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The invasive marsh frogs advancing into The Netherlands carry a diverse mix of mtDNA

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Abstract. The marsh frog (*Pelophylax ridibundus* complex) has been dubbed the number one amphibian invader in Western Europe. In The Netherlands, marsh frogs are native to the north, centre and west of the country, whereas pool frogs (*P. lessonae*) are mainly found in the south and east; their hybridogenetic hybrid, the edible frog (*P.* klepton *esculentus*), is distributed throughout The Netherlands. Over the past decades, the southeastern province of Limburg has been overrun by marsh frogs. To expose the invasion history of marsh frogs in The Netherlands, we mtDNA barcoded hundreds of water frogs from across the country. Throughout The Netherlands, we primarily detect pool frog mtDNA: the only mtDNA type that is expected to occur here in any native water frog, regardless of taxon, due to asymmetric introgression. By contrast, we identify four distinct marsh frog mtDNA haplotypes in Limburg, originating from four regions of the native marsh frog range: (1) Central, Eastern and Southeastern Europe, (2) western Balkan Peninsula, (3) western and northern Turkey, Iran and eastern Ukraine into Russia, up to the Ural Mountains and (4) central southern Turkey. Additionally, two presumably introduced Central, Eastern and Southeastern European marsh frog haplotypes are detected locally elsewhere in The Netherlands, within the native marsh frog range. Our results align with the broader pattern that invasive marsh frogs constitute a 'cocktail' of genetically diverse lineages. As the invasion front in Limburg approaches native water frog populations, we urge that conservation measures be taken promptly.

Keywords: citizen science, invasive species, mtDNA barcoding, *Pelophylax*, swabbing.

Introduction

Invasive species constitute one of the main threats to amphibians worldwide (Luedtke et al., 2023). Many amphibians also act as invasive species themselves (Kraus, 2015). The marsh frog (*Pelophylax ridibundus* complex sensu Denoël and Dufresnes, 2025; Dufresnes et al.,

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2024) is considered one of the most important amphibian invaders (Dufresnes et al., 2018; Dufresnes et al., 2024; Denoël and Dufresnes, 2025). Introduced marsh frogs prey on, compete with, and spread disease to native species and also hybridize with native *Pelophylax* taxa (Pille et al., 2021; Jakóbik et al., 2024; Denoël and Dufresnes, 2025). Most introductions occurred in the second half of the 20th century. The frog leg industry has been identified as the primary introduction pathway, but other drivers of introduction are e.g., the use of frogs as ornamental species in garden ponds (reviewed by Denoël and Dufresnes, 2025). The *P. ridibundus* complex comprises 17 mtDNA phylogeographical lineages, some with and some without taxonomic names, and some of these lineages have previously been treated as distinct species (Dufresnes et al., 2024). Nine *P. ridibundus* complex mtDNA phylogeographical lineages, originating from three different continents, are involved in hundreds of individual introduction events throughout Europe.

Within The Netherlands, the marsh frog is considered native to the northern, central and western parts of the country (Blommers-Schlösser, 1990; Blommers-Schlösser, 1992; Creemers and van Delft, 2009). Marsh frogs favour relatively nutrient-rich habitats like river deltas, reclaimed land and peat meadows. Two other water frog taxa also occur natively in The Netherlands. In the southern and eastern parts, the pool frog (*P. lessonae*) is found in nutrient poor habitats, mainly on Pleistocene sandy soils. The hybridogenetic hybrid between the marsh and the pool frog, the edible frog (*P.* klepton *esculentus*), is distributed throughout The Netherlands and is a habitat generalist.

In the western part of the native range, including The Netherlands, marsh frogs carry pool frog mtDNA (Dubey et al., 2019; Dufresnes et al., 2024; Meilink et al., 2024; van Veldhuijzen et al., 2025). This cytonuclear discordance is explained by the local hybridogenetic system in which, every generation, edible frogs typically backcross with pool frogs, while also excluding the genome of that species. The repeated crossing with pool frogs rather than marsh frogs makes it likely that all offspring eventually inherit pool frog mtDNA, while marsh frog mtDNA is lost from the population. If female edible frogs carrying pool frog mtDNA mate with male edible or male marsh frogs, this results in marsh frog offspring possessing pool frog mtDNA (Plötner et al., 2008).

