

Barcoding Anurans from Brazilian Atlantic Forest

Mariana L. Lyra, Célio F. B. Haddad & Ana Maria L. de Azeredo-Espin

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Capes/CNPq Prodoc nº 563.975/05-9





UNICAMP

Laboratório Genética
Animal (UNICAMP)

Prof^a Ana Maria L. Azeredo-
Espin

Molecular Markers
mtDNA



unesp 

Laboratório de Herpetologia
(UNESP-Rio Claro)
Prof. Célio F. B. Haddad

Biggest tissue and
specimens collection
of Anurans (Brazil)



Pilot Project: Testing COI DNA
Barcode in Anurans from
Brazilian Atlantic Forest

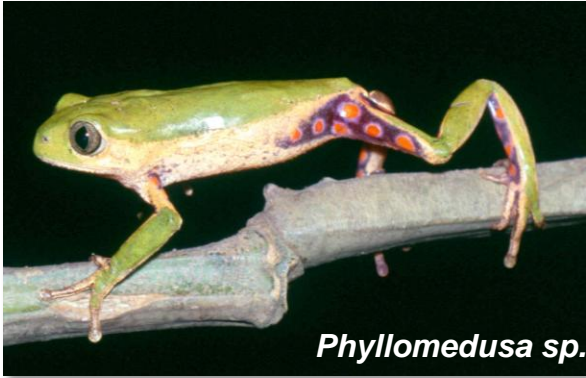
Barcoding Anurans from Brazilian Atlantic Forest

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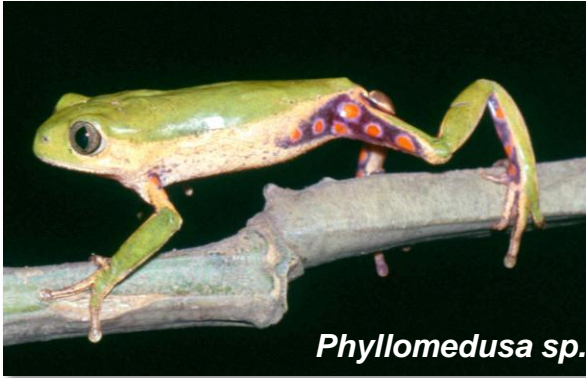
Amphibia- 6433 sp.



Anura- 5679 sp.

Caudata- 580 sp.

Gymnophiona- 174 sp.



Amphibia- 6433 sp.



Anura- 5679 sp.

Caudata- 580 sp.

Gymnophiona- 174 sp.

Amphibia- 857 sp.



Anura- 823 sp.

Caudata- 2 sp.

Gymnophiona- 33 sp.



Decline in amphibian populations worldwide



National Geographic Brazil – April 2009



Decline in amphibian populations worldwide



National Geographic Brazil – April 2009

1- Habitat Loss 2- Climate changes 3- Spread of microbial pathogens



Decline in amphibian populations worldwide



National Geographic Brazil – April 2009

1- Habitat Loss 2- Climate changes 3- Spread of microbial pathogens

Brazilian Atlantic Forest

Original area

16% of the Brazilian territory

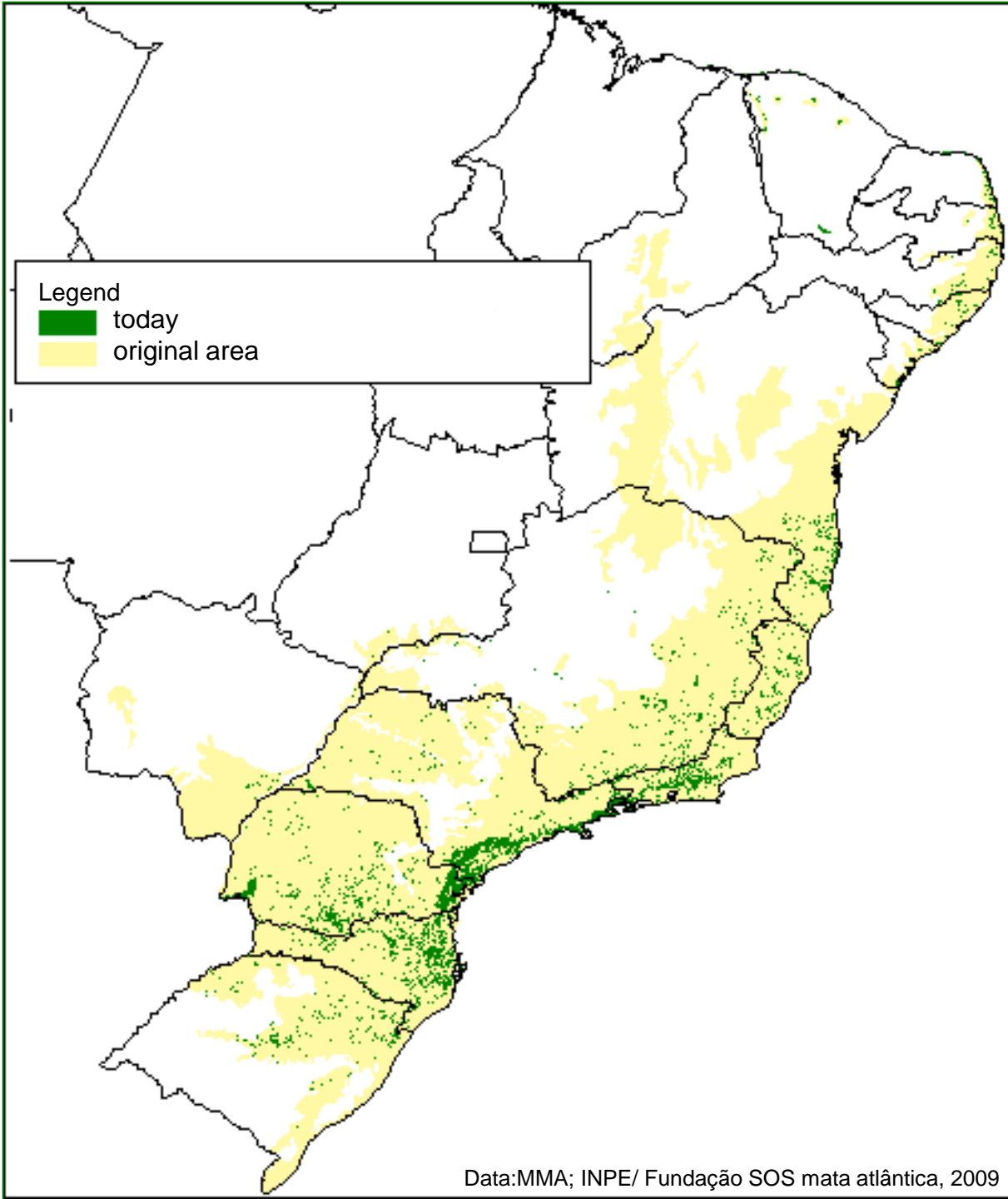
Today:

~7-8%

~1,2-0,8% intact

Legend

- today
- original area



Brazilian Atlantic Forest

Original area

16% of the Brazilian territory

Today:

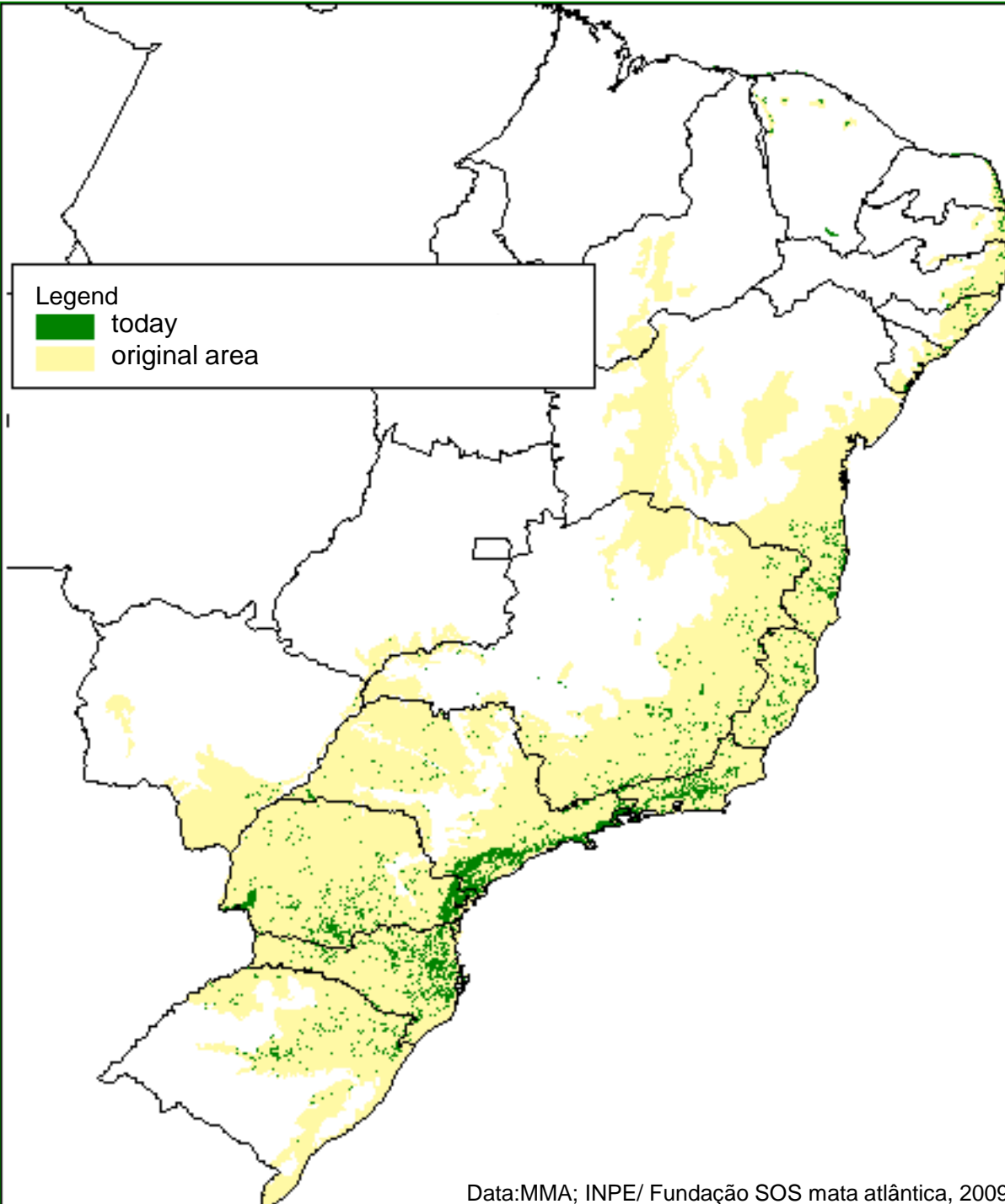
~7-8%

~1,2-0,8% intact

Biodiversity hotspots

460 amphibian species

~90 endemic species-Bioma



Brazilian Atlantic Forest

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16% of the Brazilian territory

