Research Article

First record of black bullhead (*Ameiurus melas* Lesueur, 1819*)* and the occurrence of hybridization with brown bullhead (*A. nebulosus* Rafinesque, 1820) in Belgium

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Abstract

Ictalurid bullheads are freshwater fish species native to North America that were introduced to Europe in the late 19th century, where they negatively impact native aquatic species. In Belgium, brown bullhead (*Ameiurus nebulosus*) was introduced decades ago, and is already established and widespread. Here, we report the first occurrence of a congeneric non-native invasive bullhead species in Belgium, the black bullhead (*Ameiurus melas*). In early May 2023, electric fishing in the Zonderikbeek, a stream situated in the northeast of Flanders (Northern region of Belgium), led to the capture of a suspicious bullhead specimen. Diagnostic PCR analysis identified this specimen as a hybrid between black and brown bullheads. The following spring, in 2024, 21 *Ameiurus* specimens were additionally captured in a nearby lake that is connected to the Zonderikbeek. Diagnostic PCR revealed that 17 of the captured individuals were pure black bullheads, three were pure brown bullheads, and one specimen appeared to be a hybrid. Morphological examination of the main distinguishing key features of these bullhead species, such as the pigmentation of the anal and caudal fin membrane, gill-raker count, and the serration of the pectoral spine, were in line with these molecular analyses. Notably, hybrid individuals were morphologically characterized with features of both *A. melas* and *A. nebulosus*. The discovery of multiple individuals of *A. melas*, along with hybrids with the already established *A. nebulosus* is concerning especially given the extensive network of lakes, creeks, and small rivers in vicinity of these observations. This interconnected system of water bodies could facilitate the further dispersal of both species and their hybrids. Given the significant potential ecological and socio-economic impacts of both species, urgent management efforts are needed to remove or contain not only the brown bullhead, but also the black bullhead as emerging non-native invasive species in Belgium.

Key words: biological invasion, fish introduction, invasive non-native species, hybrid, morphological identification, spread, diagnostic PCR

Introduction

Since the late 19th century, the brown bullhead catfish (*Ameiurus nebulosus* Lesueur, 1819; further denoted as brown bullhead) and black bullhead catfish (*Ameiurus melas* Rafinesque, 1820; further denoted as black bullhead)

Figure 1. (a) Brown bullhead (*Ameiurus nebolosus)* and (b) black bullhead (*Ameiurus melas*). Photographs by Jelger Herder (Ravon).

have been introduced to Europe (Carlander 1977; Walter et al. 2014) (Figure 1). Originally native to subtropical and temperate zones of North America, these non-native invasive fish species were first introduced to France from the Mississippi Basin in 1871 (Vivier 1951; Wheeler 1978). Subsequent secondary introductions (i.e., post-introduction human-mediated displacement) occurred across European waters resulting from unintentional releases during baiting, intentional stocking for recreational fishing, and escapes from aquaculture facilities. Following primary and secondary introduction, both bullhead species have dispersed naturally throughout Europe (Kalinowska et al. 2023). Their success can be attributed to several characteristics, including resilience to drought through sediment burrowing, tolerance to pollution and low oxygen levels, strong parental care, and protective spines (Scott and Crossman 1973). Once established, both bullhead species can outcompete native species for resources, predate on native species, and bioaccumulate toxic substances, thereby disrupting the structure and functioning of native aquatic ecosystems (Vila-Gispert et al. 2005; Leunda et al. 2008; Ribeiro et al. 2009; Savini et al. 2010).

In Belgium, non-native *Ameiurus* species were introduced in 1882 to populate the very polluted waters in the vicinity of cities as almost all native fish species had disappeared (Lefebvre 1882). At least for the last 50 years, the brown bullhead is the only species of the *Ameiurus* genus known to occur in the wild in Belgium (Verreycken et al. 2007), with initial sightings dating back to the early 1900s (Rousseau et al. 1915). Since then, this species has expanded into new habitats and has become a predominant member of various fish communities in lakes and ponds in Belgium, especially in the northeast of Flanders. In lotic systems, such as streams and canals, brown bullhead seems to show a decrease in numbers and occurrence sites in the last decades (Verreycken et al. 2007). A similar declining trend appears to be occurring in several other regions, including parts of Poland and the Netherlands (Kalinowska et al. 2023; Groen M. et al. *unpubl. results*). Due to political and jurisdictional reasons, only the black bullhead has been listed among the non-native invasive species of Union Concern (EU Regulation 2014/114).