Marsh frogs are assumed to have been historically absent from the south of the province of Limburg (Blommers-Schlösser, 1990; Blommers-Schlösser, 1992). They were first reported in this region, right on the border with Belgium, in the period 1980-1990 (Wijnands, 1992), and subsequently spread (fig. 1). In 2009–2010, water frogs from the southern part of Limburg were mtDNA barcoded (Felix et al., 2012). In addition to the presumed native pool frog mtDNA, four introduced haplotypes were reported: three marsh frog haplotypes from the Balkan Peninsula and Central Europe and one pool frog haplotype from Italy (belonging to the Italian pool frog, *P. l. bergeri*). Since then, marsh frogs have rapidly expanded their range northwards (fig. 1). Furthermore, elsewhere in The Netherlands introductions have been reported that were either unsuccessful or whose outcomes remain unclear (e.g., on the Wadden Sea isles and near the city of Rotterdam; Creemers and van Delft, 2009). The high frequency of known marsh frog introductions suggests that more introductions may have gone unnoticed.

The marsh frog case raises several conservation-related questions. How far have marsh frogs that invade The Netherlands from the south expanded their range? Is there evidence for further marsh frog introductions in The Netherlands? Which mtDNA phylogeographic lineages are involved in marsh frog introductions in The Netherlands? To address these questions, we mtDNA barcode water frogs from over 200 localities throughout The Netherlands. Any marsh frog mtDNA detected would

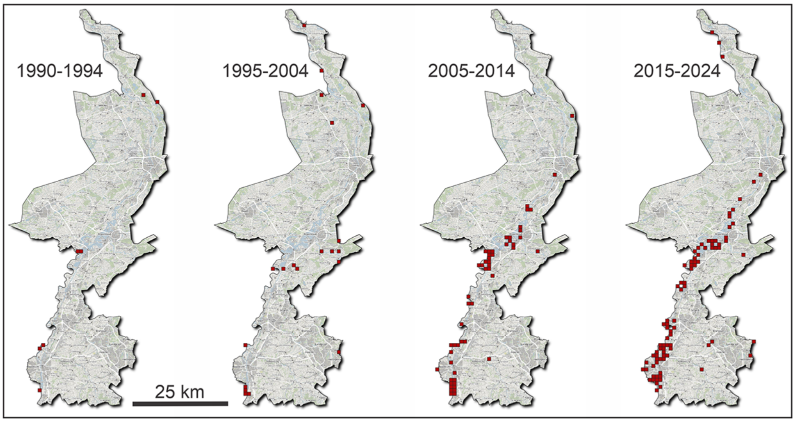

Figure 1. A time-structured overview of marsh frogs (*Pelophylax ridibundus* complex) invading the province of Limburg in the southeast of The Netherlands. Note that the oldest period spans five instead of ten years because records do not extend further back. Occurrence data are taken from the Nationale Databank Flora en Fauna (NDFF). See fig. 2 for geographic context.

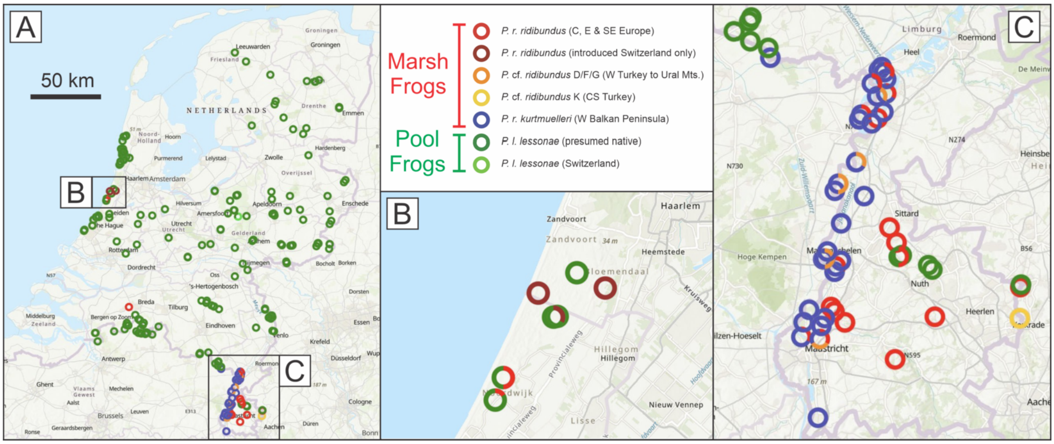

Figure 2. Distribution map of mtDNA phylogeographical lineages of water frogs (*Pelophylax*) in The Netherlands based on 530 bp of cyt-b. Two areas in panel A are shown in detail as panels B and C. Panel B shows the boundary of the provinces of Zuid-Holland and Noord-Holland. Panel C shows the southern half of the province of Limburg.

point towards an introduction event and a comparison with the natural distribution of marsh frog mtDNA would shed light on the geographical origin of these introductions.

