



# Identification of Invasive Alien Species using DNA barcodes

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## General introduction to this factsheet

The Barcoding Facility for Organisms and Tissues of Policy Concern (BopCo) aims at developing an expertise forum to facilitate the identification of biological samples of policy concern in Belgium and Europe. The project represents part of the Belgian federal contribution to the European Research Infrastructure Consortium LifeWatch.

Non-native species which are being introduced into Europe, whether by accident or deliberately, can be of policy concern since some of them can reproduce and disperse rapidly in a new territory, establish viable populations and even outcompete native species. As a consequence of their presence, natural and managed ecosystems can be disrupted, crops and livestock affected, and vector-borne diseases or parasites might be introduced, impacting human health and socio-economic activities. Non-native species causing such adverse effects are called Invasive Alien Species (IAS). In order to protect native biodiversity and ecosystems, and to mitigate the potential impact on human health and socio-economic activities, the issue of IAS is tackled in Europe by EU Regulation 1143/2014 of the European Parliament and Council. The IAS Regulation provides for a set of measures to be taken across all member states. The list of Invasive Alien Species of Union Concern is regularly updated. In order to implement the proposed actions, however, methods for accurate species identification are required when suspicious biological material is encountered.

Because morphology-based species identifications are not always possible (e.g. cryptic species, trace material, early life-stages), the purpose of the present work is to investigate and evaluate the usefulness of DNA sequence data to identify each of the IAS included in the EU Regulation. The results are presented as factsheets (one per IAS) compiled using publicly available DNA sequence data and information aggregated from various sources. Each factsheet consists of two major parts; (i) a short introduction to the specific IAS compiling information on its taxonomy and current occurrence/distribution in Europe; (ii) an investigation with respect to the usefulness of publicly available DNA sequences to identify this IAS to the taxonomic level stated in the EU list using DNA barcoding. For further information about the reasoning behind the applied approach and details on the materials and methods utilised, please see below and Smits *et al.* [1].

More info about BopCo on <http://bopco.myspecies.info/> or contact us via [bopco@naturalsciences.be](mailto:bopco@naturalsciences.be).

More info on the EU Regulation on [http://ec.europa.eu/environment/nature/invasivealien/index\\_en.htm](http://ec.europa.eu/environment/nature/invasivealien/index_en.htm).

## *Lithobates catesbeianus*

(Shaw, 1802)

Common names:

English: American bullfrog, common bullfrog, bullfrog

French: grenouille-taureau

German: (Nord)Amerikanische Ochsenfrosch

Dutch: Amerikaanse stierkikker, Amerikaanse brulkikker

Last update: January 2019



## General information on *Lithobates catesbeianus*

### Classification

Kingdom	Phylum	Class	Order	Family	Genus
Animalia	Chordata	Amphibia	Anura	Ranidae	<i>Lithobates</i>

### Species in the same genus: N = 51 [2-4]

Note: The phylogeny of the family Ranidae is still a matter of discussion. The genus *Lithobates* as defined by Frost *et al.* [3] is not commonly accepted and the name *Rana catesbeiana* is often used [e.g. 4]. We follow the classification of the Amphibian of the World online reference database [2].

### Infra-species level: N = 0 [2]

Note: To our knowledge, no subspecies have been described.



### Native range: [5-7]

Central and eastern part of North America (Canada, Mexico, USA).

### Invasive range: [7-10]

#### Europe (geographical):

Belarus, Belgium, Denmark, France, Germany, Greece, Italy, Netherlands, Russia, Slovenia, Spain (incl. Canary Islands), United Kingdom.

**For more detailed locality information and the most recent distribution updates, please visit:**

<http://alien.jrc.ec.europa.eu/SpeciesMapper>

<https://www.gbif.org/species/2427091> (*Lithobates catesbeianus*)

<https://www.gbif.org/species/2427092> (*Rana catesbeiana*)

#### Outside Europe (geographical):

Asia, Caribbean, Namibia, South and Central America, western part of North America (including Hawaii).

### Morphology, biology, invasion, negative effects and remedies

For more information on *Lithobates catesbeianus* please see the references and online information listed at the end of this document.



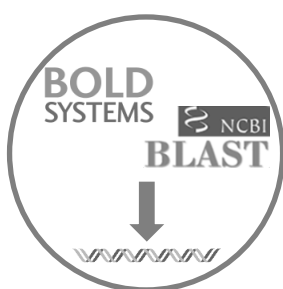
## Species identification based on DNA barcodes

### Introduction

DNA barcoding is a species identification method that uses a short genetic sequence (DNA barcode) to compare an unknown sample to a database of reference sequences with known species affiliations. The underlying rationale is that the divergence of nucleotide sequences among different species is larger than the nucleotide divergence between sequences within a species. DNA barcoding can facilitate the identification of IAS samples, especially when morphological characteristics are absent or useless. To assure correct species identifications, however, reference libraries need to include a sufficiently large number of sequences of (i) the IAS under investigation, in order to assess the intraspecific genetic divergence; (ii) the closely related species, in order to evaluate the interspecific genetic divergence; (iii) the different geographical areas covering the distribution range (native and invasive) of the IAS in order to detect potential population structure or local hybrids.

