Alytes, 2024, **41** (1–4): 49–55.

Difficulties in species identification in water frogs (genus *Pelophylax*) using morphological and molecular markers in The Netherlands

Willem R. M. MEILINK[1,2,3,*], Francis D. VAN BEEK[3], Ieke VAN DE BREEVAART[3], Samuel DE GEUS[3], Cato HELLENDOORN[3], Farzaan JAHANGIER[3], Arslan LIM[3], Michel SLUIMERS[3], Ahmed M. WALI[3], Jeroen J. C. W. VAN DELFT[4], Jelger E. HERDER[4], James FRANCE[1,2] & Ben WIELSTRA[1,2]

[1] Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA, Leiden, The Netherlands.

[2] Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, The Netherlands.

[3] Portus Groene Hart, Barendrecht, P.O. Box 13, 2990 AA, The Netherlands.

[4] Reptile, Amphibian and Fish Conservation Netherlands (RAVON), P.O. Box 1413, 6501 BK Nijmegen, The Netherlands.

*Corresponding author: Willem MEILINK <w.r.m.meilink@gmail.com>

Several molecular protocols have been published to rapidly identify water frog species (genus *Pelophylax*) at low costs by genotyping without sequencing. Here, we compare identifications based on one morphological character (the shape of the metatarsal tubercle) with identifications based on one mitochondrial (*COI*) and one nuclear marker (*SAI*-1) – for which the PCR products shows species-diagnostic length polymorphism – for three populations (19 individuals) representative of *P. lessonae*, *P. ridibundus*, and their hybrid *P. esculentus*; the three species present in The Netherlands. Species identification based on the shape of the metatarsal tubercle is consistent with the known distribution of water frogs in the country. However, species identification based on genetic markers yielded discordant patterns, namely that *P. lessonae* occurs in the western Netherlands, where it is presumably absent. We discuss potential natural (introgression of mtDNA) and technical (unsuitability of *SAI*-1 for species identification in The Netherlands) reasons that could explain the discrepancies. Before reliable molecular identification based on genome-wide loci data becomes easily accessible, morphological and acoustic characteristics remain the most reliable way to identify *Pelophylax* populations in The Netherlands.

Keywords: hybridogenesis, metatarsal tubercle, *COI*, *SAI*-1

urn:lsid:zoobank.org:pub: 7FB89594-E7A7-4914-AA9E-F442CF6FA507

INTRODUCTION

Hybridogenesis is a phenomenon that is common in water frogs, genus *Pelophylax* Fitzinger, 1843 (Dufresnes & Mazepa 2020). In this system, hybrids (or hemiclones) produce gametes in which they exclude one parental genome, and that contain only the unrecombined genome of the other parental species (Berger 1967). By mating with the parental species of which the genome was excluded, a new generation of hemiclonal hybrids is produced. The continuous hybrid line perpetuates a mixture of morphological and genetic traits intermediate between the two parental species, which complicates species identification (Hauswaldt *et al.* 2012). The most studied example of hybridogenesis is between the pool frog *Pelophylax lessonae* (Camerano, 1882) (genomes LL) and the marsh frog *Pelophylax ridibundus* (Pallas, 1771) (genomes RR), which leads to the hybrid edible frog *Pelophylax esculentus* (Linnaeus, 1758) (genomes LR) (Dufresnes *et al.* 2024*b*). In addition to diploid hybrids, hybrid individuals with higher levels of ploidy exist in the northern and eastern parts of the *Pelophylax* range, especially two different types of triploids (LLR and LRR) (Christiansen 2009; Hoffmann *et al.* 2015; Dufresnes & Mazepa 2020).