Materials and methods

Sampling, DNA extraction, PCR and sequencing

We studied 548 individuals from 218 localities (fig. 2, supplementary table S1). Samples consisted of 286 buccal swabs, 253 skin swabs and 9 tissue samples (taken from deceased individuals) of which many were collected by citizen scientists. We used Copan 155C Rayon swabs. DNA was extracted using the Wizard® Genomic DNA purification kit (Promega). We amplified a 530 bp fragment of the cyt-b (cytochrome b) mtDNA gene, because this mtDNA gene has been sampled most comprehensively across the Western Palearctic (Dufresnes et al., 2024). We used the primers Cytb-F2 (5′-TTAGTAATAGCCACAGCTTTTGTAGGC-3′) and Cytb-R2 (5′-AGGGAACGAAGTTTGGAGGTGTGG-3′) from Dubey et al. (2019). PCRs were performed in 12 μl reactions, containing 0.06 μl of both forward and reverse primer (0.05 μM end concentration of each primer), 6 μl QIAGEN multiplex PCR master mix, 4.88 μl purified water and 1 μl of DNA extract. PCR conditions were: a hot start for 15 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 1 minute at 55°C and extension for 1 minute at 72°C, and extension at 72°C for ten minutes. Sanger sequencing was outsourced to Macrogen and sequences were edited and trimmed in Geneious Prime 2025.0.2 (https://www.geneious.com).

Reference database of mtDNA barcodes

We compiled a database of 75 cyt-b haplotypes of 530 bp in length, found across 1003 *Pelophylax* individuals from the Western Palearctic. This database provides a comprehensive overview of genetic variation across the region and allows us to trace introduced populations back to their origin within the natural distribution range. We based our database on a longer, rather gappy, 1143 bp alignment of 261 cyt-b haplotypes found across 1598 Western Palearctic *Pelophylax* individuals presented in Dufresnes et al. (2024). We trimmed off the part of the alignment extending beyond our targeted 530 bp cyt-b segment and removed any sequence that did not fully cover the targeted segment. We follow the haplotype coding system from Dufresnes et al. (2024), but, because we focus on a shorter section, some haplotypes have been collapsed (since they were identical for the targeted segment). Details are in supplementary tables S2 and S3.

Genetic analyses

We used the ‘DNA to haplotype collapser and converter’ function in FaBox (Villesen, 2007) to determine the corresponding haplotypes (if any) of the newly sequenced individuals in our database. To determine the phylogenetic position of newly identified haplotypes, we conducted maximum likelihood phylogenetic inference using the IQ-TREE webserver (Trifinopoulos et al., 2016). We used *Amolops ricketti* as an outgroup (GenBank Accession KJ008461; taken from Lu et al., 2014). We used ModelFinder (Kalyaanamoorthy et al., 2017) to determine the most appropriate partitioning scheme and model of sequence evolution (HKY+I for codon 1 and codon 2, and TIM3+F+G4 for codon 3). We used 1000 ultrafast bootstrap replicates (Hoang et al., 2017) to determine branch support.

Results

We identify 19 cyt-b haplotypes in 548 water frogs (218 localities) from The Netherlands (fig. 2; supplementary table S1). Five of these haplotypes could be matched to the Western Palearctic *Pelophylax* database (supplementary table S2). The remaining 14 haplotypes (with haplotype codes COB.920-COB.933 and GenBank accession Nos PV862272–PV862285) are newly identified and are allocated to a taxon/mtDNA lineage based on phylogenetic analysis (fig. 3). Of the 19 haplotypes found in The Netherlands, five belong to marsh frogs and 14 to pool frogs.