Against this background, BopCo evaluated the inclusion of the IAS and their close relatives in both publicly available reference libraries BOLD ([www.boldsystems.org/](http://www.boldsystems.org/)) and GenBank ([www.ncbi.nlm.nih.gov/nucleotide/](http://www.ncbi.nlm.nih.gov/nucleotide/)) to estimate the reliability with which a species identification can be obtained using DNA barcoding.

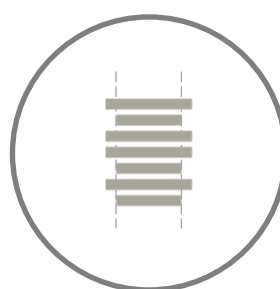
### Material and Methods [1]



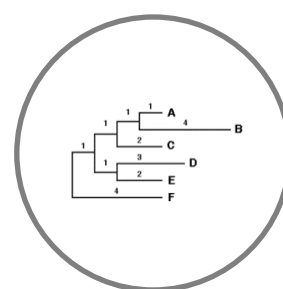
Download all sequence data available for the genus



Filtering the data and selecting 'promising' markers



Aligning and trimming of the sequences



Building Neighbour-Joining tree with Bootstrap support

### Conclusion

**Based on the present evaluation of the available sequence data, 12S and 16S are the most reliable DNA markers for the identification of *Lithobates catesbeianus*. To allow for a better evaluation of the performance of these markers, the missing species as well as additional sequences for the species now represented by one unique sequence only should be added.**

### Discussion

DNA markers for which *Lithobates* sequences were available, including those listed as *Rana*, were downloaded from GenBank and BOLD for all represented species of the genus *Lithobates* as defined by Frost [2] and Frost *et al.* [3]. Ten DNA markers were evaluated (Table 1).

At first glance **COI**, **cytb**, **12S**, **16S** and **ND2** look promising, since for each of these markers *L. catesbeianus* sequences form highly supported clusters and many of the other species represented by  $\geq 2$  sequences also cluster. For **cytb**, **12S**, **16S** and **ND2**, however, around half of the species are represented by one unique sequence only in the final NJ-trees. To allow for a better evaluation of the performance of these markers for species identifications, the missing species (Table 2) as well as more sequences per species should be added. The number of species currently represented in GenBank and BOLD suggests that **12S** and **16S** are the most suitable and require the least amount of additional work to complete the dataset for this genus.

**MHC II $\beta$**  and **RAG-1** recover *Lithobates catesbeianus* as a supported cluster, but suffer from various issues (Table 1). It is not advisable to apply these markers for species identification.

For **rho**, **RAG-2** and **NF** only one or two species (including *L. catesbeianus*) are represented by two or more sequences. Therefore it is currently impossible to assess the ability of these markers to identify *L. catesbeianus*.

**Table 1:** Overview of the encountered issues concerning the DNA-based identification of the IAS [1]: (1) Insufficient publicly available DNA sequences of the IAS to capture the intra-species divergence; (2) Poor geographical coverage of the IAS sequences (native or invasive range missing); (3) The IAS sequences do not form supported clusters; (4) Potential misidentification of a specimen which influences the clustering of the IAS sequences; and (5) Not all congeneric species are represented in the final NJ-tree. An 'X' indicates that the issue was encountered.

Markers analysed	1	2	3	4	5
COI					X



cytb						X
12S						X
16S						X
ND2						X
MHC IIβ	X	X				X
rho		X	X			X
RAG-1	X	X				X
RAG-2	X	X	X			X
NF	X	X	X			X

**Table 2:** Publicly available sequences downloaded (January 2019) from BOLD and GenBank (including sequences extracted from mitochondrial genomes) which were withheld as reliable and informative in the final alignment that was used for building the NJ-trees. The species names follow [2]. An 'X' indicates that at least one sequence was used in the final alignment, an 'S' indicates that the species was only represented in the alignment of trimmed fragments.