Brown and black bullheads are morphologically very similar, making it challenging to distinguish between them based on morphology alone, particularly in juvenile specimens (Rutkayová et al. 2013; Dumke et al. 2020). The most commonly used distinguishing feature is the development of the pectoral spine serrae: in the brown bullhead, they are well-developed along the full length of the spine, while in the black bullhead, these spine serrae are small or absent. However, a study executed on specimens from Belgium, the Netherlands and France, illustrated that for those specimens the distinction based on pectoral spine serration was not always straightforward, especially for smaller specimens (Decru and Snoeks 2011). Other distinguishing features include gill-raker count, anal fin ray count, pigmentation of the anal-fin membrane, and body colouration (Dumke et al. 2020; Nowak et al. 2010; Scott and Crossman 1973) (Figure 1). Yet again, in the study of Decru and Snoeks (2011), the anal fin count was not a diagnostic trait for those specimens as a range of 16–23 was found in *A. nebulosus,* which overlapped completely with the range of 18–21 found in *A. melas.* The only clear diagnostic characteristics found here were the number of gill rakers (11–15 in *A. nebulosus* vs 16–20 in *A. melas*), and the presence of a black-white radiation in the anal and caudal fin (absent in *A. nebulosus* and present in *A. melas).* In addition, pectoral serrations were indeed mostly stronger in *A. nebulosus;* colour pattern was mostly mottled in *A. nebulosus,* while this was not the case in *A. melas;* and the vertical band on the caudal fin was mostly light of colour in *A. melas,* while darker coloured in *A. nebulosus.* Due to the complexity and variability of these morphological traits, some historical records and citizen science observations may require further verification by expert judgement.

In the last decade, DNA-based methods have emerged as reliable alternatives for morphological identification of fish species. Various molecular markers, such as primers for sequencing the mitochondrial cytochrome *c* oxidase subunit I (COI) (Béres et al. 2017; Hubert et al. 2008; Zangl et al. 2022) and cytochrome *b* (cyt *b*) (Béres et al. 2017; Rutkayová et al. 2013), have been employed to distinguish closely related *Ameiurus* species. Unfortunately, several of the published reference sequences appear to be misidentified, likely due to errors in morphological identification, making accurate identification based on the public reference databases challenging (Ward et al. 2009; Kutsokon et al. 2024). To address these issues, non-sequencing methods such as species-specific markers in duplex PCR (Béres et al. 2017) and Restriction Fragment Length Polymorphism (RFLP) assays (Walter et al. 2014) have proven to be effective alternative molecular tools for accurately identifying pure *Ameiurus* species and their resulting hybrids, that are independent of DNA sequences, and therefore straightforward and relatively inexpensive.

In this study, we report the first occurrence of black bullhead in the wild in Belgium since their introduction in Europe and provide evidence of hybridization with brown bullhead at two locations in Belgium. To this end, we used both genetic analyses and morphological characterization for taxonomic identification, and compared their efficiency in identifying pure black bullheads, pure brown bullheads, and hybrids.

Materials and methods

Study area

In May 2023, a 100 meter transect of the stream Zonderikbeek, near the cities of Zonhoven and Genk, Belgium, was electrically fished for routine monitoring purposes (see Figure 2). Electrofishing was conducted using a 5 kW generator with an adjustable output voltage of 300–500 V (RUD 24.xx), moving upstream along both riverbanks across the entire transect (for details, see also Belpaire et al. 2000). The following year, in early March 2024, a nearby lake, within the Vijvers Midden-Limburg area, and connected to the Zonderikbeek was drained, and nets were simultaneously placed at the outlet to prevent fish from escaping. When all fish had gathered around this outlet basin, all individuals were caught using electrofishing and drag-netting with a 1 cm mesh size. All fish collected from both fishing events were identified to species level, counted, measured for length and weight, and bullhead specimens were set aside for further molecular and morphological identification.

