The introduction of three cryptic tree frog species in the Dutch coastal dunes challenges conservation paradigms

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Abstract. Invasive species are considered one of the main drivers of the sixth mass extinction. Conservation solutions depend on whether a species is also indigenous to the country it invades (i.e., beyond its native range). In the case of invasive cryptic species, genetic tools are required to establish their identity. We illustrate these issues with the human-mediated colonization of the Dutch coastal dunes by *Hyla* tree frogs. Although previously assumed to concern the indigenous common tree frog *H. arborea*, European tree frogs comprise a complex of allopatric cryptic species, meaning the taxonomic identity of introduced Dutch populations warrants investigation. We sequence mtDNA for 173 individuals from native and introduced populations across the Netherlands and compare our dataset with hundreds of *Hyla* haplotypes previously barcoded in the Western Palearctic. Two of the dune populations carry an mtDNA haplotype of the native species *H. arborea* that occurs naturally elsewhere in the Netherlands. In contrast, mtDNA assigned to the eastern tree frog *H. orientalis* was detected in all three other dune populations. In one of these populations mtDNA of the Italian tree frog *H. intermedia* was also found. Not one, but three species of tree frogs have thus been introduced to the Dutch coastal dunes, only one of them being native to the Netherlands. This situation causes a conservation conundrum as some introduced populations are lawfully protected but could pose a threat to local biodiversity. Regarding the 'true' exotic tree frog species, all conservation options should be considered.

Keywords: alien species, cryptic species, DNA barcoding, exotic species, *Hyla*, invasion genetics, phylogeography.

Introduction

One of the main drivers of extinction is the homogenization of the world's biodiversity by invasive species (Bellard et al., 2016; Diagne et al., 2021). Invasive species can corrode natural communities, e.g., through predation, competition, disease transmission and hybridization (Simberloff, 2013; Pyšek et al., 2020). The legal status of an introduced population determines what kind of conservation action could be taken. In Europe, this legal status is typically influenced by both the national legislation (e.g., Wet Natuurbescherming in the Netherlands) and the legislation imposed by the European Union (EU Habitat Directive), and whether the introduced species is also indigenous to the country where it is found (Simberloff, 2013).

Determining the species identity of introduced populations is not always straightforward, especially when dealing with cryptic species complexes (Bickford et al., 2007; Pfenninger and Schwenk, 2007), in which case, genetic tools are required. In most instances, DNA barcoding is a cheap and efficient way to obtain the required information (Hebert et al., 2003; Mir et al., 2021). Cryptic species complexes have typically been identified through phylogeographic surveys (Avise, 2000), so matching sequences obtained from introduced populations against published phylogeographic datasets can reveal the species involved (and whether it is an indigenous species or not) as well as its putative geographic origin (which might well be outside of the country concerned).

Western Palearctic tree frogs of the genus *Hyla* provide a case in point. Continental Europe is inhabited by four closely related species that lack any diagnostic criteria for field identification, both morphologically and acoustically (Speybroeck et al., 2016; Dufresnes et al., 2020): the common tree frog *H. arborea*, the Italian tree frog *H. intermedia*, the eastern tree frog *H. orientalis*, and the Iberian tree frog *H. molleri*. The latter two were elevated to species level by the Taxonomic Committee of the Societas Europaea Herpetologica as recently as 2020 (Speybroeck et al., 2020), following phylogenomic and hybrid zone analyses (Dufresnes et al., 2015a; Dufresnes et al., 2018; Dufresnes et al., 2020).

Importantly, tree frogs are known to travel easily with humans. Some species are frequently transported from south-western to north-western Europe with the horticultural and agricultural trade (Dufresnes and Alard, 2020), and established populations can become a threat to local taxa. In Western Switzerland, introduced *H. intermedia* (from the subspecies *H. i. perrini*) have replaced a once large population of the autochthonous *H. arborea* through introgressive hybridization, despite tremendous conservation efforts (Dufresnes et al., 2015b).