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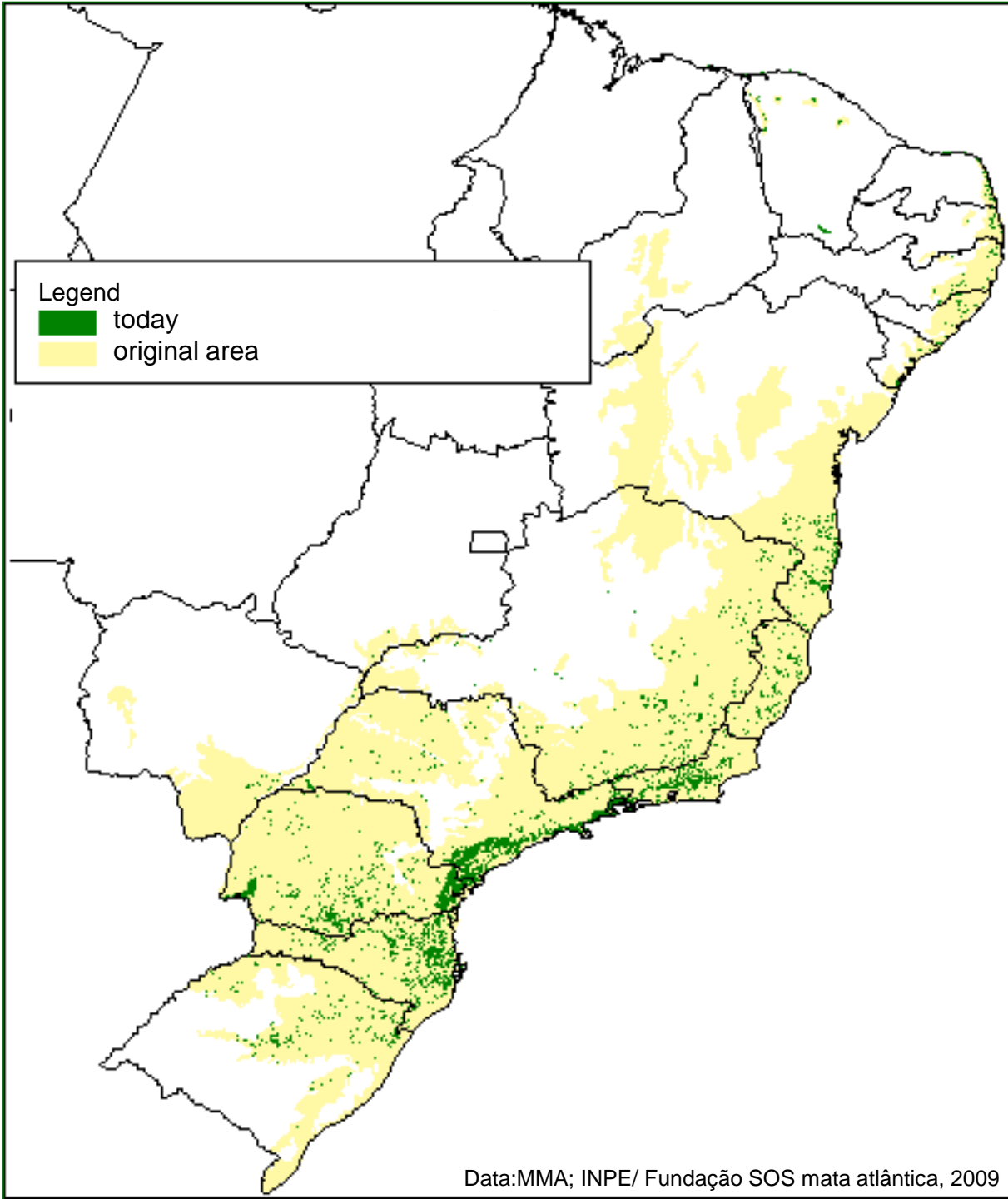
Biodiversity hotspots

460 amphibian species

~90 endemic species-Bioma

New species are described
every year

How many are
disappearing?





We need to know the species to conserve

DNA barcoding

has emerged at the forefront of efforts to accelerate the rates of taxonomic discovery



We need to know the species to conserve

DNA barcoding

has emerged at the forefront of efforts to accelerate the rates of taxonomic discovery

Amphibians COI DNA barcoding main problems:

- 1- the high variability of priming sites that hinder the application of universal primers
- 2- the observed distinct overlap of intraspecific and interspecific divergence values, which implies difficulties in the definition of threshold values to identify candidate species



Objectives

- 1- Design new primers for standard COI amplifications in Anurans**
- 2- Preliminary analysis of inter and intraspecific diversity in anurans from Brazilian atlantic forest: COI and 16S**
- 3- Case study in *Leptodactylus ocellatus* (Anura: Leptodactylidae)**
- 4- Case study in *Scinax fuscovarius* (Anura: Hylidae)**



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**CFBH tissue collection
(UNESP-Rio Claro)
1994-2009**

58 anuran species

14 different families



Aparasphenodon brunoi



Aplastodiscus callipygius



Xenohyla truncata



Proceratophrys appendiculata



Physalaemus atlanticus



Flectonotus sp.



Ceratophrys aurita



Phyllomedusa sp.



Arcovomer passarellii



Hypsiboas prasinus

Images: Célio F B Haddad



Ischnocnema sp.



Brachycephalus epphipium

**CFBH tissue collection
(UNESP-Rio Claro)
1994-2009**

58 anuran species

1 Caudata



Aparasphenodon brunoi



Aplastodiscus callipygius



Xenohyla truncata



Bolitoglossa paraensis



Flectonotus sp.



Phyllomedusa sp.



Arcovomer passarellii



Hypsiboas prasinus

Images: Célio F B Haddad



Ischnocnema sp.



Brachycephalus epphipium

**CFBH tissue collection
(UNESP-Rio Claro)
1994-2009**

58 anuran species

1 Caudata

3 Gymnophiona



Aparasphenodon brunoi



Aplastodiscus callipygius



Xenohyla truncata



Siphonops paulensis



Flectonotus sp.



Phyllomedusa sp.



Arcovomer passarellii



Hypsiboas prasinus



Ischnocnema sp.



Brachycephalus epphipium

Images: Célio F B Haddad



Primers design

1- Previous works in Amphibian Barcode

Vences et al. 2005 – (Amphibians from Madagascar)

Smith et al. 2008 (Holoartic amphibians)

2 – 33 complete COI sequences (GeneBank)



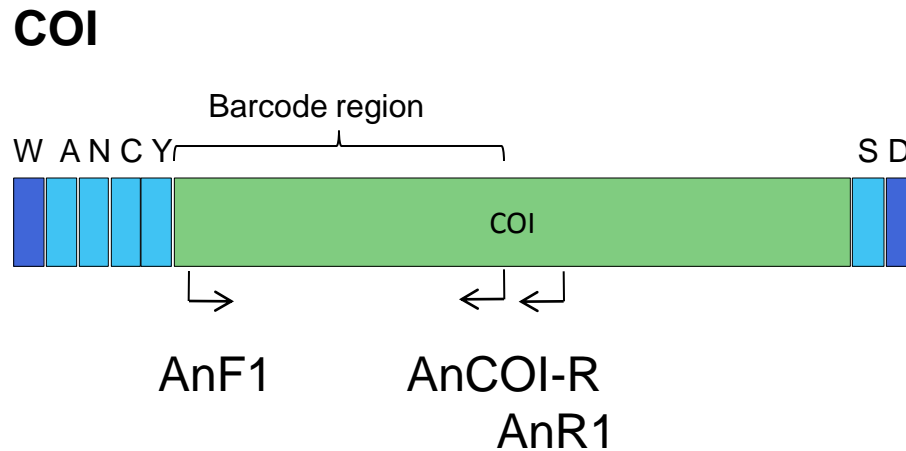
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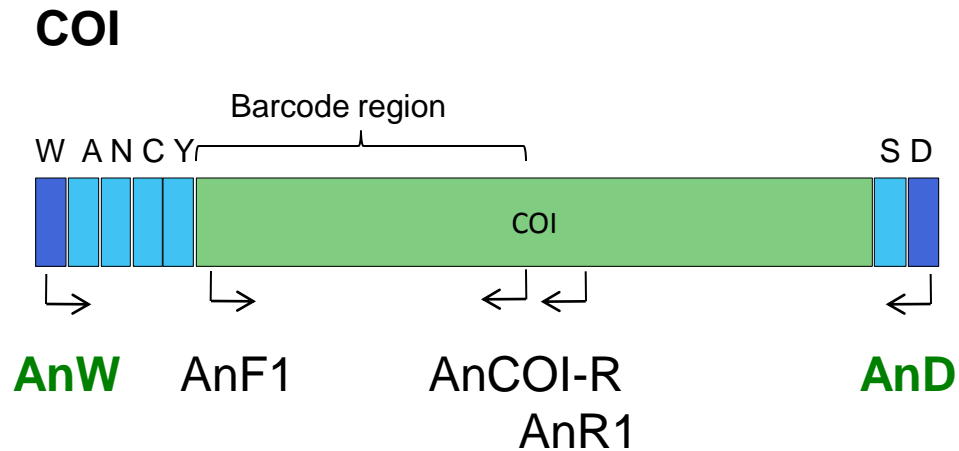
Primers design