All three water frog species, and the two types of triploids, occur in The Netherlands. While *P. lessonae* is distributed predominantly in the higher sandy areas of the southern and eastern parts of the country, *P. ridibundus* occurs mostly in the lowland wet areas (peaty and clay soils) of the northern and western part, and *P. esculentus* is found across practically the entire country (Creemers & van Delft 2009). Species identification is generally conducted at the level of the population based on multiple morphological and acoustical traits (Creemers & van Delft 2009). Elucidating species distribution and ploidy level in The Netherlands has historically been based on laboratory methods such as examining erythrocyte length or allozyme analysis (Blommers-Schlösser 1990). Quick and cheap genotyping without sequencing PCR protocols, involving both mitochondrial and nuclear markers, have since become available (Hauswaldt *et al.* 2012; Ermakov *et al.* 2019; Cuevas *et al.* 2022), prompting us to assess their reliability through comparison with phenotypic data. Here, we compare species identification based on one morphological character, the shape of the metatarsal tubercle (Fig. 1), with species identification based on one mitochondrial and one nuclear marker, for three populations in The Netherlands, together representing all three species.

METHODS

Nineteen adult water frogs were caught at nighttime at three locations: Meije (ditch, 52.120° N, 4.795° E; west Netherlands; n = 3), Zevenhuizen (ditch, 52.009° N, 4.575° E; west Netherlands, n = 9) and Rheeze (pond, 52.556° N, 6.562° E; east Netherlands; n = 7). Meije and Zevenhuizen are in the western province Zuid-Holland, where *P. ridibundus* is typically distributed, while Rheeze is in the eastern province Overijssel, where *P. lessonae* is typically distributed; *P. esculentus* is expected to occur at all sites.

Fieldwork was conducted under collection permits and guidelines for the prevention of disease spreading, as stipulated by the amphibian conservation agency of

The Netherlands (RAVON), were respected. Frogs were handled with gloves during measurements as well as skin or buccal swabbing; nets and boots were cleaned and disinfected between sites. Sex was established in the field based on the presence of nuptial pads. Snout-vent length (SVL) was measured, and the shape of the metatarsal tubercle was examined. Frogs were sampled for DNA using Copan 155C Rayon swabs. All individuals were skin swabbed, and all except three (because the mouth would not open, see Table 1) were also buccal swabbed.

Morphological species identification was performed by determining the shape of the metatarsal tubercle (Fig. 1) as follows: large and symmetrical for *P. lessonae*; large and asymmetrical for *P. esculentus*, small and asymmetrical for *P. ridibundus*.

For genetic species identification, DNA was extracted with the Wizard® Genomic DNA purification kit (Promega). Two markers that feature species-diagnostic length polymorphism distinguishable by agarose gel electrophoresis were amplified by PCR, namely the nuclear marker serum albumin intron 1 (*SA*I-1; with primers Pel-SA-F1 and Pel-SA-R2) and the mitochondrial marker *cytochrome c oxidase* subunit I (*CO*I with primers COIR-Pu, COIF-Pl, COIF-Pr and COIF-Pb) (Hauswaldt *et al.* 2012; Ermakov *et al.* 2019). PCRs were conducted with the QIAGEN multiplex PCR master mix in 12 µl reactions containing 0.6 µl of each nuclear primer or 0.3 µl of each mitochondrial primers (end concentrations of 0.5 µM), 6.0 µl QIAGEN multiplex PCR master mix, 3.8 µl purified water and 1 µl of DNA extract. The PCR protocol consisted of 95°C for 15 minutes for activation, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C (*SA*I-1) or 57°C (*CO*I) for 60 seconds, elongation at 72°C for 60 seconds, completed with a final elongation at 72°C for 10 minutes.

RESULTS AND DISCUSSION

Morphological species identification based on the shape of the metatarsal tubercle are in line with the expected distributions of the species (Cremers & van Delft 2009). All nine individuals from the eastern Rheeze population were assigned to *P. lessonae* (Table 1). The three individuals form Meije and the nine individuals from Zevenhuizen were assigned to either *P. esculentus* or *P. ridibundus*.

Both genetic markers amplified in all 19 *Pelophylax* samples (Table 1), which were assigned to either of the three species based on PCR amplicon length (Fig. 2). For the nuclear *SA*I-1, we did not retrieve the length polymorphism expected for *P. ridibundus* (a single band at ±840 bp), even in western populations of Meije and Zevenhuizen where this species was expected. Instead, all individuals were assigned to either *P. lessonae* (a single band at ±300 bp) or *P. esculentus* (one band at ±840 bp and one band at ±300 bp). For the mitochondrial *CO*I, all but three individuals feature *P. lessonae* electrophoresis profile (a 294 bp band). One individual (swab_1520) from Zevenhuizen was assigned to *P. ridibundus* (a 214 bp band), whereas two individuals from this location (swab_1521 and swab_1522) had both bands.