The natural distribution range of the common tree frog *H. arborea* extends to the southern and eastern parts of the Netherlands (Creemers and van Delft, 2009). The species is considered 'threatened' at the national level (van Delft et al., 2007). Tree frogs have also been introduced in five distinct regions of the coastal dunes in the west of the country, which can be considered separate populations, 50-100 kilometres away from the closest native populations (fig. 1). These introduced populations are separated from the native range, and from each other, by unsuitable habitat (often urban area).

The earliest of these introductions was in the late 1980s at Kop van Schouwen (Musters, 2000; van der Molen, 2001). Since 2000, tree frogs have been recorded from Meijendel (Oppentocht, 2002; Zuiderwijk, 2004); this population has expanded exponentially and since 2005 also covers the directly adjacent areas Berkheide and Lentevreugd (Teunissen, 2007). From 2003 onwards, tree frogs have been present in the Amsterdamse Waterleidingduinen (van Deursen, 2010; Lenders and van Delft, 2011). Starting 2017, tree frogs have been reported from Westduinpark, and since 2019 from Solleveld (National Database Flora & Fauna, NDFF). All these introductions were illegal and therefore little to no information is available on the introduction history.

Given the introduced nature of the tree frog populations in the Dutch coastal dunes it is unclear whether they belong to the indigenous *H. arborea* (and, if so, if they originate from the Netherlands or beyond), or whether, perhaps, any of the aforementioned cryptic tree frog species are involved. We conduct mtDNA barcoding to determine which tree frog species inhabit the Dutch coastal dunes, and to get an insight into their putative origins, in the light of the phylogeographic resources available for the genus in Europe (Dufresnes et al., 2020). Our findings call to revisit the management of these introduced populations, in respect to their unexpected diversity and taxonomic identity.
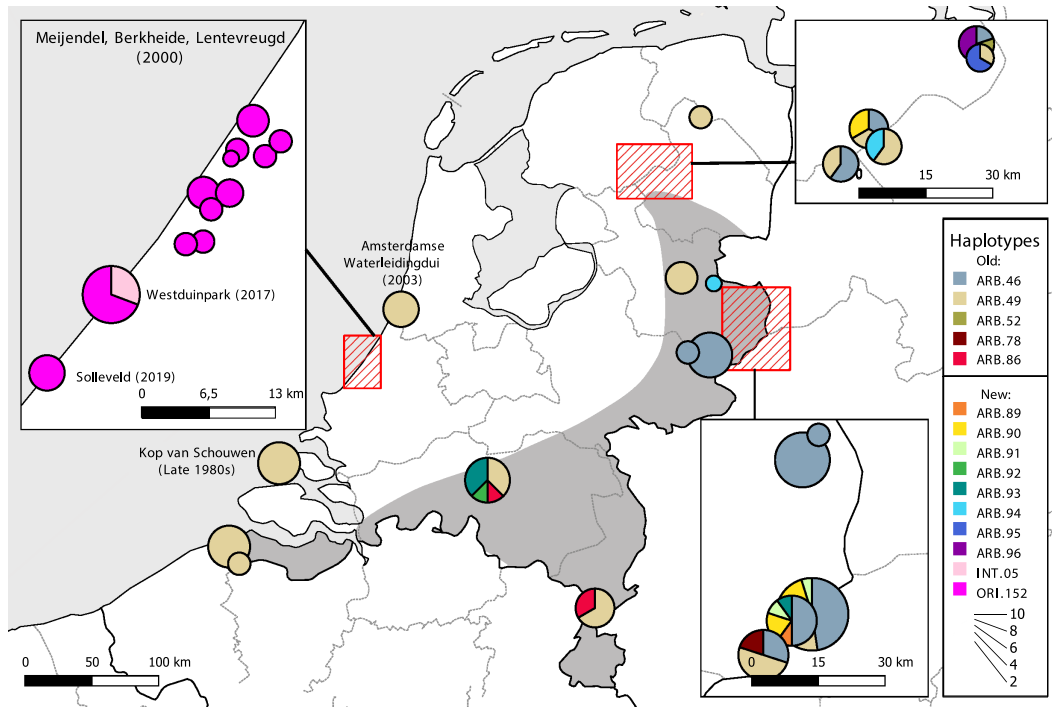
Figure 1. Map of the Netherlands showing sampled localities for *Hyla* tree frogs. A rough outline of the natural tree frog distribution range is shaded grey. Pies are sampled localities. Pie slices are coloured according to haplotype and pie sizes reflect sample sizes. Previously identified haplotypes are labelled 'old' and those newly identified in this study 'new'. The five populations introduced in the coastal dunes are labelled with the (approximate) date of appearance. Sampling details are in supplementary table S1.