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Nested or Semi-nested PCR- Museum specimens



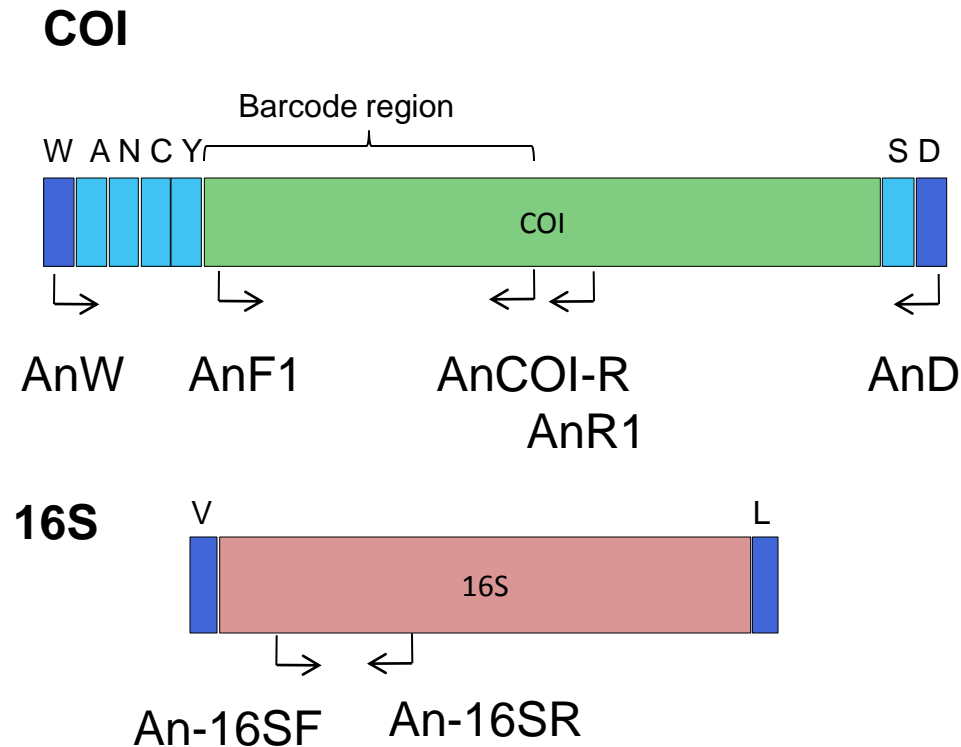
Primers design

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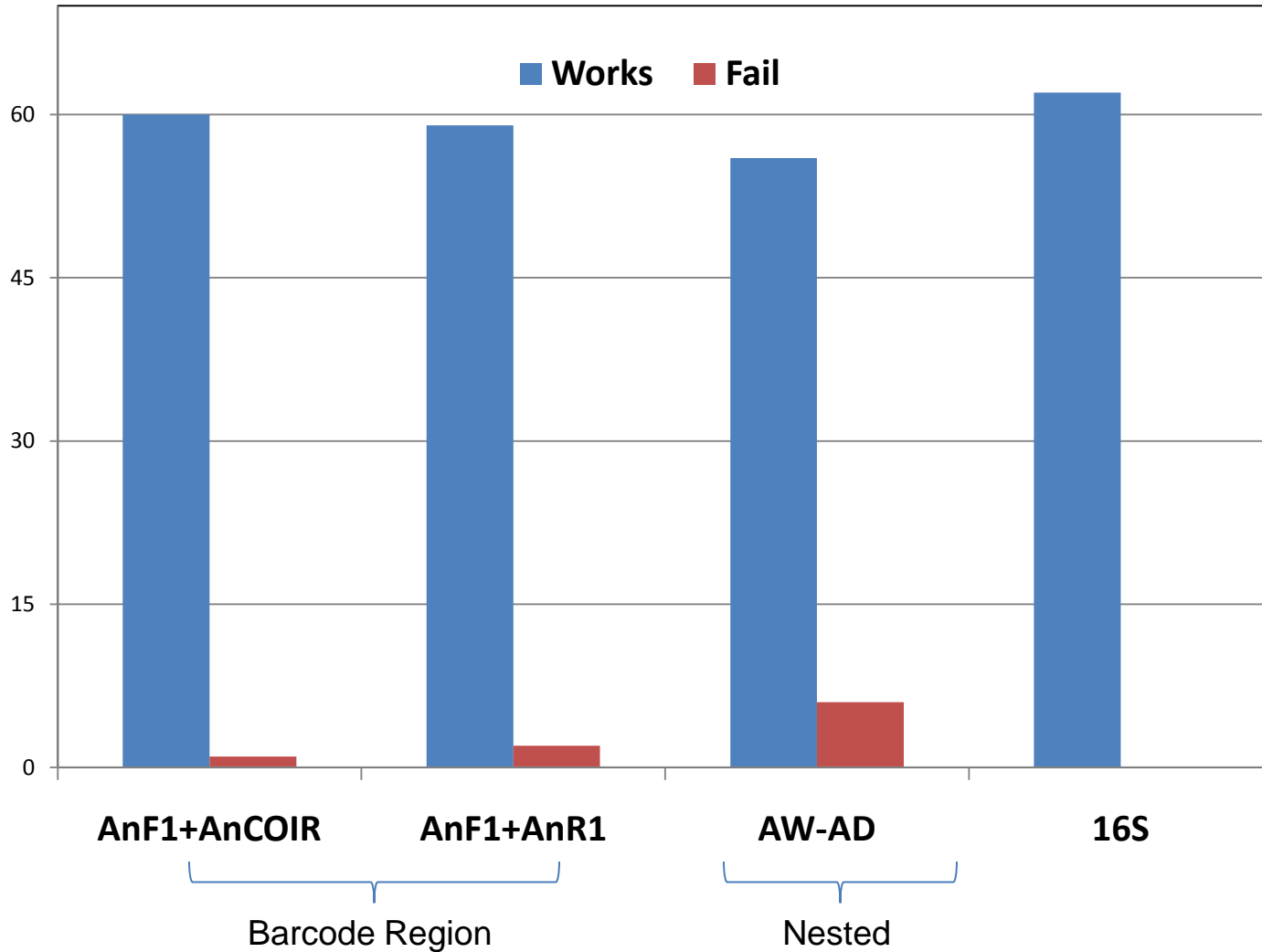
2 – 33 complete COI sequences (GeneBank)





Primers design: Results

COI and 16S - All species amplified



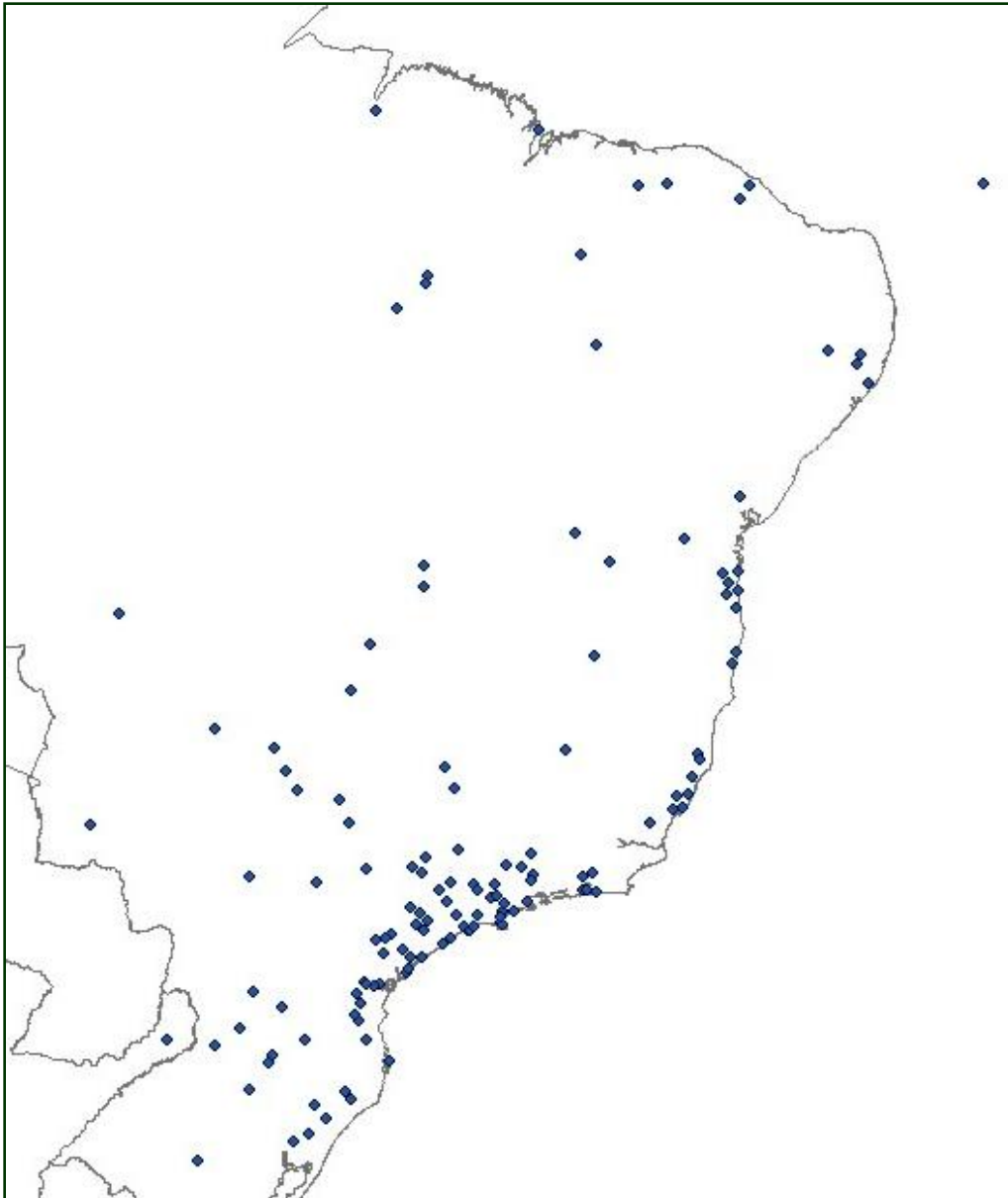


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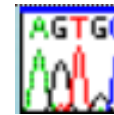