Species in genus	COI	cytb	12S	16S	ND2	MHC IIβ	rho	RAG-1	RAG-2	NF
<i>Lithobates areolatus</i>										
<i>Lithobates berlandieri</i>		X	X	X	X		X	X	X	X
<i>Lithobates blairi</i>	X		S	X		X				
<i>Lithobates brownorum</i>			X							
<i>Lithobates bwana</i>			S	X						
<i>Lithobates capito</i>			X	X	X		X			
<b><i>Lithobates catesbeianus</i></b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>
<i>Lithobates chichicuahutla</i>										
<i>Lithobates chiricahuensis</i>	X	X	X	X	X	X	X	X		X
<i>Lithobates clamitans</i>	X	X	X	X	X	X	X	X	X	X
<i>Lithobates dunni</i>		X	S	X	X			X	X	X
<i>Lithobates fisheri</i>			S				X			
<i>Lithobates forreri</i>	X	X	X	X	X	X	X	X	X	X
<i>Lithobates grylio</i>		X	X	X	X		X			
<i>Lithobates heckscheri</i>	X	X	X	X	X		X			
<i>Lithobates johnei</i>										
<i>Lithobates juliani</i>			S	X	X			X	X	X
<i>Lithobates kauffeldi</i>			S	X						
<i>Lithobates lemosespinali</i>										
<i>Lithobates lenca</i>										
<i>Lithobates macroglossa</i>		X	S	X	X			X	X	X
<i>Lithobates maculatus</i>										
<i>Lithobates magnaocularis</i>		X	X	X	X			X	X	X
<i>Lithobates megapoda</i>										
<i>Lithobates miadis</i>										
<i>Lithobates montezumae</i>		X	S	X	X			X	X	X
<i>Lithobates neovolcanicus</i>		X	X	X	X			X	X	X
<i>Lithobates okaloosae</i>	X	X	X	X	X					
<i>Lithobates omiltemanus</i>		X	S	X	X			X	X	X
<i>Lithobates onca</i>		X	S	X	X					
<i>Lithobates palmipes</i>	X		X	X			X			
<i>Lithobates palustris</i>	X	X	S	X	X	X	X	X	X	X
<i>Lithobates pipiens</i>	X		X	X	X		X			
<i>Lithobates psilonota</i>		X	S	X	X			X	X	
<i>Lithobates pueblae</i>										
<i>Lithobates pustulosus</i>										
<i>Lithobates septentrionalis</i>	X	X	S	X	X			X	X	X
<i>Lithobates sevosus</i>			S	X						
<i>Lithobates sierramadrensis</i>		X	S	X	X			X	X	X
<i>Lithobates spectabilis</i>		X	X	X	X			X	X	X
<i>Lithobates sphenoccephalus</i>	X					X				
<i>Lithobates sylvaticus</i>	X	X	X	X	X	X				
<i>Lithobates tarahumarae</i>		X	S	X	X		X	X	X	X
<i>Lithobates taylori</i>	X		S	X						
<i>Lithobates tlaloci</i>		X	S	X	X			X	X	X
<i>Lithobates vaillanti</i>	X		S	X	X	X		X	X	X
<i>Lithobates vibicarius</i>		X	S	X	X			X	X	X
<i>Lithobates virgatipes</i>	X	X	S	X	X	X		X	X	
<i>Lithobates warszewitschii</i>	X	X	X	X	X		X	X	X	X
<i>Lithobates yavapaiensis</i>	X	X	X	X	X	X	X	X	X	X



<i>Lithobates zweifeli</i>		X	X	X	X			X	X	X
<b>TOTAL (trimmed) species</b>	<b>18/51</b>	<b>28/51</b>	<b>19(40)/51</b>	<b>38/51</b>	<b>32/51</b>	<b>11/51</b>	<b>15/51</b>	<b>25/51</b>	<b>24/51</b>	<b>23/51</b>

For a more elaborate discussion of the available databases, the sequence selection process, the outcome of the NJ-tree analyses, the usefulness of the investigated DNA sequences for species identification, as well as information on how to send samples for analyses please contact BopCo directly.



## References and online information

### Online information

<https://www.cabi.org/isc/datasheet/66618>  
<http://www.nonnativespecies.org/factsheet/factsheet.cfm?speciesId=2040>  
<https://www.invasivespeciesinfo.gov/aquatics/bullfrog.shtml>  
<https://nas.er.usgs.gov/queries/factsheet.aspx?SpeciesID=71>  
<https://nrm.dfg.ca.gov/FileHandler.ashx?DocumentID=86493>  
<http://species.biodiversityireland.ie/profile.php?taxonId=6998&taxonGroupName=amphibian&keyword=Invasive>  
<http://ias.biodiversity.be/species/show/88>  
<https://www.nvwa.nl/documenten/dier/dieren-in-de-natuur/exoten/risicobeoordelingen/factsheet-amerikaanse-stierkikker-brulkikker> [NL]

### Picture credits

Page 1: *Lithobates catesbeianus* - Bullfrog By Brian Gratwicke [CC BY 2.0]  
Page 2 (left): American bullfrog (*Lithobates catesbeianus*) By Thomas Brown [CC BY 2.0]  
Page 2 (right): *Lithobates catesbeianus* By Andrew DuBois [CC BY-NC 2.0]

### References

- [1] N. Smits, S. Gombeer, K. Meganck, A. Vanderheyden, Y. R. Van Bourgonie, T. Backeljau, and M. De Meyer, "Identifying IAS based on DNA barcoding using currently available sequence data: details on applied material and methods." 2019. [Online]. Available from: <http://bopco.myspecies.info/content/invasive-alien-species-ias-factsheets>.
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