Buccal swabbing performed better than skin swabbing. In the three samples that were only skin swabbed (Table 1), the mitochondrial marker amplified normally, but the nuclear marker only amplified after tripling the amount of template DNA in the PCR mix.

Table 1. Water frog (*Pelophylax*) samples and their localities (see Methods for GPS coordinates), snout-vent-length (SVL, in mm), sex, and identification based on the shape of metatarsal tubercle, as well as the nuclear and mitochondrial genotypes; purple: *P. lessonae*; pink: *P. esculentus*; orange: *P. ridibundus*. *only skin swabbed.

Sample	Location	SVL	Sex	Shape of Metatarsal Tubercle	nuclear DNA	mitochondrial DNA
swab_1509*	Meije	55	Male	*P. esculentus*	*P. lessonae*	*P. lessonae*
swab_1510*	Meije	60	Male	*P. esculentus*	*P. esculentus*	*P. lessonae*
swab_1511*	Meije	60	Male	*P. ridibundus*	*P. esculentus*	*P. lessonae*
swab_1513	Zevenhuizen	78	Female	*P. ridibundus*	*P. lessonae*	*P. lessonae*
swab_1514	Zevenhuizen	84	Male	*P. ridibundus*	*P. esculentus*	*P. lessonae*
swab_1515	Zevenhuizen	83	Male	*P. ridibundus*	*P. esculentus*	*P. lessonae*
swab_1516	Zevenhuizen	72	Male	*P. esculentus*	*P. esculentus*	*P. lessonae*
swab_1518	Zevenhuizen	75	Male	*P. esculentus*	*P. lessonae*	*P. lessonae*
swab_1519	Zevenhuizen	78	Female	*P. esculentus*	*P. lessonae*	*P. lessonae*
swab_1520	Zevenhuizen	85	Female	*P. esculentus*	*P. lessonae*	*P. ridibundus*
swab_1521	Zevenhuizen	83	Male	*P. ridibundus*	*P. lessonae*	heteroplasmy?
swab_1522	Zevenhuizen	69	Male	*P. ridibundus*	*P. lessonae*	heteroplasmy?
swab_1524	Rheeze	46	Male	*P. lessonae*	*P. lessonae*	*P. lessonae*
swab_1525	Rheeze	42	Male	*P. lessonae*	*P. lessonae*	*P. lessonae*
swab_1526	Rheeze	50	Female	*P. lessonae*	*P. esculentus*	*P. lessonae*
swab_1527	Rheeze	44	Female	*P. lessonae*	*P. esculentus*	*P. lessonae*
swab_1528	Rheeze	4,4	Female	*P. lessonae*	*P. lessonae*	*P. lessonae*
swab_1531	Rheeze	40	Female	*P. lessonae*	*P. lessonae*	*P. lessonae*
swab_1532	Rheeze	43	Female	*P. lessonae*	*P. lessonae*	*P. lessonae*

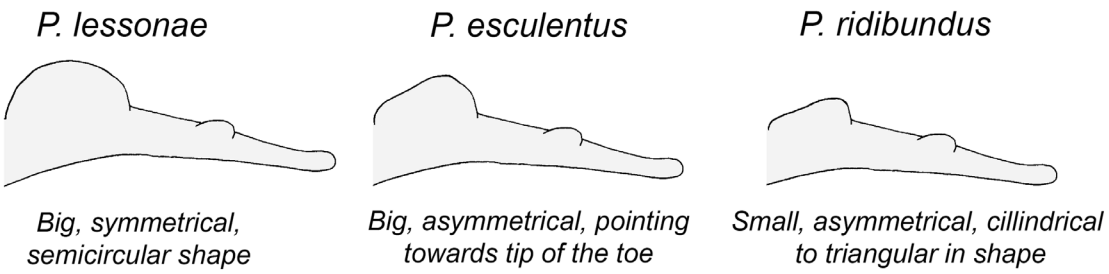

Figure 1. The shape of the metatarsal tubercle as a morphological trait for species identification in water frogs (*Pelophylax*).