Materials and methods

Sampling, DNA extraction and PCR

We sampled 173 Dutch *Hyla* individuals (fig. 1, supplementary table S1). For part of these individuals (n = 110), DNA extractions were available from a different study (Gilbert et al., 2021). For the remainder (n = 63), we took buccal swabs using 4N6FLOQSwabs (Copan) and stored these at −20°C in 96% ethanol until further use. DNA was extracted using the Wizard® Genomic DNA purification kit (Promega). Samples included 24 individuals from 10 localities in the continuous Meijendel, Berkheide and Lentevreugd area. We included 4 other introduced dune populations: 5 individuals from the Amsterdamse Waterleidingduinen, 13 from Westduinpark, 5 from Solleveld, and 7 individuals from Kop van Schouwen. Additionally, we included 119 individuals from 19 localities elsewhere in the Netherlands.

We amplified a 811 bp fragment of the *cytochrome-b* gene using the primer pair *Hyla*-L0-*Hyla*-H1046 (Stöck et al., 2012). PCRs were performed in 12 microliter reactions containing 0.06 μl of both forward and reverse primer (0.05 μM end concentration of each primer), 7.2 μl QIA-GEN multiplex PCR master mix, 3.68 μl purified water and 1 μl of DNA extract. PCR conditions were: a hot start of 15 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 48°C for 1 minute and

extension at 72°C for 1 minute, and a final extension at 60°C for 30 minutes. Sanger Sequencing was performed commercially by BaseClear B.V. Sequences were edited in Geneious Prime 2021.1.1 (https://www.geneious.com).

Genetic analyses

We built a database of *cytochrome-b* haplotypes of the 10 Western Palearctic *Hyla* taxa from the sequences published by the studies reviewed in Dufresnes et al. (2020). This alignment spanned 811bp but it should be noted that most of the *H. intermedia* haplotypes generated by Canestrelli et al. (2007) were shorter (606 bp). Sequences for the newly sequenced individuals from the Netherlands were aligned to this database. The Haplotype Collapser function in FaBox (Villesen, 2007) was used to check to which previously identified haplotypes (if any) each of the 173 Dutch *Hyla* individuals belonged. New haplotypes were identified and added to the database, which is available in supplementary table S2 (447 haplotypes, including ten newly identified in the present study).

Bayesian phylogenetic inference was conducted with MrBayes 3.2.7 (Ronquist et al., 2012). The appropriate models of sequence evolution for each codon position (supplementary table S3) were determined with jModelTest 2 (Darriba et al., 2012). Because we discovered several new haplotypes, we first determined to which *Hyla* species

these belong. This analysis included a 'backbone' dataset, including a single haplotype for each of the species in our database (*H. arborea*, *H. carthaginiensis*, *H. meridionalis*, *H. felixarabica*, *H. orientalis*, *H. savignyi*, *H. sarda*, *H. intermedia* [including *H. (intermedia) perrini*] and *H. molleri*), as well as the newly identified haplotypes. Second, we conducted intraspecific analyses that included all haplotypes of the identified species, to pinpoint the geographic origin of the Dutch haplotypes. We ran two, four-chain, two-million-generation runs, with a sampling frequency of 0.001 and a heating parameter of 0.2 in MrBayes, using a 25% burnin. We confirmed that runs converged and effective sample sizes exceeded 200 in Tracer 1.7 (Rambaut et al., 2018). A haplotype network was made for *H. arborea* (the species for which multiple haplotypes were found in the Netherlands) with TCS 1.21 (Clement et al., 2000) and tcsBU (Múrias dos Santos et al., 2015).