Intra and interspecific diversity – COI x 16S



Number species Anura:
58

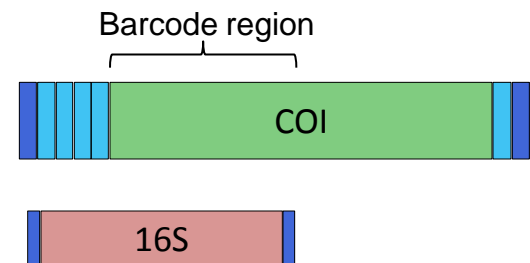
Number individuals:
170
~3 ind/ sp



BARCODE OF LIFE DATA SYSTEMS
Advancing species identification and discovery through the analysis of short, standardized gene regions

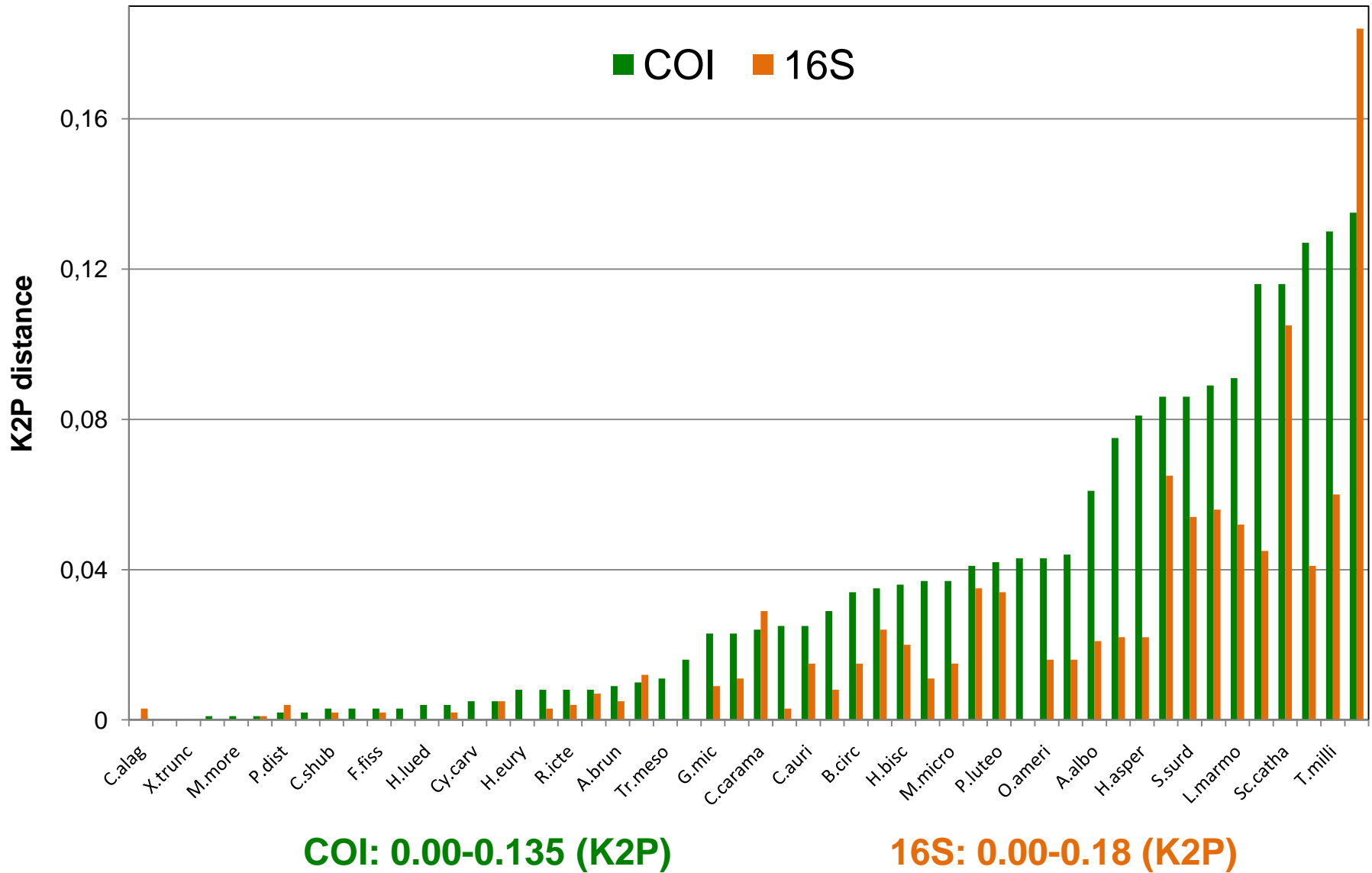
A banner for the Barcode of Life Data Systems project, featuring a row of five small images: a forest, a bee, a flower, a butterfly, and a hummingbird.

Published Projects | Taxonomy Browser | Request an Account | Identify Specimen | Introductory Tutor



