-	-	0.01	-
Gasterosteus aculeatus	4.5	+	0.38	+
Gobio gobio	0.45	-	-	-
Leucaspius delineatus	-	-	0.01	-
Pseudorasbora parva	1.2	+	0.3	-
Pungitius pungitius	0.45	+	0.03	-
Rhodeus amarus	-	-	0.19	-
Tinca tinca	2.1	+	-	-

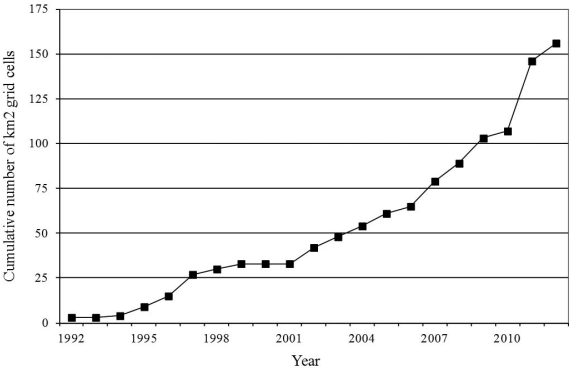

Figure 2. Time series of topmouth gudgeon (*Pseudorasbora parva*) colonizing the Netherlands, based on its presence in square kilometre grid cells (data source NDFF).

phase after the first detection, it was found across the whole Dutch part of the river Meuse basin within less than 10 years, also colonizing its tributaries. In 1995, topmouth gudgeon was detected in the Rhine basin, but further dispersal was not recorded at that site during the following years. From 2002 onwards, topmouth gudgeon rapidly expanded its range and by 2012 it had been detected in 158 square km grid cells (Figure 2). The average number of newly colonized grid cells was 3.3 per year in the 1992–2001 period, and 11.2 in the 2002–2012 period, showing no sign of saturation. Records of topmouth gudgeon in isolated locations suggest multiple introduction events.

Measured densities of topmouth gudgeon were 1.2 individuals/m^2 at the Everlose beek site and 0.3 individuals/m^2 at the Teelebeek site (Table 1). Sympatric freshwater fish populations were found at the Everlose beek site, consisting of threespine stickleback (*Gasterosteus aculeatus* Linnaeus, 1758), ninespine stickleback [*Pungitius pungitius* (Linnaeus, 1758)], gudgeon [*Gobio gobio* (Linnaeus, 1758)] and tench [*Tinca tinca* (Linnaeus, 1758)]. Species present at the Teelebeek site included bitterling [*Rhodeus amarus* (Bloch, 1782)], threespine stickleback (*G. aculeatus)*, ninespine stickleback (*P. pungitius*), carp (*C. carpio*) and sunbleak (*L. delineatus)*. Evidence of reproduction (presence of YOY) has been found for four species at the Everlose beek site (including topmouth gudgeon), and one at the Teelebeek site (Table 1).

PCR targeting the 18S rRNA gene of *S. destruens* resulted in fragments with a length of 896 basepairs (primers F2 + R1) and 588 basepairs (primers F2 + R2). At the Teelebeek site, 28 out of 38 topmouth gudgeons (74%) tested positive for *S. destruens,* while the prevalence of the parasite at the Everlose beek site was 67% (6 out of 9). PCR products from three gudgeons from each site were checked by cloning and sequence analyses. Blast search analysis gave a positive match with *S. destruens* with a maximum identity of 99% using BLASTn (E-value 0.0). Phylogenetic analysis (neighbour joining) of the sequences obtained from both sites shows that they all cluster together with *S. destruens* sequences obtained from infected Atlantic or Chinook salmon and sunbleak (Figure 3). Differences between the sequences found at our sites in the Netherlands and the known sequences of *S. destruens* from GenBank are very minor, pointing to a low genetic diversity within this species.

Discussion

We report the first evidence of *S. destruens* being present in wild populations of topmouth gudgeon, with a prevalence of 67 to 74%. Phylogenetic analysis of 18S rRNA gene sequences showed a high similarity (99%) of the samples with known sequences of *S. destruens*. The