While the shape of the metatarsal tubercle is consistent with species identification based on previous allozyme data (Blommers-Schlösser 1990), the two genetic markers tested are not. Gel electrophoresis banding patterns for nuclear and mitochondrial DNA typical of *P. lessonae* are found for samples from areas only inhabited by *P. ridibundus* and *P. esculentus* (Table 1; Creemers & van Delft 2009). It has been shown that species identification based on the *SA*I-1 nuclear DNA marker is not reliable across the entire *Pelophylax* range, because the length polymorphism is not fully species diagnostic (Dufresnes *et al.* 2024*b*). Furthermore, mtDNA shows massive introgression in some parts of the *Pelophylax* distribution (Plötner *et al.* 2008; Dufresnes *et al.* 2024*b*). Our findings show that species identification based on the two molecular markers genotyped here is unreliable in The Netherlands, while the morphological character conforms to the known species distributions.

The two bands on the *CO*I gel observed for two Zevenhuizen individuals might reflect mitochondrial heteroplasmy. This phenomenon could be caused by paternal leakage in hybrids, namely where the mitochondrial DNA of a different species than the one present in the egg cell is transferred into the offspring via the sperm (Radojičić *et al.* 2015). However, these individuals are suggested to either be *P. ridibundus* (morphology) or *P. lessonae* (*SA*I-1), but not hybrids. Sequencing *SA*I-1 would be required to clarify these conflicting patterns.

Genetic identification with one nuclear marker in a complex that hybridizes extensively is likely to be inaccurate, particularly if the marker is not fully diagnostic (Dufresnes *et al.* 2024*a*). *SA*I-1 has been used in many studies (e.g., Vucić *et al.* 2018), but its length polymorphism must be interpreted with caution, especially around the Alpine region, where similar discrepancies were reported (Dufresnes *et al.* 2024*a*). Our results thus suggest that the geographical region across which the length polymorphism in *P. lessonae* is problematic for species identification is more extensive than previously thought.

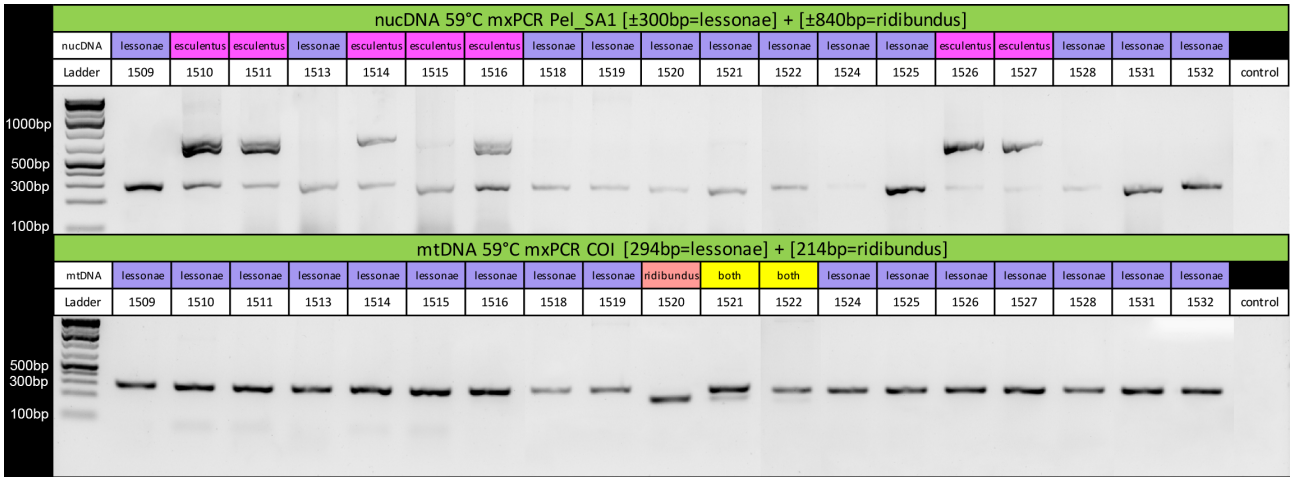

Figure 2. Gel electrophoresis profiles of the 19 *Pelophylax* individuals for the nuclear marker *SA*I-1 (top) and the mitochondrial marker *CO*I (bottom). Individuals are assigned based on the length of the PCR amplicons to *P. lessonae* (purple), *esculentus* (pink), *ridibundus* (orange). For *CO*I, the individuals with two bands are highlighted in yellow.