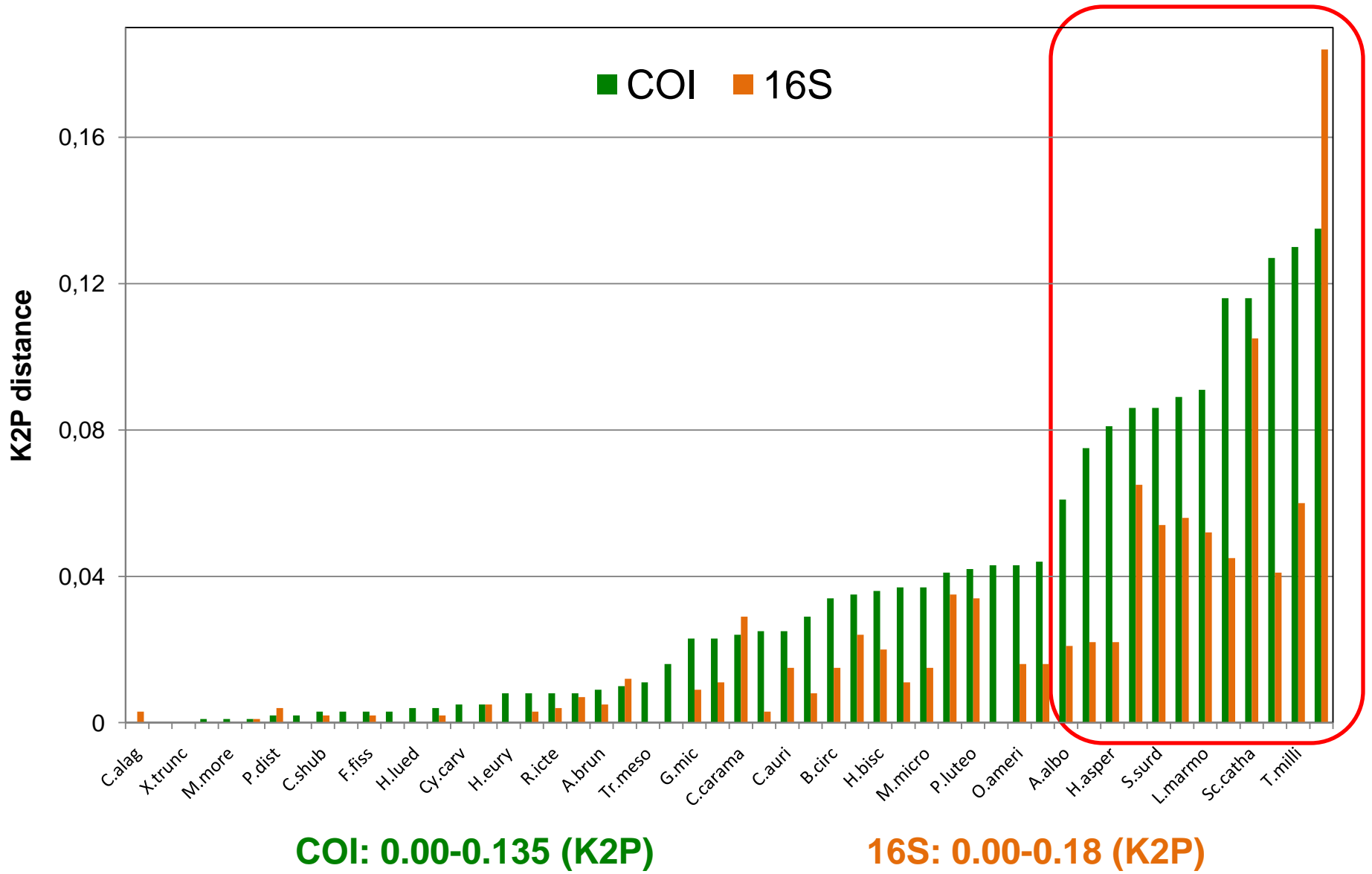


Intraspecific diversity – COI x 16S



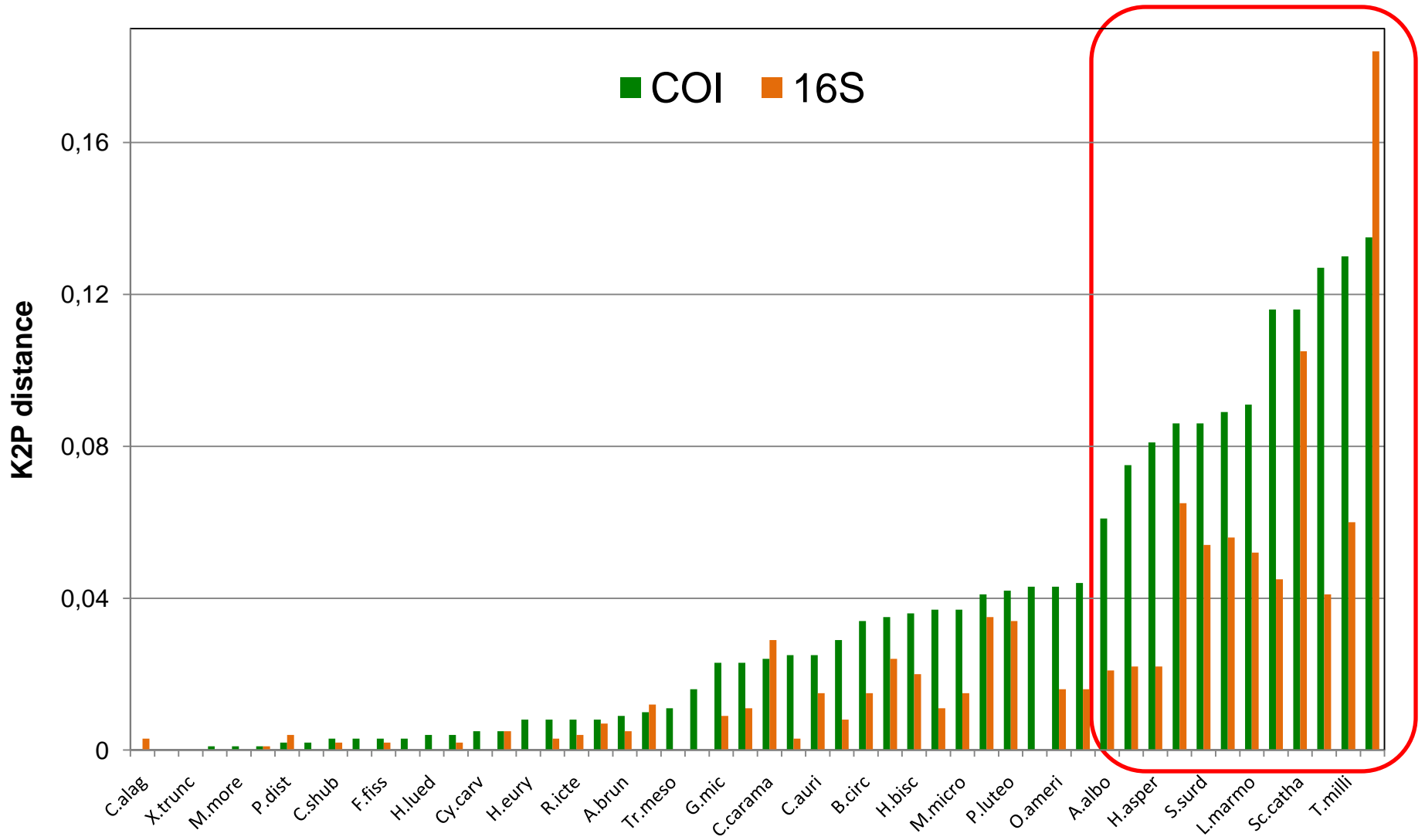


Intraspecific diversity – COI x 16S





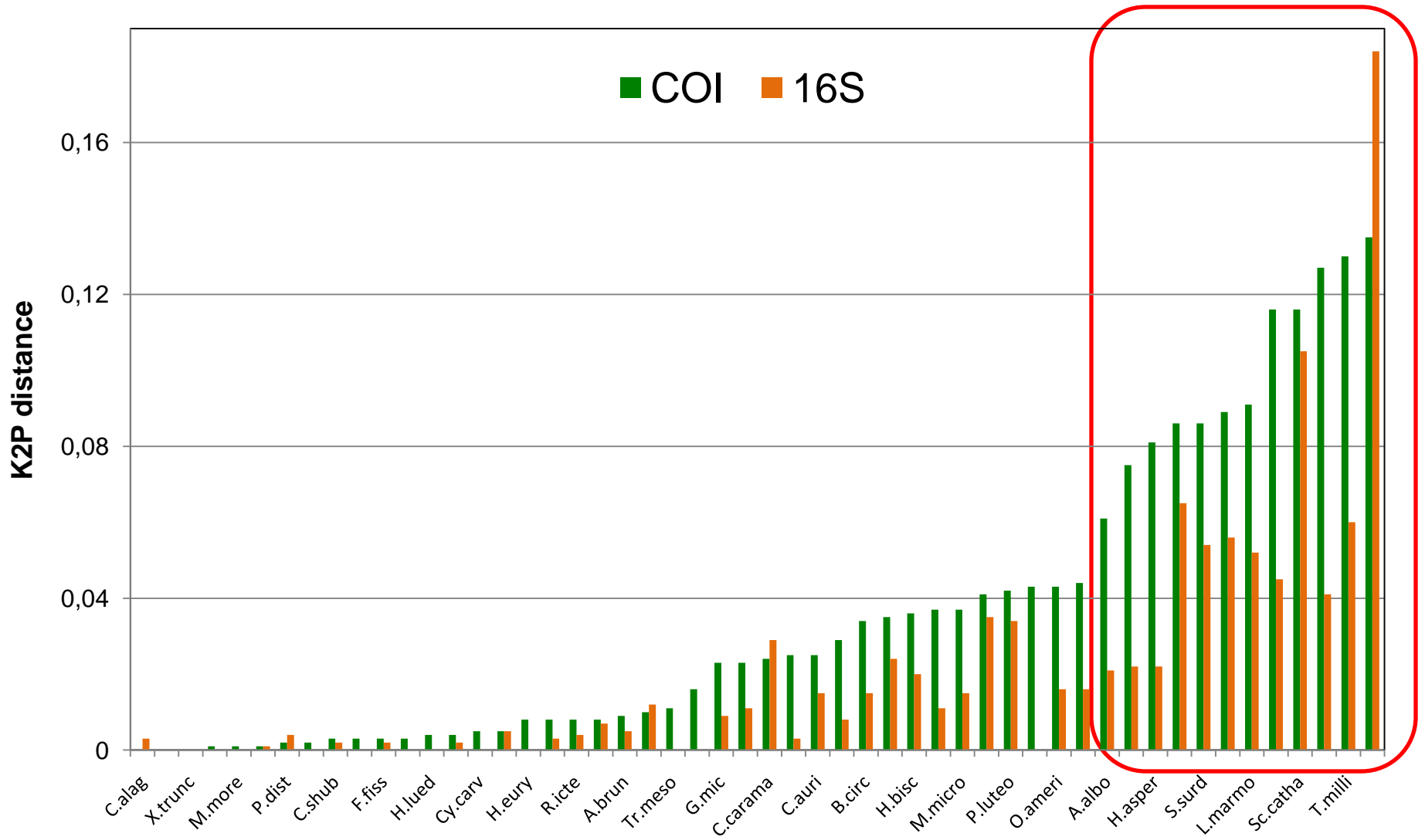
Intraspecific diversity – COI x 16S



Highly correlated with geographical distance



Intraspecific diversity – COI x 16S



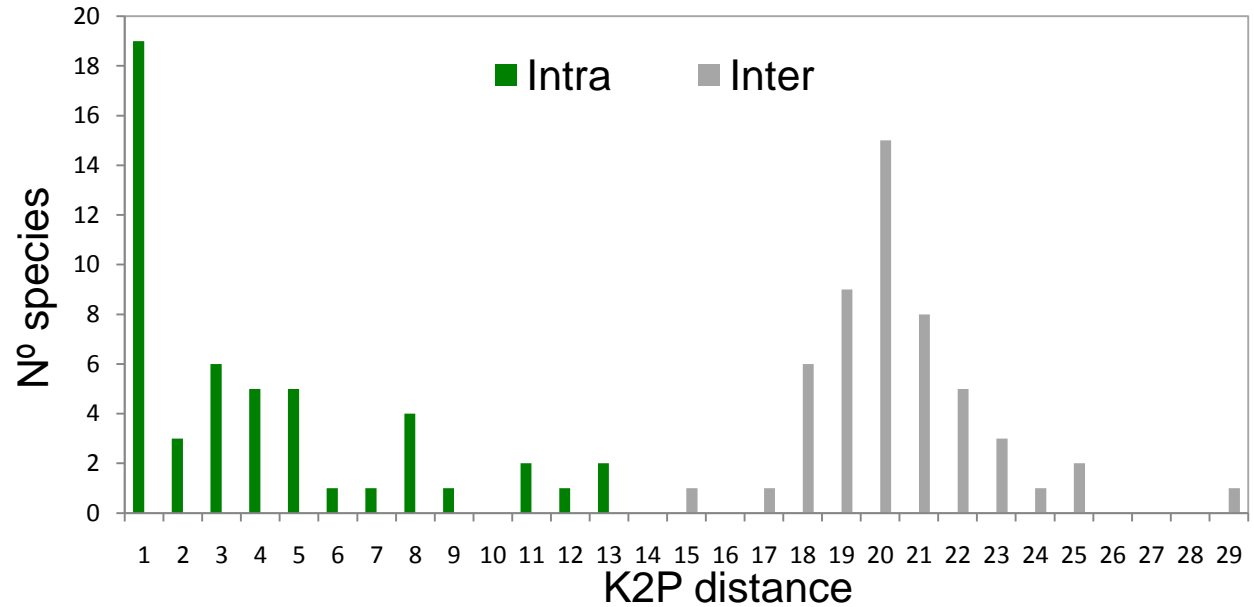
Highly correlated with geographical distance
Potential new species? – look for other evidences



