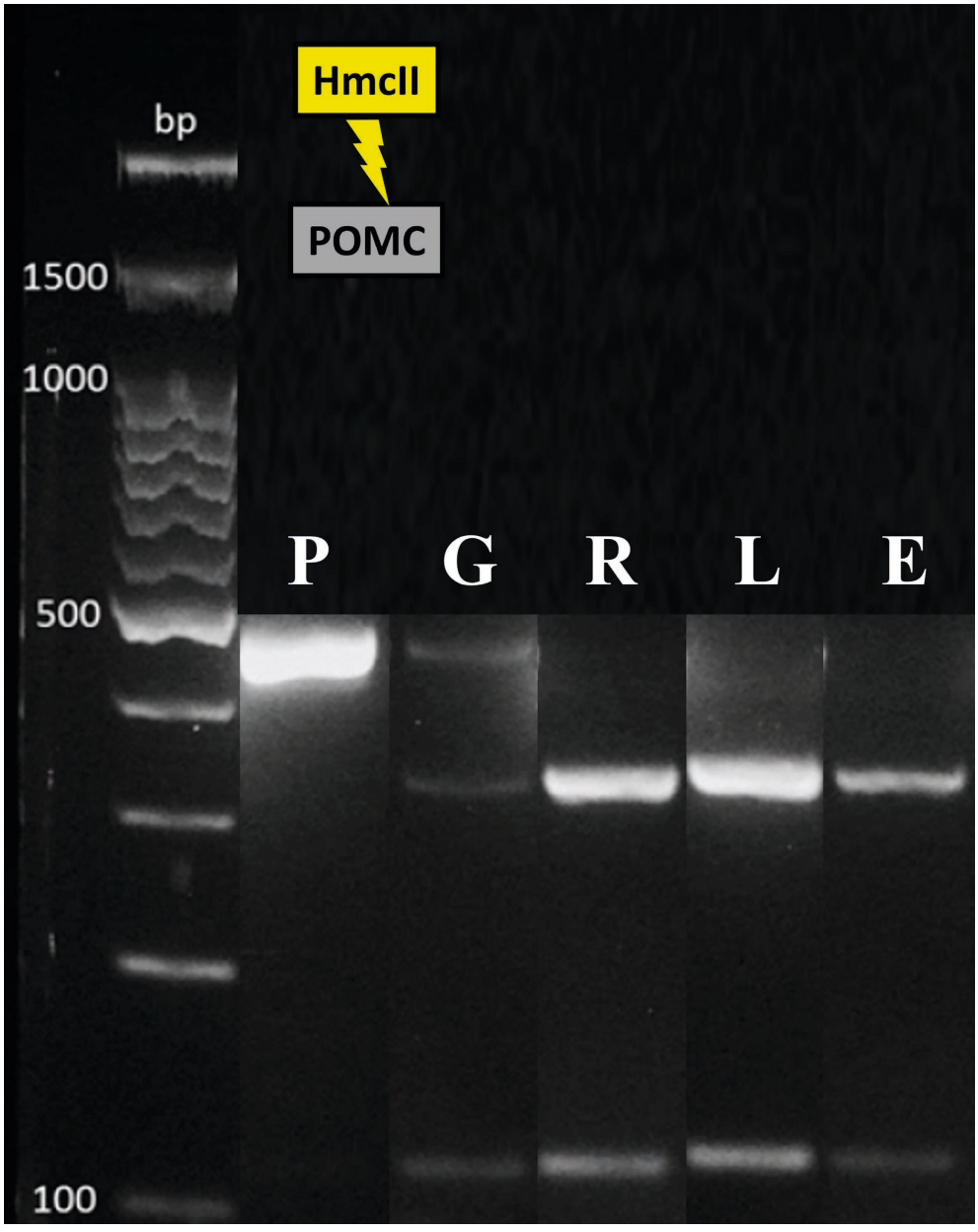
The amplification of the NTF3 fragment produces a 646-bp amplicon (589 bp aligned after removing the primer) in which sequences of *Pelophylax ridibundus* and *P. lessonae* are nearly identical while *P. perezi* presents a single near diagnostic SNP. Both *P. ridibundus* and *P. lessonae* have a XapI restriction site between position 409 and 410 of the alignment, producing two fragments of 437 and 209 bp, respectively. *Pelophylax* kl. *esculentus* produces the same profile as its parents, with two visible fragments, while *P.* kl. *grafi* presents three visible fragments, two of 437 bp and one of 209 bp, corresponding to the *P. ridibundus* genome, and a longer fragment of 646 bp for the *P. perezi* genome.

This NTF3/XapI restriction site was genotyped by RFLP or sequenced in a total of 69 specimens (see Supplementary File 1 for details): 14 *P. ridibundus*, 17 *P. perezi*, 15 *P. lessonae*, 11 *P.* kl. *grafi*, and 12 *P.* kl. *esculentus* that yielded RFLP patterns or genotyped for the restriction site as expected for their taxon. However, this pattern is not fully diagnostic, as some *P. ridibundus* share the *P. perezi* mutation at position 410 while a few *P. perezi* lack this mutation, as is visible in the NTF3 alignment for the sequenced individuals with the XapI restriction site aligned to the sequences provided in Supplementary File 3. As a consequence, we did not retain NTF3 for our final marker selection.



Supplementary File 2. Enzymatic digestion pattern of the NTF3 marker by the XapI enzyme on Western European *Pelophylax* species. DNA fragment size is determined by comparison with a 100-bp DNA Step Ladder (Promega). Species names are labelled as follows: P: *Pelophylax perezi*; G: *P.* kl. *grafi*; R: *P. ridibundus*; L: *P. lessonae*; E: *P.* kl. *esculentus*.