In the province of Limburg we find four marsh frog haplotypes, often in the same locality, although their frequencies vary (fig. 2C; supplementary table S1):

- Haplotype COB.007/008/012/014/015/016/018, belonging to *P. r. ridibundus*, has previously been reported from Bulgaria, Poland, and (western) Russia, as well as outside of the native marsh frog range in France and Switzerland (supplementary table S2).
- Haplotype COB.036/037/038, belonging to *P. r. kurtmuelleri*, has previously been reported from Albania and Greece, as well as outside of the native marsh frog range in France (supplementary table S2).
- Haplotype COB.932 is a newly identified haplotype that clusters with *P. r.* cf. *ridibundus* lineage K from central southern Turkey (haplotypes COB.056 and COB.057 in fig. 3; supplementary table S2).
- Haplotype COB.933 is a newly identified haplotype that clusters with the closely related *P. r.* cf. *ridibundus* lineages D, F and G, distributed across western and northern Turkey, Iran and eastern Ukraine into Russia, up to the Ural Mountains (haplotypes COB.114, COB.115 and COB.125 in fig. 3; supplementary table S2). Because our targeted 530 bp cyt-b segment does not fully overlap with many of the haplotypes for *P. r.* cf. *ridibundus* lineages D, F and G presented in Dufresnes et al. (2024), we also focused on a shorter, 249 bp cyt-b segment. For this segment, our Dutch haplotype is identical to multiple haplotypes from throughout the range of the *P. r.* cf. *ridibundus* lineages D, F and G, preventing us from pinpointing a more precise origin (haplotypes COB.107-108 and COB.110-113 in supplementary table S2).

In the provinces of Noord-Brabant and Zuid-Holland we find a single marsh frog haplotype,

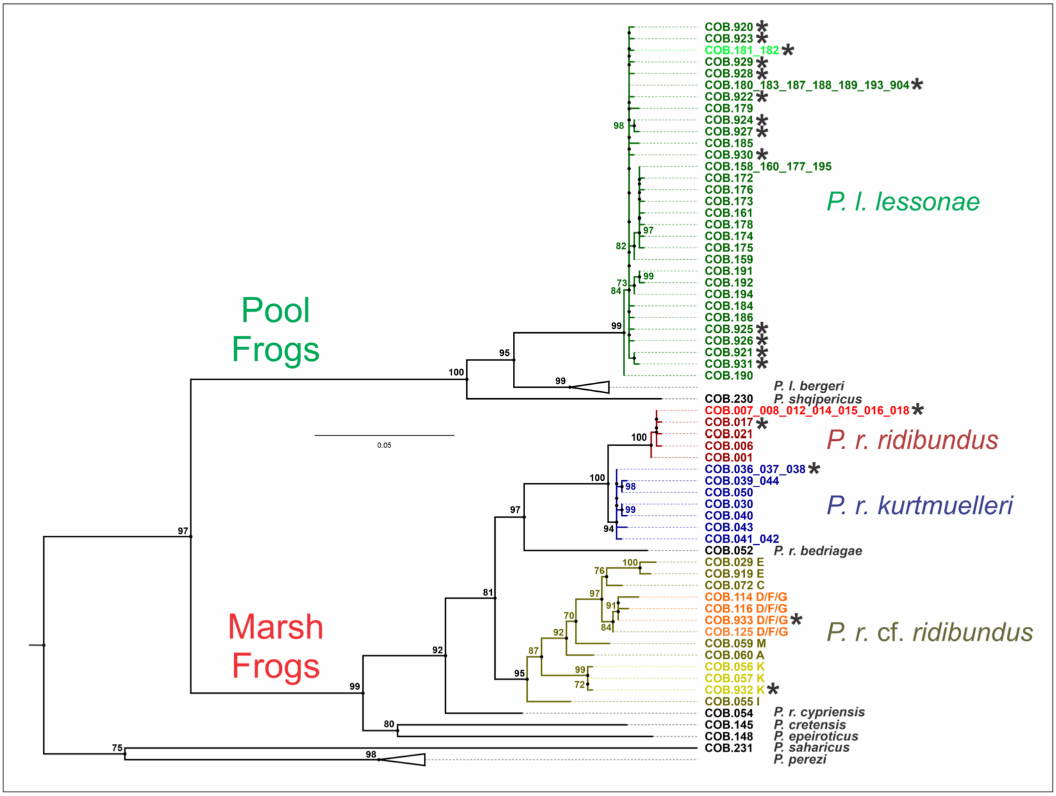

Figure 3. Maximum likelihood phylogeny of mtDNA phylogeographical lineages of Western Palearctic water frogs (*Pelophylax*) based on 530 bp of cyt-b. The outgroup is not shown. Only ultrafast bootstrap support values ⩾70 are displayed. Haplotypes found in The Netherlands are denoted with an asterisk (*). Haplotype details can be found in supplementary table S2.