Results

Fifteen haplotypes were identified among the 173 samples analysed from the Netherlands (supplementary table S1). Five of these corresponded to known *Hyla arborea* haplotypes. Haplotype ARB.49 is widely distributed across Europe, ranging from the Netherlands to France and Greece (supplementary table S2). Haplotype ARB.46 is widely distributed in the east of the Netherlands, but has never been recorded elsewhere. Haplotype ARB.86 was detected in two localities from the south of the Netherlands, and was previously reported in nearby Belgium. Haplotype ARB.78, found in one location (Witte Veen; fig. 1), was previously identified in Croatia, France and Romania. Haplotype ARB.52, also found in a single location (Fochteloërveen; fig. 1), was previously identified in Austria. Eight newly identified haplotypes also belong to *H. arborea* (fig. 2; ARB.89-ARB.96), each with local distributions (fig. 1).

The two remaining newly identified haplotypes do not belong to *H. arborea*. One belongs to *H. orientalis* (fig. 2; ORI.152). This haplotype was fixed in our samples from Meijendel, Berkheide and Lentevreugd, and Solleveld, and also segregated in Westduinpark, in nine out of 13 individuals sampled. The other haplotype belongs to *H. intermedia* (fig. 2; INT.05). It was found in the remaining four individuals sampled in Westduinpark.

A phylogeny of the *H. arborea* haplotypes confirmed shallow genetic structuring in this species (see also the haplotype network in supplementary fig. S1), and little geographic associations between the new and previously identified *H. arborea* haplotypes (fig. 3). In contrast, *H. orientalis* consists of distinct clades and our new ORI.152 haplotype is closely and robustly related to haplotype ORI.19, which corresponds to a diverged *H. orientalis* mitochondrial lineage endemic to the Greek island of Lesbos, off shore the Turkish coast in the north-eastern Aegean Sea (fig. 4). Finally, our *H. intermedia* haplotype (INT.05) is identical to the shorter (606 bp, fully overlapping with our 811 bp) haplotype hc1 from Canestrelli et al. (2007), which is widely distributed in central Italy, from Calabria to the Northern Apennines.

Discussion

Two out of five tree frog populations introduced in the Dutch coastal dunes (Kop van Schouwen and Amsterdamse Waterleidingduinen; fig. 1) consist of the indigenous species *H. arborea*. The lack of phylogeographic structure in *H. arborea*, at least with mitochondrial markers (Dufresnes et al., 2013), as well as the wide distribution of some haplotypes, hampers our ability to pinpoint from which part of the natural distribution the introduced dune populations originate. The haplotype sampled in both populations (ARB.49) is widespread from Greece to France, and also occurs naturally in the Netherlands (Dufresnes et al., 2013).