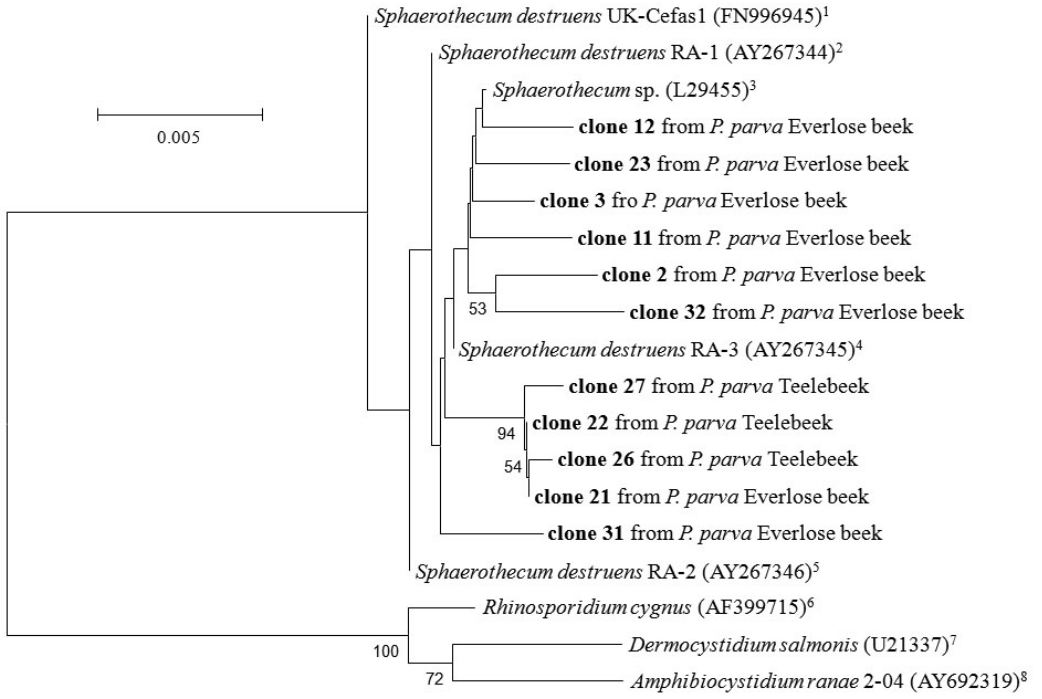

Figure 3. Phylogenetic analysis of the 18S rRNA sequences obtained from *Pseudorasbora parva* from the Teelebeek and Everlose Beek sites, together with *Sphaerothecum destruens* sequences from GenBank and some other Dermocystida (outgroup). The bar represents 0.5% sequence divergence. The Neighbour-Joining tree is drawn to scale and the evolutionary distances were computed using the Maximum Composite Likelihood method and expressed in units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. There were a total of 1826 positions in the final dataset. Additional information: [1] from sunbleak; [2] from Chinook salmon kept in seawater pens; [3] from infected Chinook salmon embryo cell culture; [4] from kidney tissue of infected winter-run Chinook salmon; [5] from Atlantic salmon kept in fresh water; [6] from swan; [7] from Chinook salmon; [8] from frog.

presence of *S. destruens* in wild populations has only been reported for sunbleak (prevalence 5%) in the UK (Andreou et al. 2011a) and Chinook salmon in the USA (prevalence 32%) (Arkush et al. 1998), while experimental research has established infection of *Leucaspius delineatus* (prevalence 28%), *Abramis brama* (prevalence 75%), *Cyprinus carpio* (prevalence 20%) and *R. rutilus* (prevalence 5%) (Gozlan et al. 2005; Andreou et al. 2012). However, no information on the prevalence of *S. destruens* in topmouth gudgeon was previously available. In the study by Gozlan et al. (2005), all topmouth gudgeon tested negative, but the authors assumed that this was due to a low pathogen concentration. Although PCR is considered to be an effective method to screen individuals for *S. destruens*, low parasite levels might remain undetected (Andreou et al. 2011a). In addition, non-homogeneous distribution of *S. destruens* in organs may lead to false-negative PCR results (Andreou et al. 2011b). *S. destruens* has been shown to appear in two forms in *L. delineatus*, a nodular form causing an inflammatory response and a disseminated form causing limited host response (Andreou et al. 2011a). Histological techniques, such as Gram stain, as well as light microscopy, need to be used alongside PCR amplification of *S. destruens* DNA to increase the detection rate of low-level infections (Andreou et al. 2011a) and also to clarify the status of topmouth gudgeon as a healthy carrier. In the present study, the prevalence of 67–74% observed in topmouth gudgeon could even be an underestimation, and is thus clearly associated with the invasion of topmouth gudgeon.

In line with the pan-continental invasion of topmouth gudgeon (Gozlan et al. 2010), rapid colonization is also taking place in the Netherlands, and is considered to be still in progress. We believe not all suitable areas have as yet been occupied, and most of the Dutch water systems provide suitable habitats for the

species, further increasing the risk of pathogen spread. Sympatric populations of known susceptible fish species were found at the sites we sampled in the Netherlands, including species that feature in the national Red List (LNV 2004) (bitterling and sunbleak). No information about infection rates of these native species by *S. destruens* is available yet, nor about the effects on their population size. The confirmation of the association of *S. destruens* with a healthy and highly successful invasive species vector increases the need to implement control measures to limit the spread of topmouth gudgeon, described in this study, and thereby reduce the risk of mortality among native species. Measures to be taken should include (i) closure of the primary introduction pathways (aquaculture, recreational fishing and ornamental fish trade) through legislation, (ii) an early warning system to detect introductions, (iii) risk assessment to distinguish high-risk sites and (iv) the setup of a risk-based control programme that includes a protocol for rapid eradication (e.g. using piscicides, biomanipulation or draining of waterbodies).

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