COB.007/008/012/014.015/016/018, belonging to *P. r. ridibundus* (figs. 2A and 2B; supplementary table S1), which is also present in Limburg (see details above). In the province of Noord-Holland we find another marsh frog haplotype, COB.017, belonging to *P. r. ridibundus* (fig. 2B; supplementary table S1), which has previously been reported from Switzerland, i.e., outside the native marsh frog range (supplementary table S2).

Throughout The Netherlands we find 14 pool frog haplotypes (fig. 2A; supplementary table S1):

- Haplotype COB. 180/183/187/188/189/193/903, belonging to *P. l. lessonae*, has previously been reported from Austria, Croatia, the Czech Republic, Italy, Finland, France, Germany, Norway, Poland, Russia, Slovakia, Sweden, Switzerland, and Ukraine (supplementary table S2).
- Haplotype COB.181/182, belonging to *P. l. lessonae*, has previously been reported from Switzerland (supplementary table S2).
- Haplotypes COB.920-931 are newly identified haplotypes that cluster with *P. l. lessonae* (fig. 3).

Discussion

We present a dense phylogeographic survey of *Pelophylax* mtDNA for The Netherlands. Our results fit a general pattern of only pool frog mtDNA being present natively in The Netherlands, including in the parts of the country where pool frog should be absent (fig. 2). Because native marsh frogs in The Netherlands are predicted to carry pool frog mtDNA (van Veldhuijzen et al., 2025), any marsh frog mtDNA detected should immediately raise a red flag: marsh frog mtDNA in The Netherlands is a strong indication of introduction. While we detect pool frog mtDNA in all the Dutch provinces screened, marsh frog mtDNA is also detected in four of these: Limburg, Noord-Brabant, Zuid-Holland and Noord-Holland (fig. 2).

The four distinct marsh frog mtDNA haplotypes we identify in Limburg originate from four regions of the native marsh frog range: (1) Central, Eastern and Southeastern Europe (*P. r. ridibundus*), (2) western Balkan Peninsula (*P. r. kurtmuelleri*), (3) Central Southern Turkey (*P. r.* cf. *ridibundus* lineage K) and (4) Western and Northern Turkey, Iran and eastern Ukraine into Russia, up to the Ural Mountains (*P. r.* cf. *ridibundus* lineage D/F/G). Some of these regions correspond to known hotspots of frog harvest that are subsequently imported to Western Europe (e.g., central southern Turkey, Dufresnes et al., 2024). Since an earlier study conducted in 2009–2010 (Felix et al., 2012), we find that marsh frog mtDNA has expanded considerably further northwards. Furthermore, we show that two additional mtDNA phylogeographical lineages are involved, both from different corners of the marsh frog range (*P. r.* cf. *ridibundus* lineage K and *P. r.* cf. *ridibundus* lineage D/F/G). We do not detect Italian pool frog mtDNA (*P. l. bergeri*), which was previously reported from southernmost Limburg (Felix et al., 2012), as well as adjacent Belgium (Dufresnes et al., 2024), suggesting this mtDNA type has not expanded further into The Netherlands. We recommend further sampling in southernmost Limburg to determine if this mtDNA type is still extant in The Netherlands.

Two Central, Eastern and Southeastern European marsh frog haplotypes are detected elsewhere in The Netherlands. One haplotype, which is also present in Limburg, is found in the provinces Noord-Brabant and Zuid-Holland. The other haplotype is detected in Noord-Holland (about 5 kilometres north of the Zuid-Holland sites) and has only been reported before from the invasive marsh frog population in Switzerland. These locally restricted and low-frequency haplotypes likely signify unsuspected introductions that occurred ‘under the radar’. While over fifty years ago, when Dutch *Pelophylax* populations were at an all-time low due to environmental deterioration, large numbers of Bulgarian marsh frogs were introduced near the city of Rotterdam (Creemers and van Delft, 2009), we do not detect foreign haplotypes elsewhere in the province of Zuid-Holland (fig. 2). Potentially this introduced population has gone extinct, suggesting that, while marsh frogs introductions evidently can be highly successful (Denoël and Dufresnes, 2025) – at least from the frogs’ point of view – this is not a guaranteed outcome.