For Kop van Schouwen, anecdotal evidence suggests an introduction from Croatia (van der Molen, 2001); no such information is available for Amsterdamse Waterleidingduinen. Therefore, our mtDNA data do not conflict with, but also do not confirm a Dutch provenance for these two introduced populations. A study using genome-wide nuclear DNA (RAD-seq) on a subset of the Dutch populations studied here suggests that the Kop van Schouwen population is genetically diverged from autochthonous *H.*
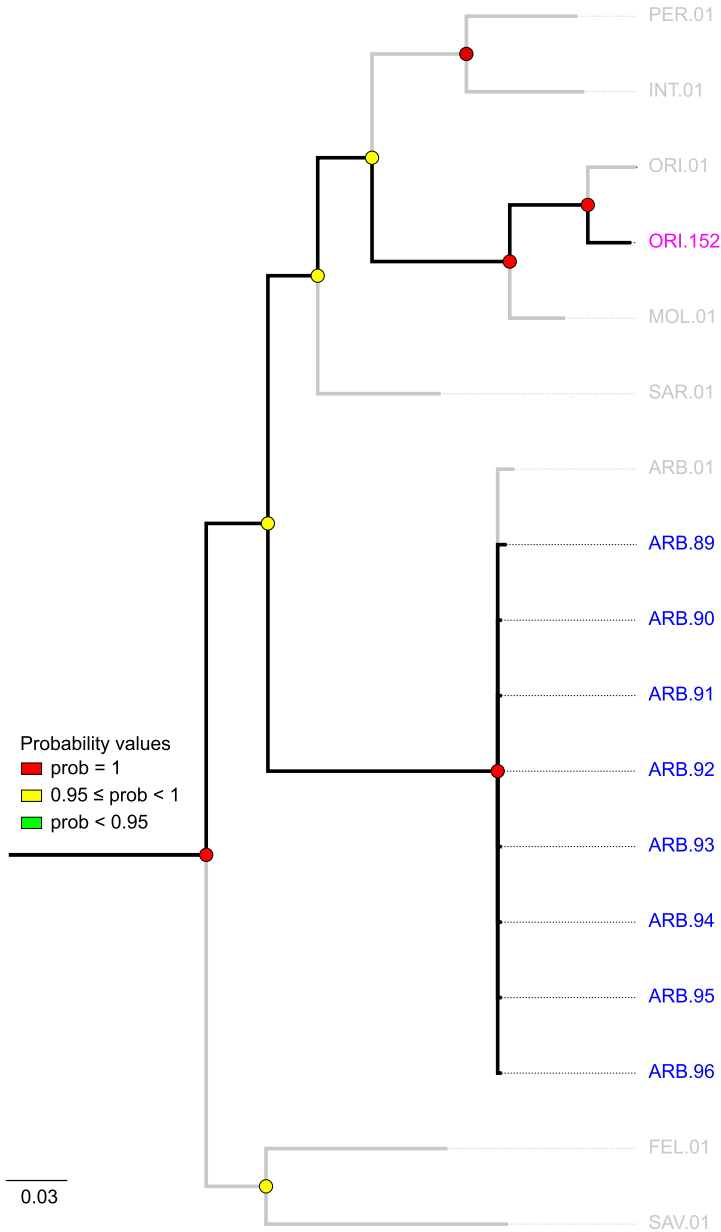
Figure 2. Majority rule consensus tree resulting from Bayesian inference to allocate new *Hyla* haplotypes to species. Grey branches indicate the backbone phylogeny with representatives for each Western Palearctic *Hyla* species; the relatively distinct *H. meridionalis* and *H. carthaginiensis* were used as outgroup and are not shown. New haplotypes are coloured blue (*H. arborea*) or pink (*H. orientalis*). Haplotype labels correspond to supplementary table S1.

arborea populations, in line with a foreign origin (Gilbert et al., 2021). In contrast, the Amsterdamse Waterleidingduinen population is similar to autochthonous *H. arborea* populations, which is compatible with a Dutch origin.