Another consideration is that triploid hybrids are common in The Netherlands (Blommers-Schlösser 1990; Blommers-Schlosser 1992), which could complicate species assignment. Newer genome wide methods, such as techniques employing many Single Nucleotide Polymorphism (SNPs), are needed to accurately accomplish species identification and calibrate new, more reliable methods. Target capture, in which fragments of thousands of genes are sequenced (Andermann *et al.* 2020), could increase the resolution to determine degree of hybridization and introgression (as well as ploidy levels). FrogCap, a target capture baitset, is already available for frogs, but has not yet been used for the genus *Pelophylax* (Hutter *et al.* 2022). When a broader genetic canvas becomes available, a new set of markers could be developed for smaller and quicker genotyping for accurate species determination in *Pelophylax*. While awaiting these developments, a recently proposed PCR-RFLP method involving two nuclear DNA markers (Cuevas *et al.* 2022) would be a useful alternative in The Netherlands, providing proper testing.

At the moment, identification based on morphological and acoustic characteristics appear the most reliable in The Netherlands, although they can be inconvenient. For instance, it is strongly advised to use a broad set of morphological characters (Creemers & van Delft 2009; Speybroeck *et al.* 2016). Additionally, the male advertisement calls can only be used for species identification during the breeding season.

ACKNOWLEDGEMENTS

The PhD position of WRMM was supported by the Dutch Research Council (NWO Promotiebeurs voor leraren 023.016.006). Other authors associated with Portus Groene Hart performed this study as part of various school activities. The authors thank Staatsbosbeheer for permission to enter the study site Rheeze. Paul Veenvliet provided the drawings of the metatarsal tubercle used in Fig. 1.

REFERENCES

Andermann, T., Torres Jiménez, M. F., Matos-Maraví, P., Batista, R., Blanco-Pastor, J. L., Gustafsson, A. L. S., Kistler, L., Liberal, I. M., Oxelman, B. & Bacon, C. D. (2020) A guide to carrying out a phylogenomic target sequence capture project. *Frontiers in Genetics,* **10** [1407]: 1–20.

Berger, L. (1967) Embryonal and larval development of F1 generation of green frogs' different combinations. *Acta zoologica cracoviensia,* **12** (7): 123–160.

Blommers-Schlösser, R. (1990) On the occurrence and identity of triploids of *Rana* kl. *esculenta* Linnaeus and *R. lessonae* Camerano in The Netherlands (Anura: Ranidae). *Bijdragen tot de Dierkunde,* **60** (3–4): 199–207.

Blommers-Schlosser, R. M. A. (1992) De groene kikkers in Nederland; samenstelling van populaties, oecologie, verspreiding en bedreiging. *De Levende Natuur,* **93** (1): 2–9.

Camerano, L. (1882) Recherches sur les variations de la *Rana esculenta* et du *Bufo viridis* dans le Bassin de la Méditerranée. *Comptes rendus de l'Association française pour l'Avancement des Sciences. Paris*, **10**: 680–690

Christiansen, D. G. (2009) Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). *BMC evolutionary Biology,* **9** [135]: 1–16.

Creemers, R. C. M. & van Delft, J. J. C. W. (2009) *Nederlandse fauna 9 – De amfibieën en reptielen van Nederland*. Leiden (Nationaal Natuurhistorisch Museum Naturalis, European Invertebrate Survey): 1–477.

Cuevas, A., Patrelle, C., Ciavatti, F., Gendre, T., Sourrouille, P., Geniez, P., Doniol-Valcroze, P. & Crochet, P.-A. (2022) A new PCR-RFLP method for the identification of parental and hybridogenetic western European Water Frogs, including the *Pelophylax perezi-grafi* system. *Salamandra,* **58** (3): 218–230.