DNA extraction and molecular species identification

Genomic DNA extraction was performed on tissue samples from caudal fin clips taken from each captured *Ameiurus* specimen and preserved on pure ethanol, comprising one individual from the Zonderikbeek (in 2023) and 21 individuals from the nearby lake (in 2024). The DNA extraction was conducted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Additionally, genomic DNA from archived reference material at the Research Institute for Nature and Forest (INBO) was

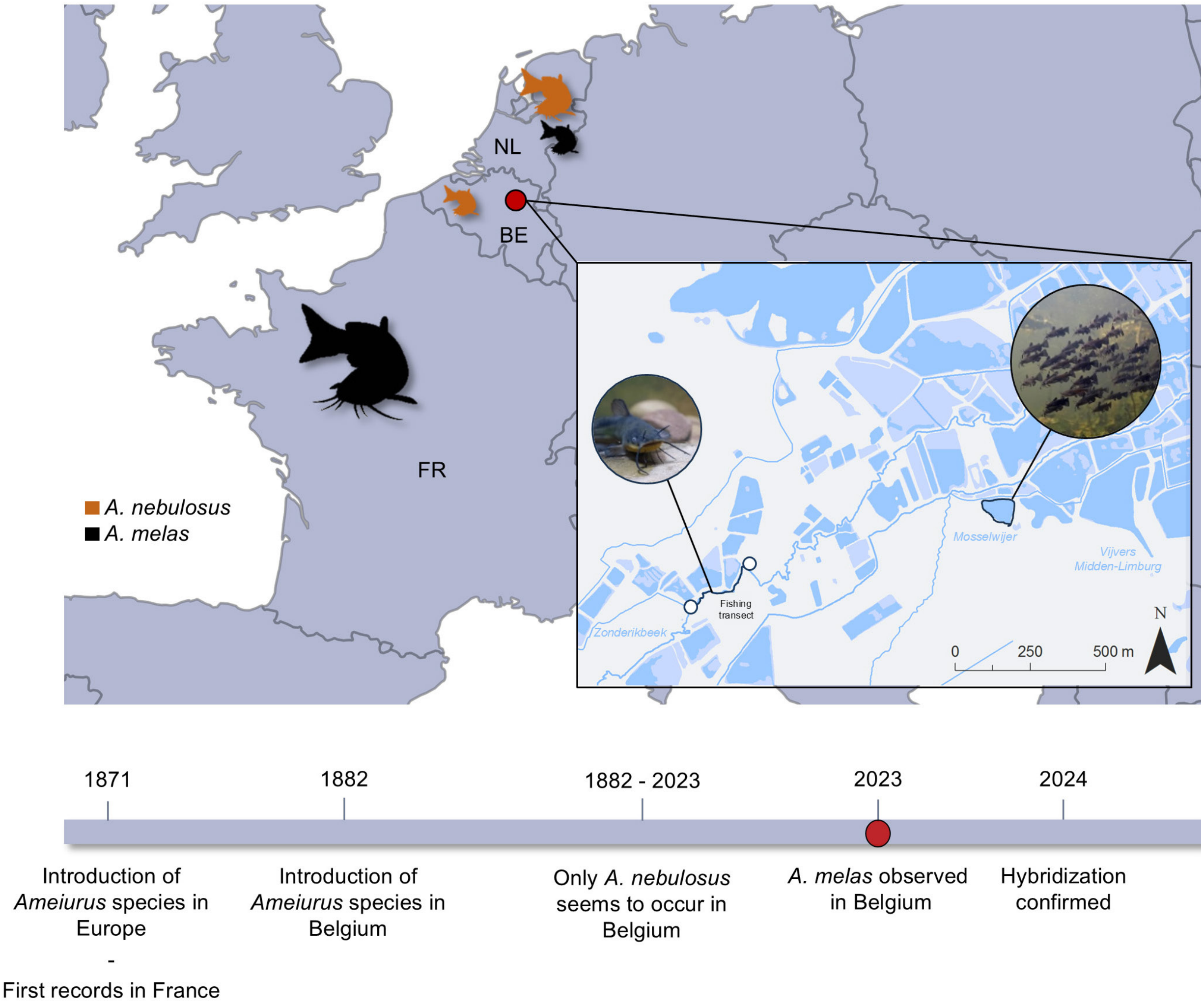

Figure 2. Colonization history of brown bullhead (*Ameiurus nebolosus)* and black bullhead (*Ameiurus melas*) in Western Europe, with brown and black bullhead silhouettes representing the previously documented occurrences of each species, respectively. The study area is located in the eastern region of Flanders, Belgium, and the inset map shows the specific locations of the 100-meter stream transect in the Zonderikbeek and the lake, that were subjected to electrofishing and netting in early spring 2023 and 2024, respectively. Photographs by Jelger Herder (Ravon).