The remaining three Dutch coastal dune populations (Solleveld, Westduinpark, and Meijendel/Berkheide/Lentevreugd; fig. 1) consist of the eastern tree frog *H. orientalis*, in particular a distinct mtDNA clade that is
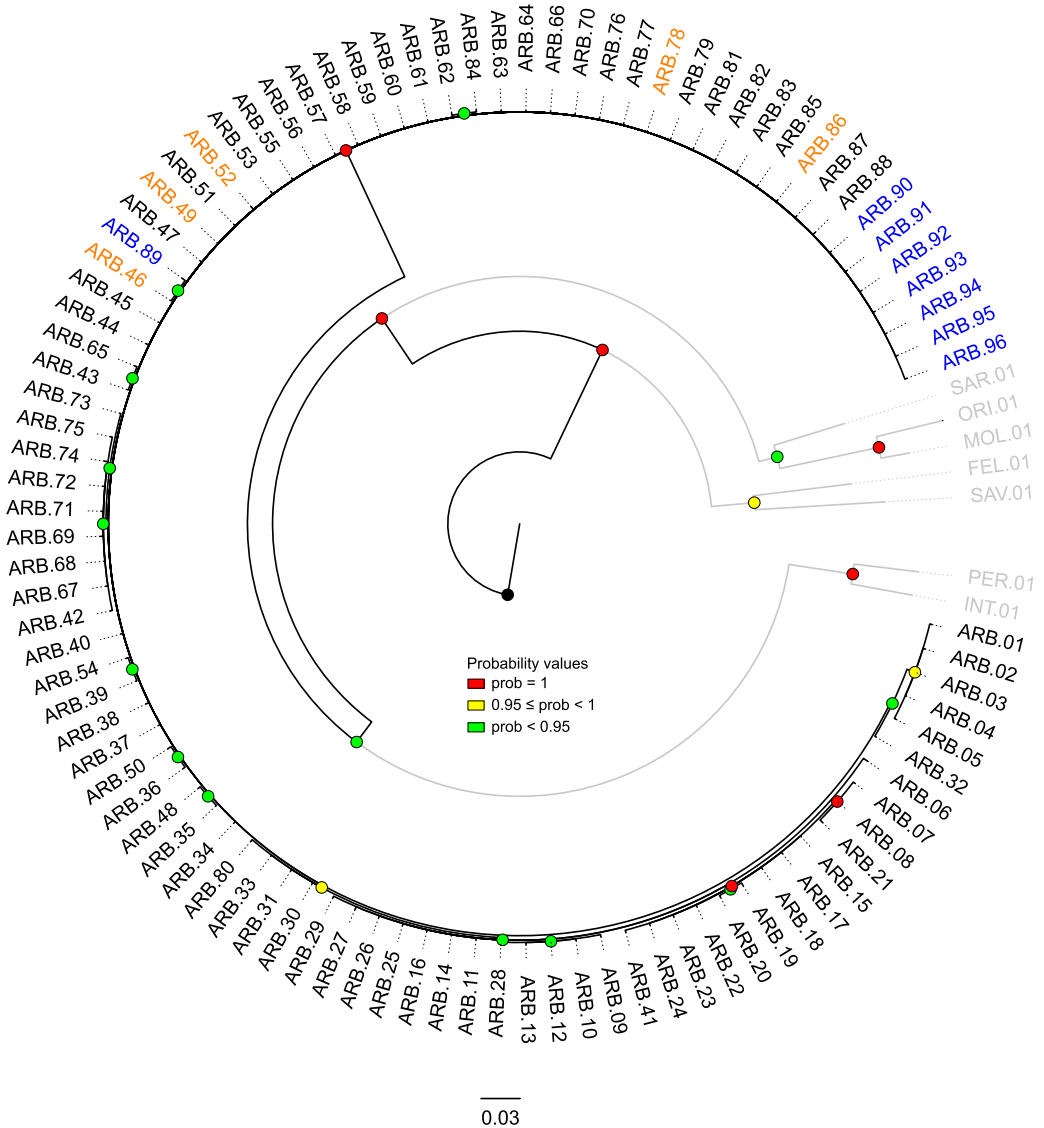
Figure 3. Majority rule consensus phylogenetic tree resulting from Bayesian inference for *Hyla arborea* haplotypes. Grey branches indicate the backbone phylogeny with representatives for each Western Palearctic *Hyla* species; the relatively distinct *H. meridionalis* and *H. carthaginiensis* were used as outgroup and are not shown. Blue haplotypes are newly identified and orange haplotypes were previously reported haplotypes in the Netherlands; black haplotypes have not been reported in the Netherlands. Haplotype labels correspond to supplementary table S1.

endemic to the island of Lesbos in Greece (Dufresnes et al., 2016). The very same haplotype is present in all three populations, suggesting that the Solleveld and Westduinpark populations, which appeared only in the last few years, were sourced from the older Meijendel/Berkheide/Lentevreugd population (established by the early 21st century). Given

that the area in between is highly unsuitable (densely populated built-up area including the city of The Hague), we consider additional human-mediated dispersal to be responsible.