Interspecific diversity – COI x 16S

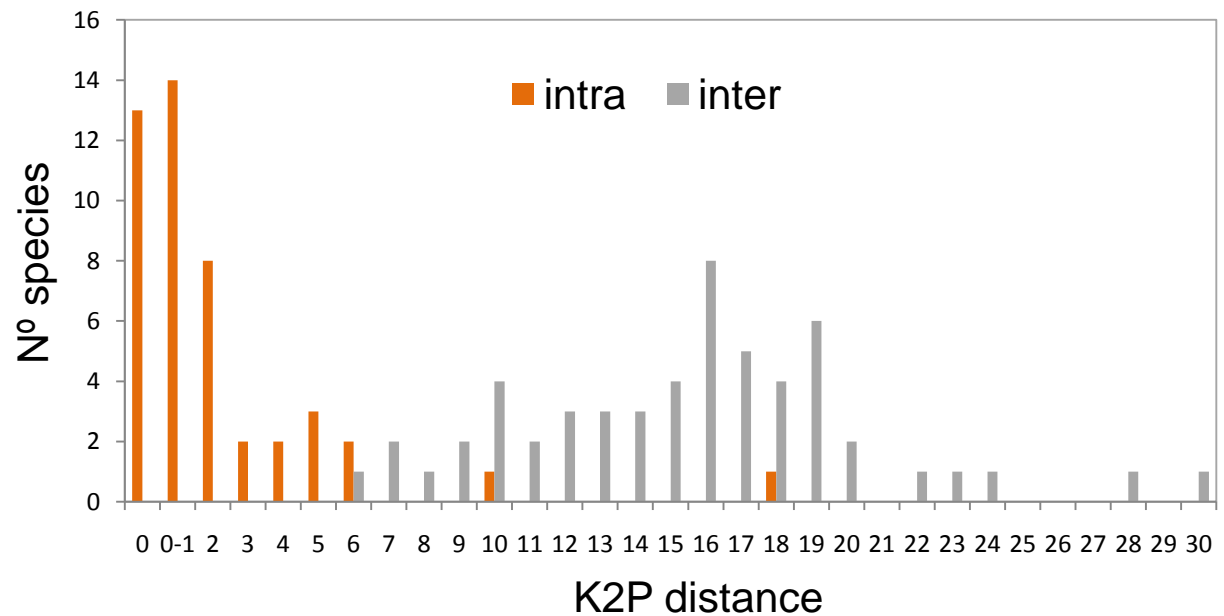
COI

**Interspecific variation
15-29%**



16S

**Interspecific variation
6-30%**



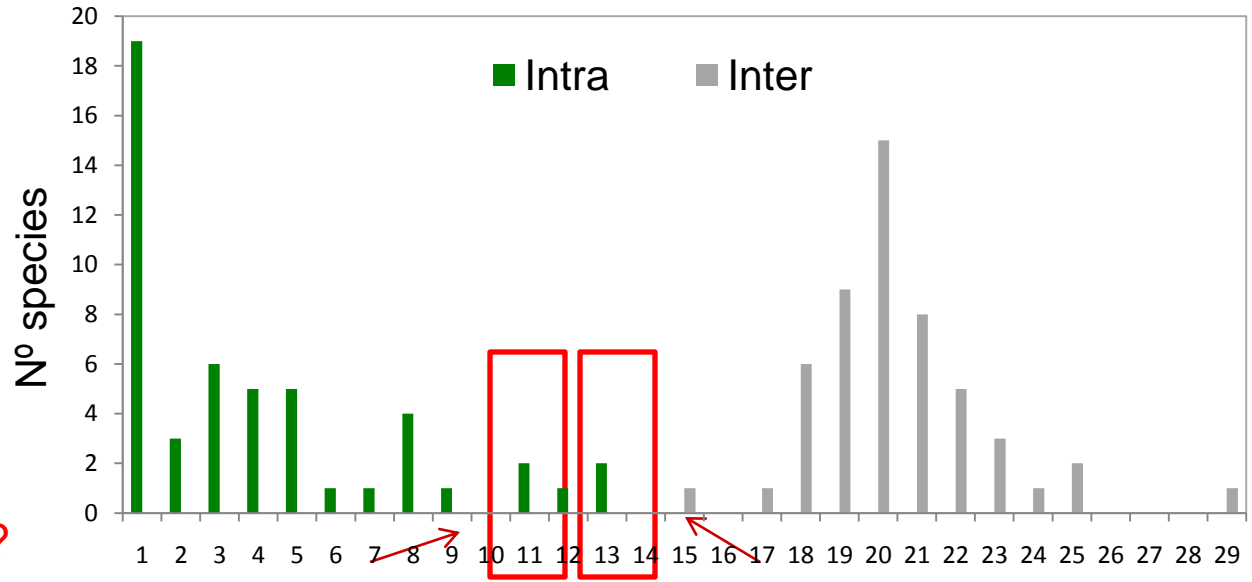


Interspecific diversity – COI x 16S

COI

**Interspecific variation
15-29%**

Candidate new species?