used as a benchmark in the subsequent PCR protocols. These included four *A. melas* and four *A. nebulosus* specimens from The Netherlands, Belgium, and France (see Supplementary material Table S1). We employed multiplex PCR reactions using a specialized primer pair (Neb_F: CTGCTACATGC TAAGGCTAACG and Neb_R: GGATTATTGTGGCGATTGGT, as described in Béres et al. 2017), on the 22 undefined *Ameiurus* specimens and the 8 reference samples. These primers amplify genomic fragments of different sizes for each *Ameiurus* species, enabling clear differentiation between pure *Ameiurus nebulosus* and *A. melas* specimens during gel electrophoresis, eliminating the need for DNA sequencing. Specifically, the used protocol amplifies a 591 bp fragment specific to *A. nebulosus* and a 457 bp fragment specific to *A. melas* (see Figure 3). In the case of hybrids between the two species, both fragments are equally amplified, resulting in the simultaneous detection of both fragment sizes in a single individual (Béres et al. 2017) (Figure 3).

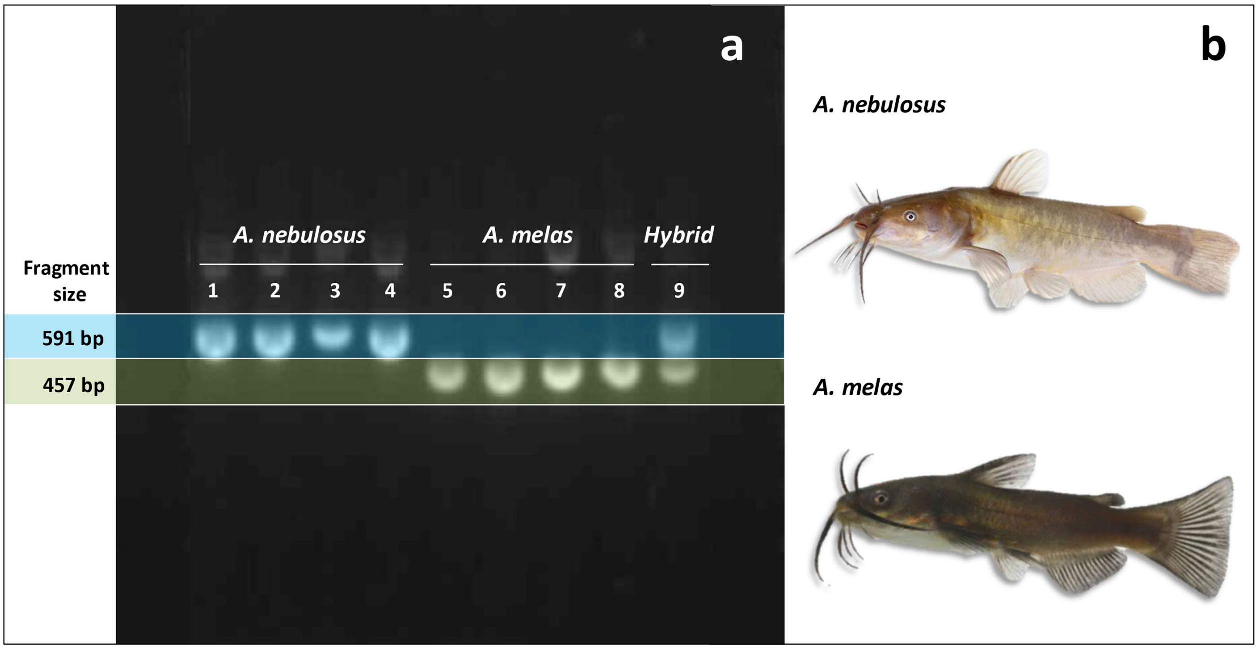

Figure 3. (a) Duplex PCR-based molecular identification of *Ameiurus nebulosus* and *A. melas*. The PCR amplifies a 591 bp species-specific fragment from four pure *A. nebulosus* specimens and a 457 bp fragment from four pure *A. melas* specimens. Both fragments are clearly amplified in a resulting hybrid between both pure species. (b) Photographs of brown and black bullhead specimens (photographs by Jelger Herder (Ravon) and Frank Spikmans respectively).