We observe that multiple marsh frog mtDNA haplotypes regularly co-occur in the same locality, a pattern that appears to be a feature of many marsh frog invasions (Denoël and Dufresnes, 2025). Mixed genetic ancestry has also been observed in other introduced taxa such as banded newts (*Ommatotriton*), where admixture was not evident from mtDNA, but was confirmed with nuclear DNA markers (van Riemsdijk et al., 2018). While the ‘cocktail’ of marsh frog mtDNA could potentially reflect repeated introductions from multiple sources, a more parsimonious explanation is that such introductions originate from ‘hybrid swarms’ of marsh frogs from multiple genetic lineages that were mixed in captivity (Denoël and Dufresnes, 2025).

The marsh frog mtDNA found in the west of The Netherlands, in Zuid-Holland and Noord-Holland, must derive from highly localized introductions. However, this is not necessarily the case in Limburg. Invasive marsh frogs are omnipresent in neighbouring Belgium and have similar geographical origins (Percsy and Percsy, 2009; Denoël and Dufresnes, 2025), meaning that the Belgian marsh frog population may have simply expanded its range into The Netherlands. The fact that the first marsh frogs in the south of Limburg were reported from right on the border with Belgium (Wijnands, 1992) supports this scenario – although there are also anecdotal reports of additional direct introductions by people (T. Starq, pers. comm.). Similarly, Noord-Brabant borders Belgium and, while we only find marsh frog mtDNA in a single locality, denser sampling is required to determine how widespread marsh frog mtDNA truly is in this area.

We identify several new pool frog mtDNA haplotypes. Due to the lack of geographical mtDNA structure in *P. l. lessonae*, these do not cluster with haplotypes that are unambiguously foreign, so there is no evidence suggesting that these new pool frog mtDNA haplotypes derive from an introduction. However, we do find one pool frog haplotype that was previously only reported from the native pool frog range in Switzerland, which might indicate an introduction – or alternatively an underestimated range or independent evolution of the haplotype (Elfering et al., 2024). Furthermore, genetically confirmed pool frogs (carrying an mtDNA haplotype also found natively in The Netherlands) have recently been reported in the Dutch coastal dunes in the province Zuid-Holland (van Veldhuijzen et al., 2025), outside of the assumed natural distribution range in The Netherlands (Creemers and van Delft, 2009). While this could reflect an underestimation of the native pool frog range, an introduction would not be farfetched, given that many amphibians have been introduced into the Dutch coastal dunes – including pool frogs in the province Noord-Holland (Herder et al., 2010; de Brouwer et al., 2023; Koster et al., 2023; Kuijt et al., 2023; Robbemont et al., 2023; Vliegenthart et al., 2023). A closer look at the introduction history of pool frogs, with nuclear DNA, seems warranted.

Our results highlight the necessity of genetic screening in exposing water frog introductions. MtDNA barcoding provides valuable first insights into the distribution of invasive marsh frogs. Introduced marsh frogs negatively impact local biodiversity, with the most severe invasion in Limburg raising the particular concern of predation on threatened amphibian species such as yellow-bellied (*Bombina variegata*) and common midwife (*Alytes obstetricans*) toads (Felix et al., 2012; Denoël and Dufresnes, 2025). The colonization of these critical sites, e.g., quarries, by *Pelophylax* may have (unintentionally) been facilitated by the creation and toleration of permanent (i.e., non-pioneer) breeding waters in which the invaders can reproduce. We urge that conservation measures be taken promptly.

Because the invasion front in Limburg approaches native water frog populations, hybridization between native and invasive water frogs will take place, and subsequent genetic pollution – gene flow from the invasive to the native species – can be expected (Theodoropoulos et al., 2025). Native and invasive marsh frogs can be expected to admix freely. While the hybridogenetic reproduction mode should limit gene flow towards the pool frog, this genetic barrier may not be absolute (Mezhzherin et al., 2024; van Veldhuijzen et al., 2025). Furthermore, novel marsh frog lineages may upset the interaction with pool frogs as not all combinations undergo hybridogenesis in the same manner (Hotz et al., 1985). We recommend a study consulting genome-wide data to untangle the intricacies of the Dutch marsh frog invasion. The target sequence capture protocol FrogCap (Hutter et al., 2022) would be suitable to obtain such data (van Veldhuijzen et al., 2025).

Supplementary materials. Data is available on https://doi.org/10.1163/15685381-bja10239 under Supplementary Materials.

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