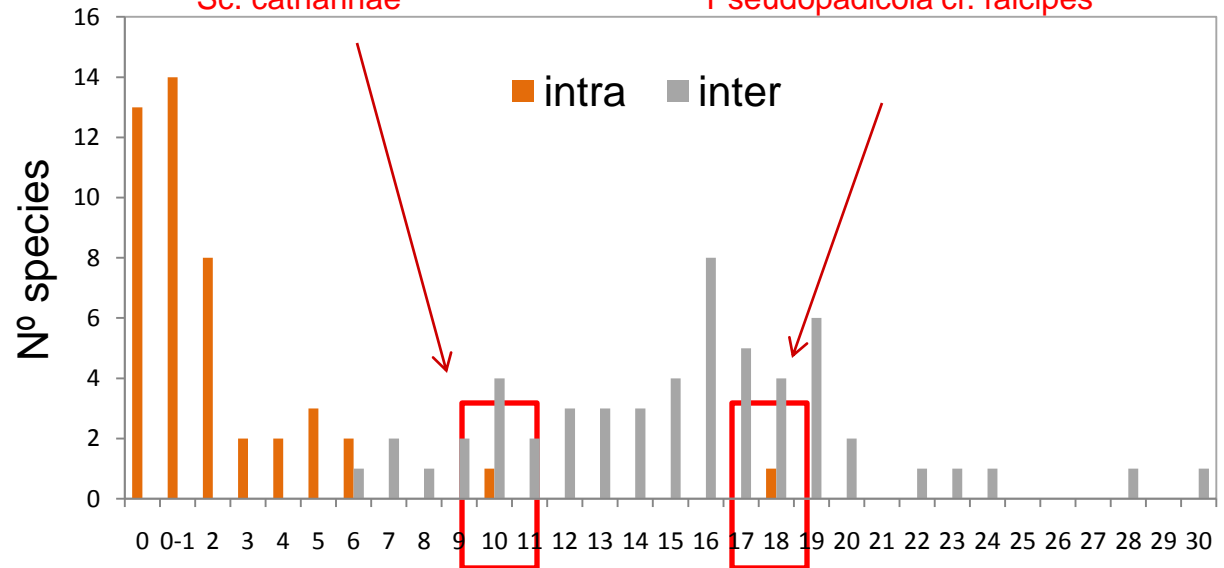


Sc. catharinae

Pseudopadicola cf. falcipes

16S

**Interspecific variation
6-30%**





Interspecific diversity – COI x 16S

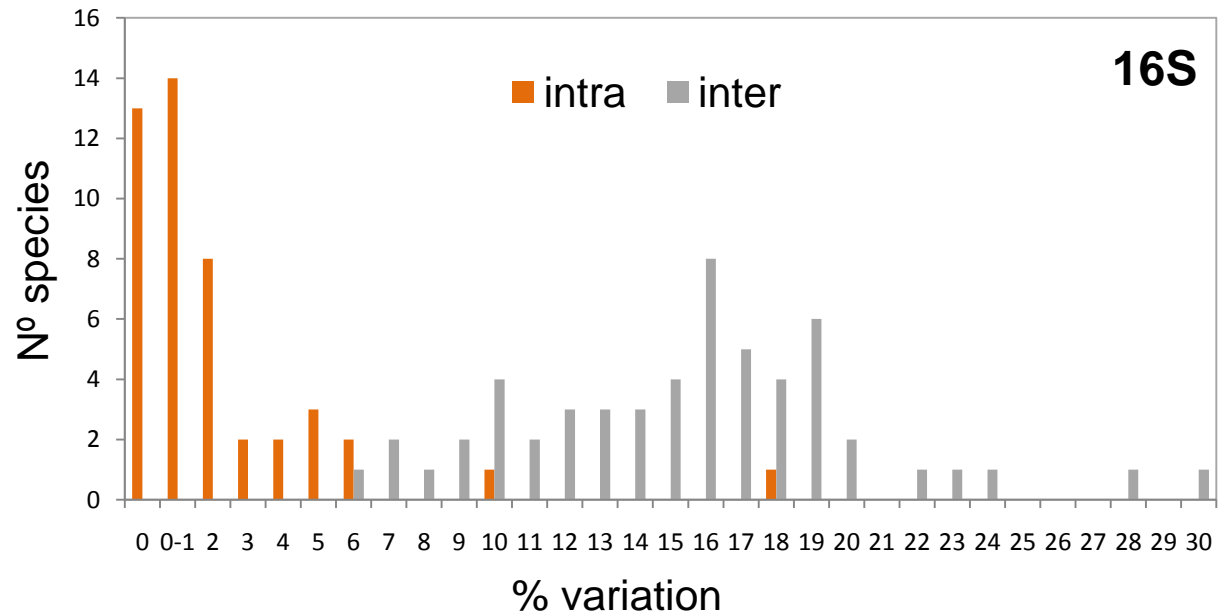
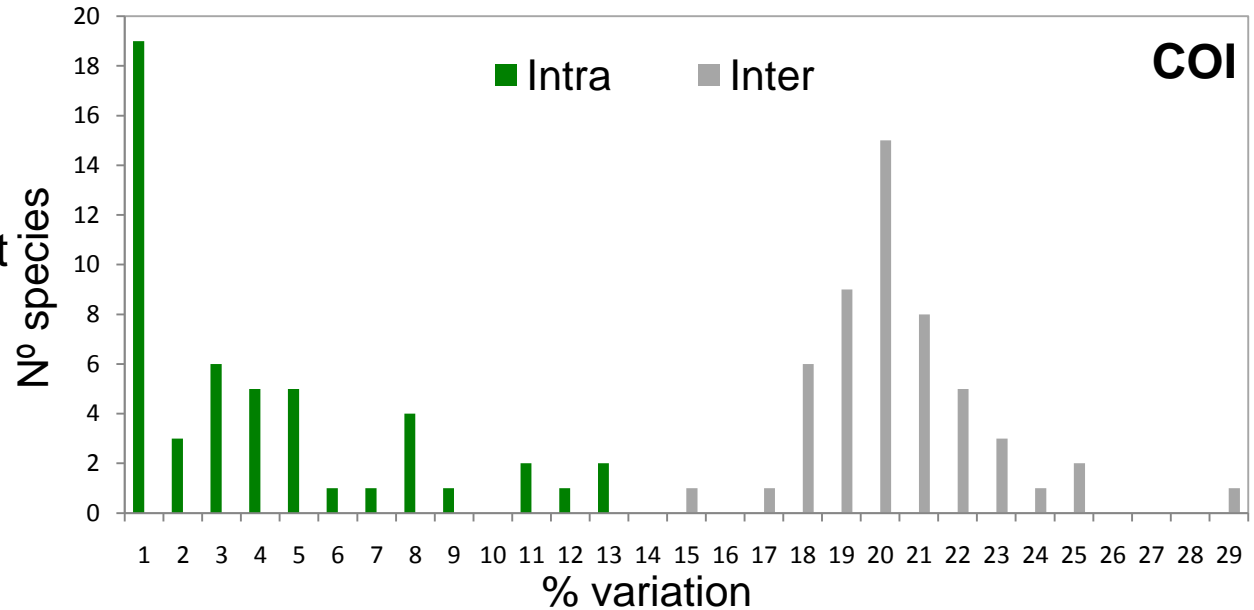
BUT

Data came from very distinct anuran families

Interspecific variation are probably over-estimated

We need to include more congeneric species to understand intra and interspecific variation in anurans

Can not suggest a threshold values to identify candidate species based on this data



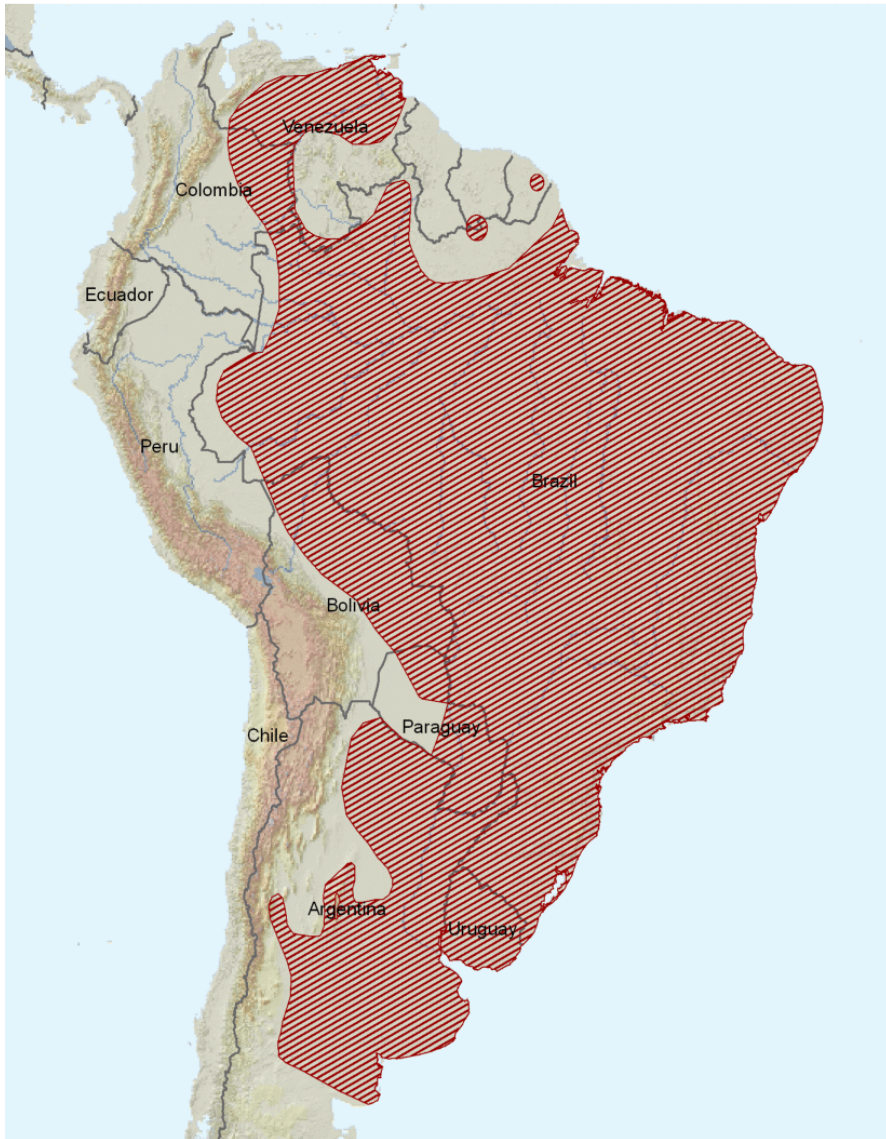


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Leptodactylus ocellatus (Linnaeus 1758)