Morphological characterization

All 22 specimens were morphologically analyzed. This characterization comprised the main diagnostic traits as found and obtained in the study of Decru and Snoeks (2011) (see Figure 4). The total number of gill rakers on the first gill arch was counted under a stereomicroscope after dissection of this first gill arch. As for colouration, the presence of a black-white striped pattern on caudal and anal fin was assessed, and whether the vertical band at the base of the caudal fin was dark or lightly coloured. In addition, the strength of the serrations on the pectoral fin was coded (almost smooth: "0", smooth to medium serrated: "0,5", medium: "1", medium to strongly serrated: "1,5", pectoral fin almost entirely serrated: "2"), and the number of clearly visible serrations was counted. For the specimen captured in 2023, only the colouration and number of gill rakers were assessed.

Results

Captured Ameiurus *specimens and molecular identification*

In the 100 m transect of the Zonderikbeek, only one *Ameiurus* specimen was captured (resulting in a density of 0.0025 individuals/m^2), representing 0.2% of the total captured fish biomass (*unpublished results*). In contrast, 21 *Ameiurus* individuals were caught in the nearby lake (resulting in a density of 0.008 individuals/m^2), which constituted 26.5% of the captured fish biomass, making them the second most dominant species within that community. Using multiplex PCR with the Neb_F and Neb_R primer pair,