Dufresnes, C., Dubey, S., Wielstra, B., Jablonski, D. & Denoël, M. (2024*a*) Panacea or nemesis? Re-assessing the reliability of serum albumin intron 1 for genotyping Western Palearctic water frogs. *Alytes,* **41** (1–4): 5–17.

Dufresnes, C. & Mazepa, G. (2020) Hybridogenesis in water frogs. *eLS,* **1**: 718–726.

Dufresnes, C., Monod-Broca, B., Bellati, A., Canestrelli, D., Ambu, J., Wielstra, B., Dubey, S., Crochet, P.-A., Denoël, M. & Jablonski, D. (2024*b*) Piecing the barcoding puzzle of Palearctic water frogs (*Pelophylax*) sheds light on amphibian biogeography and global invasions. *Global Change Biology* **30** (3) [e17180]: 1–30.

Ermakov, O., Ivanov, A., Titov, S., Svinin, A. & Litvinchuk, S. (2019) New multiplex PCR method for identification of East European green frog species and their hybrids. *Russian Journal of Herpetology,* **26** (6): 367–370.

Fitzinger, L. J. F. J. (1843) *Systema Reptilium. Fasciculus Primus*. Wien (Braumüller et Seidel): 1–106.

Hauswaldt, J. S., Hoeer, M., Ogielska, M., Christiansen, D. G., Dziewulska-Szwajkowska, D., Czernicka, E. & Vences, M. (2012) A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (*Pelophylax*). *Molecular Ecology Resources,* **12** (5): 797–805.

Hoffmann, A., Plötner, J., Pruvost, N. B., Christiansen, D. G., Röthlisberger, S., Choleva, L., Mikulíček, P., Cogălniceanu, D., Sas-Kovács, I. & Shabanov, D. (2015) Genetic diversity and distribution patterns of diploid and polyploid hybrid water frog populations (*Pelophylax esculentus* complex) across Europe. *Molecular Ecology,* **24** (17): 4371–4391.

Hutter, C. R., Cobb, K. A., Portik, D. M., Travers, S. L., Wood Jr, P. L. & Brown, R. M. (2022) FrogCap: A modular sequence capture probe-set for phylogenomics and population genetics for all frogs, assessed across multiple phylogenetic scales. *Molecular Ecology Resources,* **22** (3): 1100–1119.

Linnaeus, C. (1758) *Systema Naturae per reg.na tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis.* Editio decima, reformata. Tomus I. Holmiae (Laurentii Salvii): [i–iv] + 1–824.

Pallas, P. S. (1771) *Reise durch verschiedene Provinzen des Russischen Reichs.* Theil 1. St. Pétersbourg (Kayserlichen Academie der Wissenschaften): 1–504.

Plötner, J., Uzzell, T., Beerli, P., Spolsky, C., Ohst, T., Litvinchuk, S. N., Guex, G. D., Reyer, H. U. & Hotz, H. (2008) Widespread unidirectional transfer of mitochondrial DNA: a case in western Palaearctic water frogs. *Journal of evolutionary Biology,* **21** (3): 668–681.

Radojičić, J. M., Krizmanić, I., Kasapidis, P. & Zouros, E. (2015) Extensive mitochondrial heteroplasmy in hybrid water frog (*Pelophylax* spp.) populations from Southeast Europe. *Ecology and Evolution,* **5** (20): 4529–4541.

Speybroeck, J., Beukema, W., Bok, B. & van der Voort, J. (2016) *Field guide to the Amphibians and Reptiles of Britain and Europe*. London (Bloomsbury Publishing): 1–432.

Vucić, M., Jelić, D., Klobučar, G. I., Prkljačić, B. & Jelić, M. (2018) Molecular identification of species and hybrids of water frogs (genus *Pelophylax*) from Lake Skadar, Southeast Adriatic drainages (Amphibia: Ranidae). *Salamandra,* **54** (2): 147–157.

Submitted: 7 April 2024.
Accepted: 26 August 2024.
Published: 5 November 2024.

Corresponding Editor: Christophe DUFRESNES.