Intriguingly, an eDNA metabarcoding study previously revealed the presence of *H. orientalis*, next to *H. arborea*, in Drenthe, in
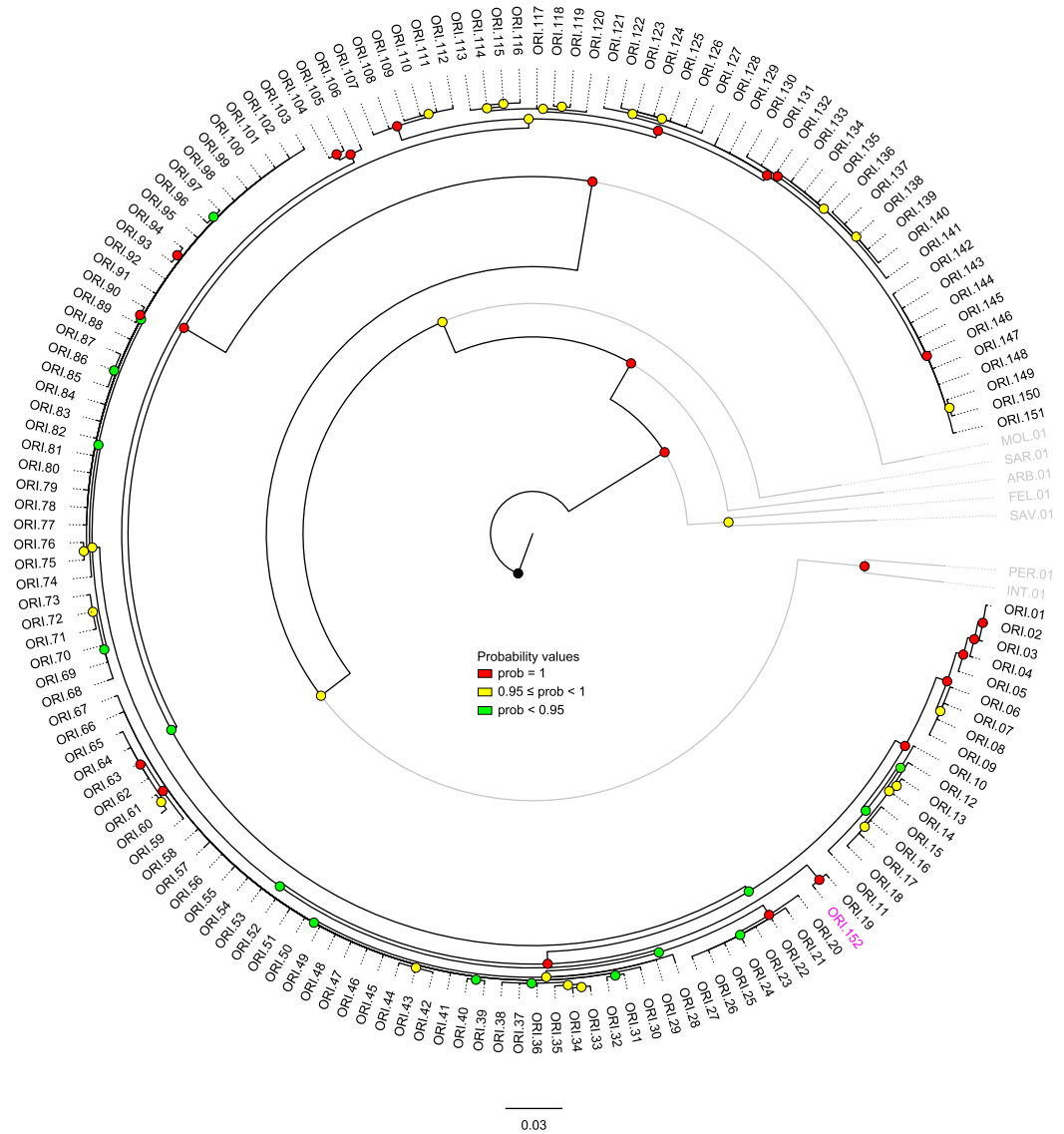
Figure 4. Majority rule consensus phylogenetic tree resulting from Bayesian inference for *Hyla orientalis* haplotypes. Grey branches indicate the backbone phylogeny with representatives for each Western Palearctic *Hyla* species; the relatively distinct *H. meridionalis* and *H. carthaginiensis* were used as outgroup and are not shown. The pink haplotype is newly identified in the Netherlands; black haplotypes have not been reported in the Netherlands. Haplotype labels correspond to supplementary table S1.

the northeast of the Netherlands (van Delft and Herder, 2017). While the origin of this population is unclear, as only short sequences were used and limited reference sequences are available, the person responsible for the Drenthe introduction has indicated (on condition of anonymity) that founders originated from France (Creuse department) and Northern Germany (Mecklenburg-Vorpommern), which would belong to *H. arborea*, and an unknown locality (purchased from a foreign, private hobbyist). We only found *H. arborea* haplotypes in the general region (top right cut-out in fig. 1).