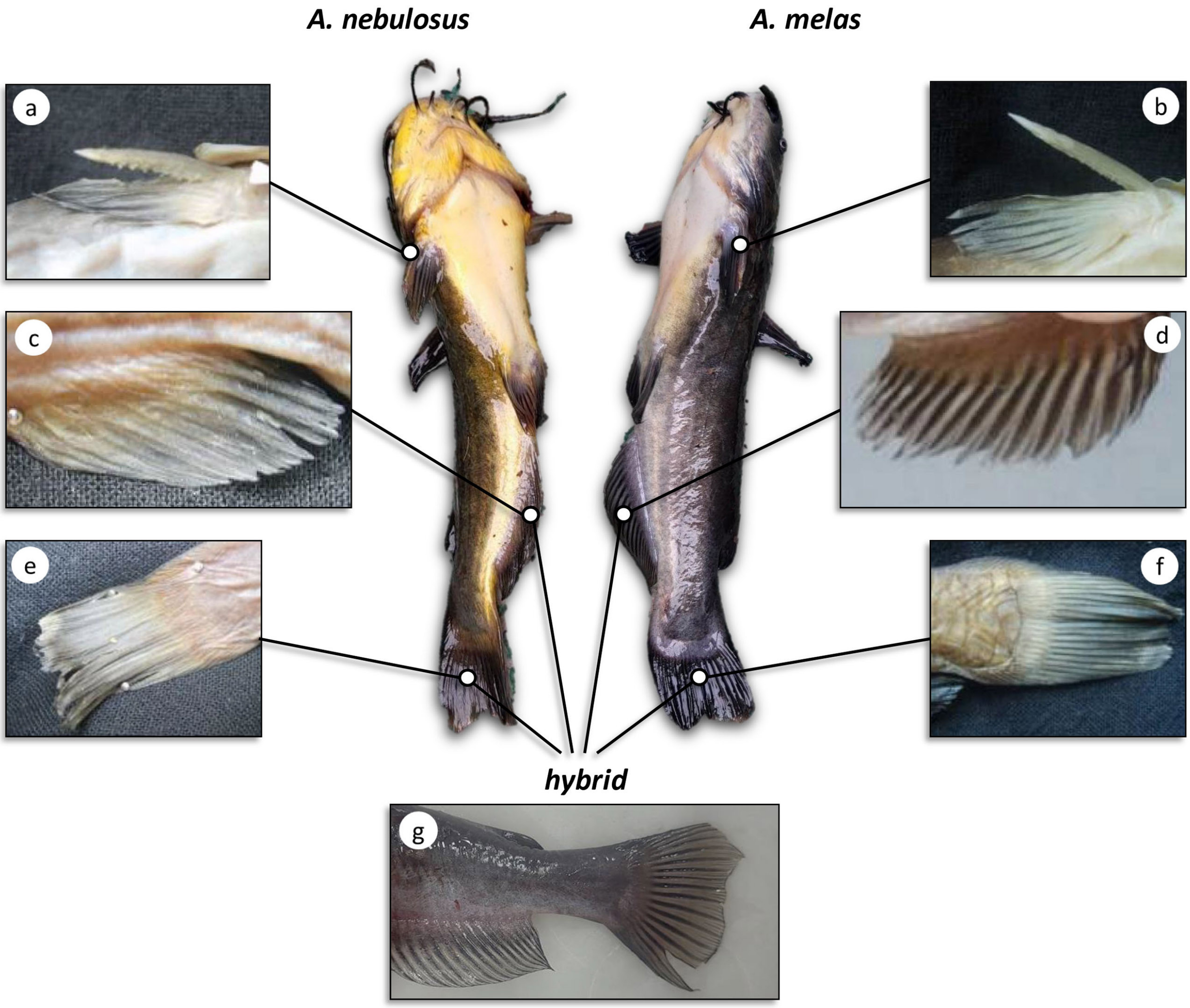

Figure 4. Brown bullhead specimens, *Ameiurus nebulosus* (left), black bullhead specimens, *A. melas* (right) and hybrid (below), captured in the lake of the Vijvers Midden-Limburg, with details of the main morphological diagnostic traits: (a–b) pectoral spine, and black-white striped pattern on anal (c–d) and caudal fin (e–g). Photographs by Rein Brys (INBO) and Eva De Cru (KU Leuven).

we successfully amplified differently sized fragments for the reference samples for each *Ameiurus* species, the 591 bp fragment for *A. nebulosus* and 457 bp fragment for *A. melas*, enabling accurate identification of both pure and hybrid individuals (see Figure 3; Table S1), resulting in a correct classification of the four *A. nebulosus* and four *A. melas* individuals. Running this analysis on the 22 specimens collected from the Zonderikbeek stream and the nearby lake, three were identified as pure *A. nebulosus*, 17 as *A. melas*, and two as putative hybrids between both species (one per sampling location).

Morphological features of Ameiurus *specimens*

Morphological identification was executed independently from the genetic results, to avoid observer bias. Subsequent comparison indeed revealed that morphological identifications matched the results of the molecular identification (Tables 1, S1). Interestingly, the two molecularly-indicated

Table 1. Morphological characterization and identification of the 22 captured *Ameiurus* specimens in this study. For details: see material and methods.

Specimen ID	Morphological diagnostic characteristics					Morphological identification
	black-white pattern	*band on caudal base*	*serration code*	*#serrations*	*Gill rakers*	
INBO_F2023E10001	present	light			12	hybrid
INBO_F2024E10001	present	light	0.5	4	17	*A. melas*
INBO_F2024E10002	present	dark	0	0	13	hybrid
INBO_F2024E10003	present	light	0.5	6	16	*A. melas*
INBO_F2024E10004	present	light	1.5	13	17	*A. melas*
INBO_F2024E10005	present	light	0.5	6	17	*A. melas*
INBO_F2024E10006	present	light	0.5	4	16	*A. melas*
INBO_F2024E10007	present	light	0.5	6	16	*A. melas*
INBO_F2024E10008	absent	dark	0.5	6	13	*A. nebulosus*
INBO_F2024E10009	present	light	1	11	17	*A. melas*
INBO_F2024E10010	present	light	0.5	5	16	*A. melas*
INBO_F2024E10011	present	light	0.5	5	16	*A. melas*
INBO_F2024E10012	present	light	0.5	3	16	*A. melas*
INBO_F2024E10013	present	light	0.5	4	16	*A. melas*
INBO_F2024E10014	absent	dark	2	11	10	*A. nebulosus*
INBO_F2024E10015	absent	dark	1	9	13	*A. nebulosus*
INBO_F2024E10016	present	light	0.5	5	18	*A. melas*
INBO_F2024E10017	present	light	0.5	3	16	*A. melas*
INBO_F2024E10018	present	light	0.5	5	16	*A. melas*
INBO_F2024E10019	present	light	0.5	6	16	*A. melas*
INBO_F2024E10020	present	light	1	12	18	*A. melas*
INBO_F2024E10021	present	light	1	12	17	*A. melas*

hybrid specimens exhibited traits of both *A. nebulosus* and *A. melas*. The putative hybrid specimen from the Zonderikbeek (INBO_F2023E10001; Table S1) clearly exhibited the black-white pigmentation on the caudal and anal fin, and a light vertical band on the caudal fin like in *A. melas*, but had 12 gill rakers on the first gill arch, which falls within the range of *A. nebulosus*. The putative hybrid specimen from the nearby lake (INBO_F2024E10002; Table S1) also exhibited the black-white pigmentation on the caudal and anal fin and had a completely smooth pectoral spine like in *A. melas*, but had a darker vertical band on the caudal fin base and 13 gill rakers, which are traits that match *A. nebulosus*. Seventeen specimens were confidently identified as *A. melas*, exhibiting distinctive morphological features such as black-white pigmentation on the caudal and anal fin, a light vertical band on the caudal fin, and a range of 16–18 gill rakers on the first arch; three specimens were clearly *A. nebulosus*, lacking the black-white pigmentation, having a dark vertical band on the caudal fin base, and a range of 10–13 gill rakers on the first arch. Like previously found in Decru and Snoeks (2011), the serrations were not always clearly diagnostic as some specimens of *A. melas* had quite serrated pectoral spines (Table 1).

Discussion

The introduction of brown bullhead (*Ameiurus nebulosus*) and black bullhead (*Ameiurus melas*) to Europe dates back to the 1880s. Since then, brown bullhead has successfully spread across the continent (both naturally and following secondary human-mediated introductions), establishing selfsustaining populations in numerous countries, including Belgium, as well as neighbouring countries such as the Netherlands, France, and Germany (Copp et al. 2005; Rutkayová et al. 2013; Béres et al. 2017). Similarly, the black bullhead (*Ameriurus melas)* expanded its distribution range in Europe following its introduction due to a combination of secondary introductions and natural dispersal. However, the black bullhead has historically been reported in fewer regions and countries compared to the brown bullhead. To date, it has not been recorded in Belgium (Kottelat and Freyhof 2007; Rutkayová et al. 2013) (Figure 2). This study presents the first documented occurrence of pure black bullhead individuals in the wild in Belgium, along with empirical evidence of hybridization with its close relative, the brown bullhead. Our findings confirm that the use of the non-sequencing molecular method described by Béres et al. (2017), is an effective and accurate diagnostic molecular tool for distinguishing between brown and black bullheads. Genetic and morphologic lines of evidence agreed that three specimens were pure brown bullheads and 17 specimens were pure black bullheads, with additionally two specimens being identified as putative hybrids between the two species.

The exact invasion pathway of black bullheads into Belgium remains unclear. Assuming the detected populations entered Belgium via secondary spread, it is plausible that they originated from the southern part of the Netherlands. Several known black bullhead populations have been reported since many years at various locations near the Belgian border. A more likely explanation, however, might be found in the unintentional introduction of the species through aquaculture facilities in the surrounding area, leading to potential escapes from breeding ponds. To gain better insights into the origin of these newly discovered black bullhead population and the extent of their distribution, further monitoring across a broader geographic range in this areas is necessary.

Although non-native brown bullheads are currently more widespread in Europe, recent trends suggest that non-native black bullhead populations are expanding more rapidly, potentially displacing brown bullhead in certain regions (Gante and Santos 2002; Nowak et al. 2010; Movchan et al. 2014). Indeed, observations suggest that in areas where both species are detected, black bullheads often outcompete brown bullheads (Movchan et al. 2014). Similarly, in the newly discovered sympatric population in Belgium, black bullheads are the predominant species, comprising 85% of the bullheads present, while brown bullheads account for only 15%. While we acknowledge that the potential dominance of black over brown bullheads is based on just a single population, it may confirm the rising dominance of black bullheads in Europe. Remarkably, in their native North American ranges, both species coexist stably without one displacing the other (Scott and Crossman 1973). This raises the question whether the black bullhead is indeed consistently displacing the brown bullhead within its invasive range in Europe as suggested, and what factors might contribute to this potential dynamic.