In Westduinpark mtDNA of the Italian tree frog, *H. intermedia* (ssp. *intermedia*), was additionally discovered (i.e., next to *H. orientalis*). We could not pinpoint the exact geographic origin since the recovered haplotype is widespread in central Italy (Canestrelli et al., 2007; Dufresnes et al., 2018). Previously (around 1990), *H. intermedia* was introduced on the Dutch Wadden Sea isle of Terschelling, but this population has not been recorded in the past 15 years and is presumably extinct (Lenders and van Delft, 2011; National Database Flora & Fauna, NDFF).

The case of the introduced Dutch *Hyla* echoes the general situation in Western Europe, where regional translocations of herpetofauna leading to sustainable populations appear common (reviewed by Dubey et al., 2019). South-Alpine species seem to be particularly involved. In Switzerland, *H. intermedia* was introduced from Ticino (ssp. *perrini*) into western Switzerland, which led to the extinction of a highly endangered and protected native population of *H. arborea* through genetic introgression, which potentially led to hybrid breakdown (Dufresnes et al., 2015b; Dubey et al., 2019).

All *Hyla* species detected in the Netherlands likely have the propensity to hybridize, and all share roughly the same amount of genetic divergence (Dufresnes et al., 2020). In nature, *H. arborea* hybridizes with both *H. orientalis* and *H. intermedia* where the ranges meet. We predict that the Westduinpark population concerns a hybrid swarm (a hypothesis that could be tested by nuclear DNA genotyping). If a hybrid swarm were to be confirmed, whether introduced frogs were already genetically admixed, or consisted of pure individuals of each species that hybridized after the releases, remains an open question (a situation reminiscent of the invasive banded newt population in Spain, which consists exclusively of genetically admixed *Ommatotriton nesterovi* × *O. ophryticus*; van Riemsdijk et al., 2018).

The latter explanation appears more parsimonious, given that the Dutch *H. orientalis* haplotype confidently suggests an introduction of this species from Meijendel. It is unclear if *H. intermedia* and *H. orientalis* were introduced simultaneously or not; no matter what, introduction must have happened very recently as the first tree frogs were only reported from (the intensively monitored) Westduinpark in 2017.

The Dutch tree frog case leaves us with two distinct conservation dilemmas. First, two populations of *H. arborea* have been introduced (at least one from elsewhere in Europe), but the species is indigenous to the Netherlands. The autochthonous versus allochthonous status of a population is typically assessed at the species level, so these populations are currently protected by Dutch law. However, the European Commission has recently stated that introduced populations of an indigenous species outside the natural range are not covered by the EU Habitat Directive (European Commission, 2021). Still, there is no juridical precedence in the Netherlands that could provide guidance on the type of management actions that could be considered in this situation. Therefore, the tree frog case calls to revisit protection status by considering population origin at the intraspecific level.

Second, in three populations we flagged genetic signatures of two tree frog species that are clearly not native to the Netherlands. Particularly the Meijendel/Berkheide/Lentevreugd population has exploded over the last two decades and is likely to have a negative impact on native biodiversity (Simberloff, 2013; Pyšek et al., 2020). Of particular concern is that tolerating exotic populations provides source localities for new translocations, a risk that policy makers should not underestimate (Simberloff, 2013). We show that *H. orientalis* has already been spread to the Westduinpark and Solleveld. A particular worry would be anthropogenic secondary contact with native populations of the threatened *H. arborea*, as this would likely lead to genetic assimilation, as has previously occurred in Switzerland (Dufresnes et

al., 2015b). Regardless of other conservation actions, additional translocations should be prevented, and this argument alone justifies conservation action. Because *H. orientalis* and *H. intermedia* have no legal status in the Netherlands, they could in principle be removed. While the popularity of tree frogs (as evidenced by 'ecotourism') may influence public sentiment, from a conservation point of view the fate of exotics should ultimately be a matter of wildlife professionals.

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