One possible explanation is related to global climate change and rising temperatures, which may facilitate the spread of black bullhead (Copp et al. 2016; Hartman 2017). If this is the case, it remains unclear why the brown bullhead is not exhibiting a similar trend, especially giving that significant declines have been observed in some regions (Verreycken et al. 2007; Kalinowska et al. 2023). Also the increased frequency of flooding events may have additionally promoted the range expansion of both bullhead species. In our study area in Belgium, unusual flooding events occurred in the spring of 2021 and 2024, potentially aiding the further spread of these species after their initial colonization. Similarly, Kutsokon et al. (2024) reported an increasing spread of black bullhead in Eastern Europe, likely driven by changing climate conditions and exceptional flooding events as well. An increased attention on the occurrence of the black bullhead, along with recent advancements in identification techniques for distinguishing between black and brown bullheads, may also have contributed to the apparent increase in black bullhead records in recent years.

Beyond typical interspecies competition, hybridization between brown and black bullheads may also occur, leading to hybrid offspring and even genetic introgression (Mallet 2005). Our findings indicate that hybridization has already occurred in the sampled populations in Belgium, suggesting that black bullheads may have been present in the region for at least one year. This time lag between the initial co-occurrence in a sympatric population and the documentation of effective hybridization can be partially attributed to the morphological similarity of black bullheads with the widely distributed brown bullheads, as well as the challenges associated with the early detection of aquatic invasive species. Routine monitoring of waterways using environmental DNA (eDNA) provides a promising method to overcome these challenges in terms of detection of early life stages (Everts et al. 2023) as well as upscaling of the detection resolution from traditional monitoring at limited spatial scales (Brys et al. 2021a, b; Van Driessche et al. 2022). Additionally, continuous human-facilitated introductions may further promote hybridization, with hybrid offspring potentially exacerbating the ecological disruption caused by invasive bullheads (Madeira et al. 2005).

To date, only the black bullhead has been listed as a non-native invasive species of Union Concern under the EU Regulation 2014/114. Consequentially, EU member states are required to prevent new introductions and manage established populations of the black bullhead, making the possession and trade of this species illegal. However, this list is species-specific and does not cover congeneric invasive species, such as the brown bullhead, nor their potential hybrids. As a result, EU member states, including Belgium, have not been incentivised to prevent the introduction of brown bullheads. This situation underscores the importance of including brown bullhead and other ictalurid species in international legislative collaborations and reveals existing shortcomings in aquatic biosecurity, such as those outlined in EU Regulation 1143/2014 (Coughlan et al. 2020). These contradictory circumstances indicate the need to maybe address entire invasive species complexes or genera on such lists rather than maintaining a species-specific approach.

The spread of *Ameiurus* species across Europe is a continental concern (Kottelat and Freyhof 2007; Nowak et al. 2010; Rutkayová et al. 2013), and their movement northward and eastward, including these recent observations in Belgium, raises significant ecological concerns. Their voracious appetite and opportunistic feeding behavior can disrupt food web dynamics, potentially causing declines in native fish populations and alterations in community compositions (Kalinowska et al. 2023; Leunda et al. 2008). Furthermore, their high reproductive rates can exacerbate their impact on local ecosystems by outcompeting native species for breeding sites and spawning resources. It remains unknown whether similar ecological impacts are already affecting Belgian freshwater ecosystems. Beyond ecological consequences, the presence of the black bullhead can also affect sport fish populations, potentially leading to economic losses. In Belgium and the Netherlands, the brown bullhead has been documented as most successful and dominant in lentic systems. However, in recent years, this species has shown a remarkable overall stagnation in frequency and local abundance, with a significant decline observed in lotic systems (Verreycken et al. 2007). With the first discovery of the black bullhead in Belgium, the highest population abundance was found in the lake rather than in the stream. Since the black bullhead is likely in the early stage of invasion in Belgium, its ecological impact is currently expected to be low (Haubrock et al. 2022). Nevertheless, the high density of lakes, streams, and ditches in this region makes this finding particularly concerning. Complete eradication in this region is still feasible, highlighting the urgency of implementing effective management measures (Mack et al. 2000).

Authors' contribution

RB – research conceptualization; RB, DH, ED, HV – sample design and methodology; RB, DV, BDB, SN, ED, HV – investigation and data collection; RB, DH – data analysis and interpretation; RB – writing – original draft; RB, DH, BDB, SN, CVD, TE, ED, HV – writing – review and editing.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Summary of the eight reference samples used as benchmarks in the diagnostic PCR for genetic identification of the 22 captured *Ameiurus* specimens in this study.

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