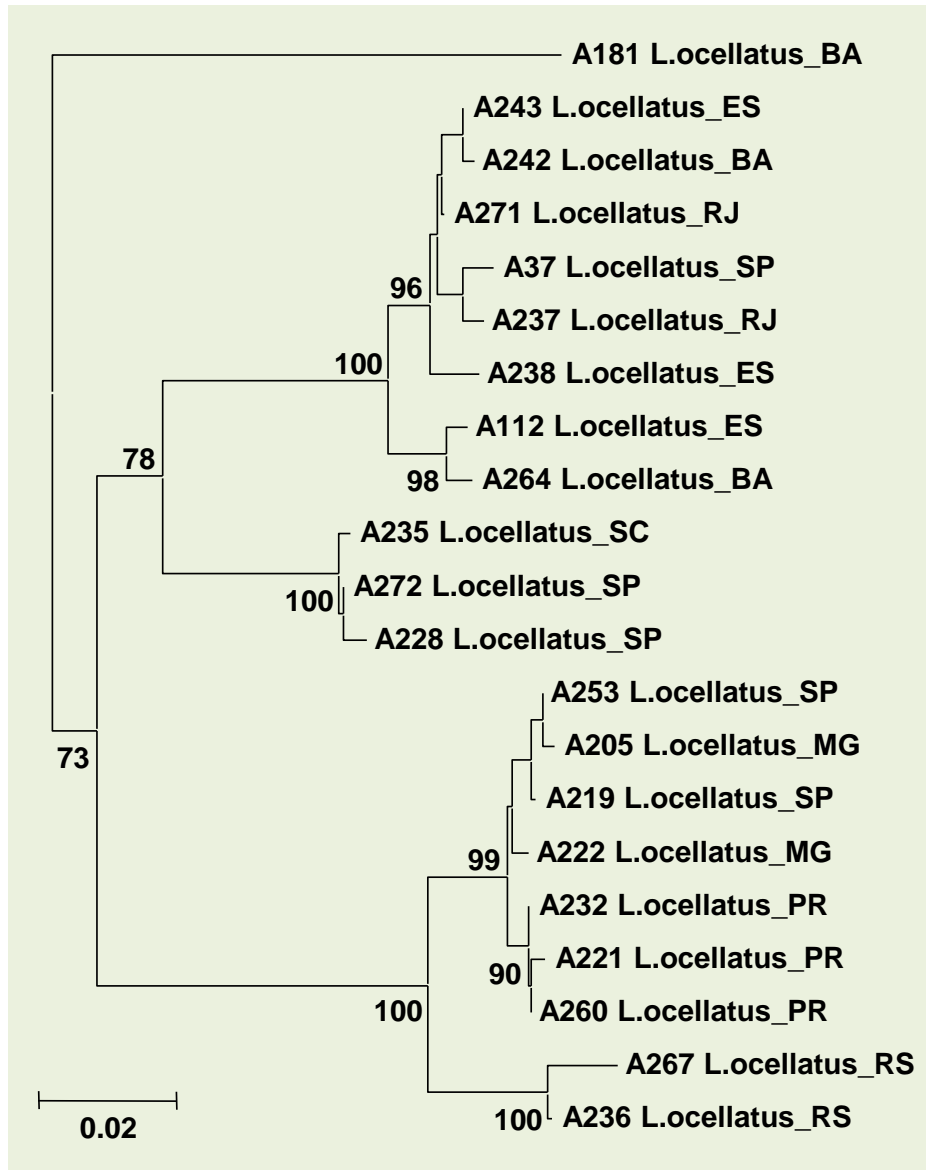


21 specimens

<http://www.iucnredlist.org/details/57151/0>



Leptodactylus ocellatus: tree

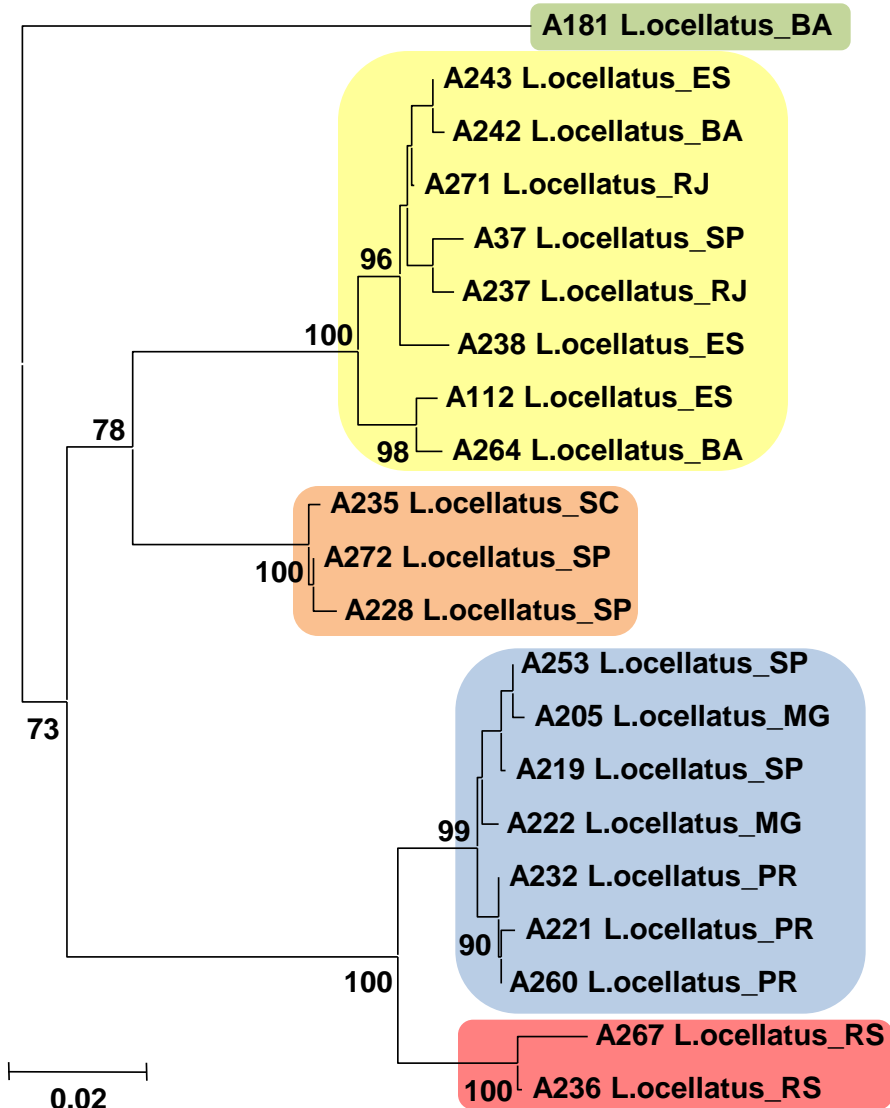


Neighbor-joining/ K2P
AND
Gen. Mix.Yule-Coalescent model – GMYC
(Bayesian analysis)



Leptodactylus ocellatus: Clades

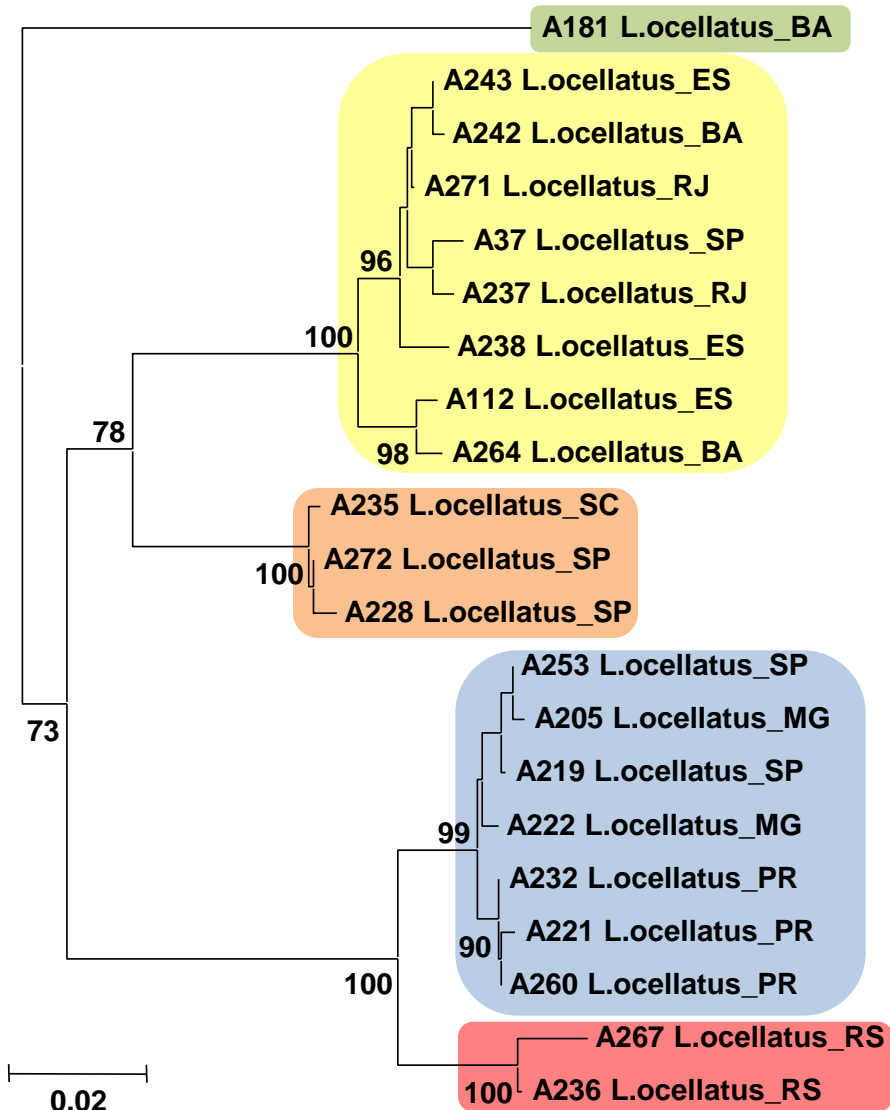
COI – NJ K2P



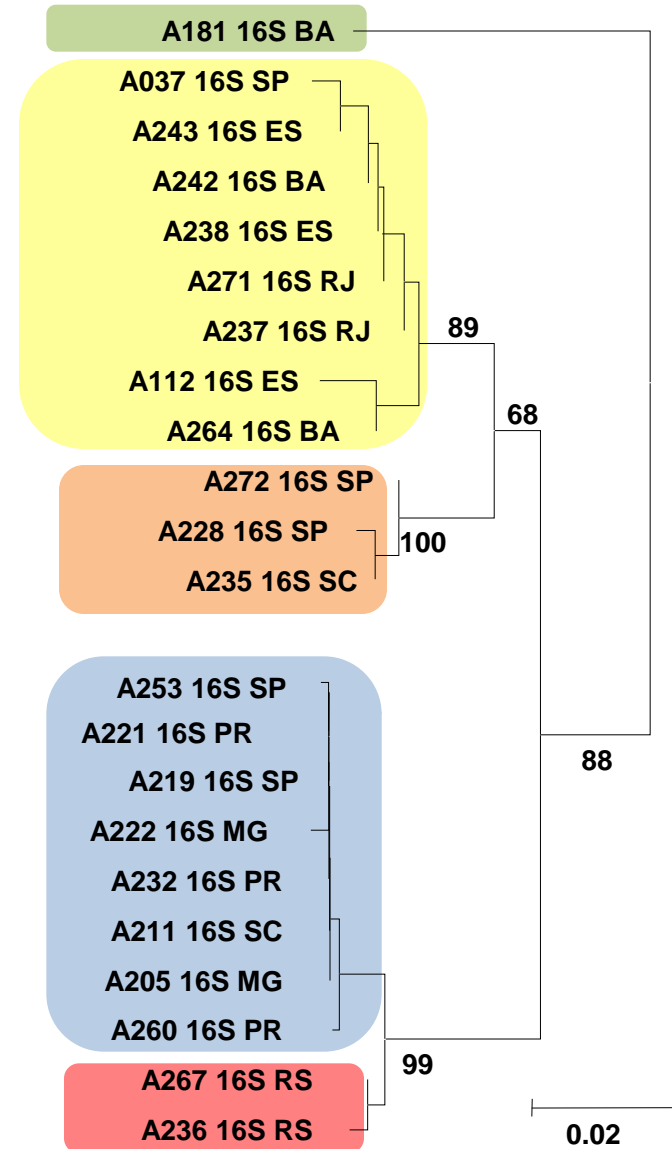


Leptodactylus ocellatus: clades COI x 16S

COI – NJ K2P

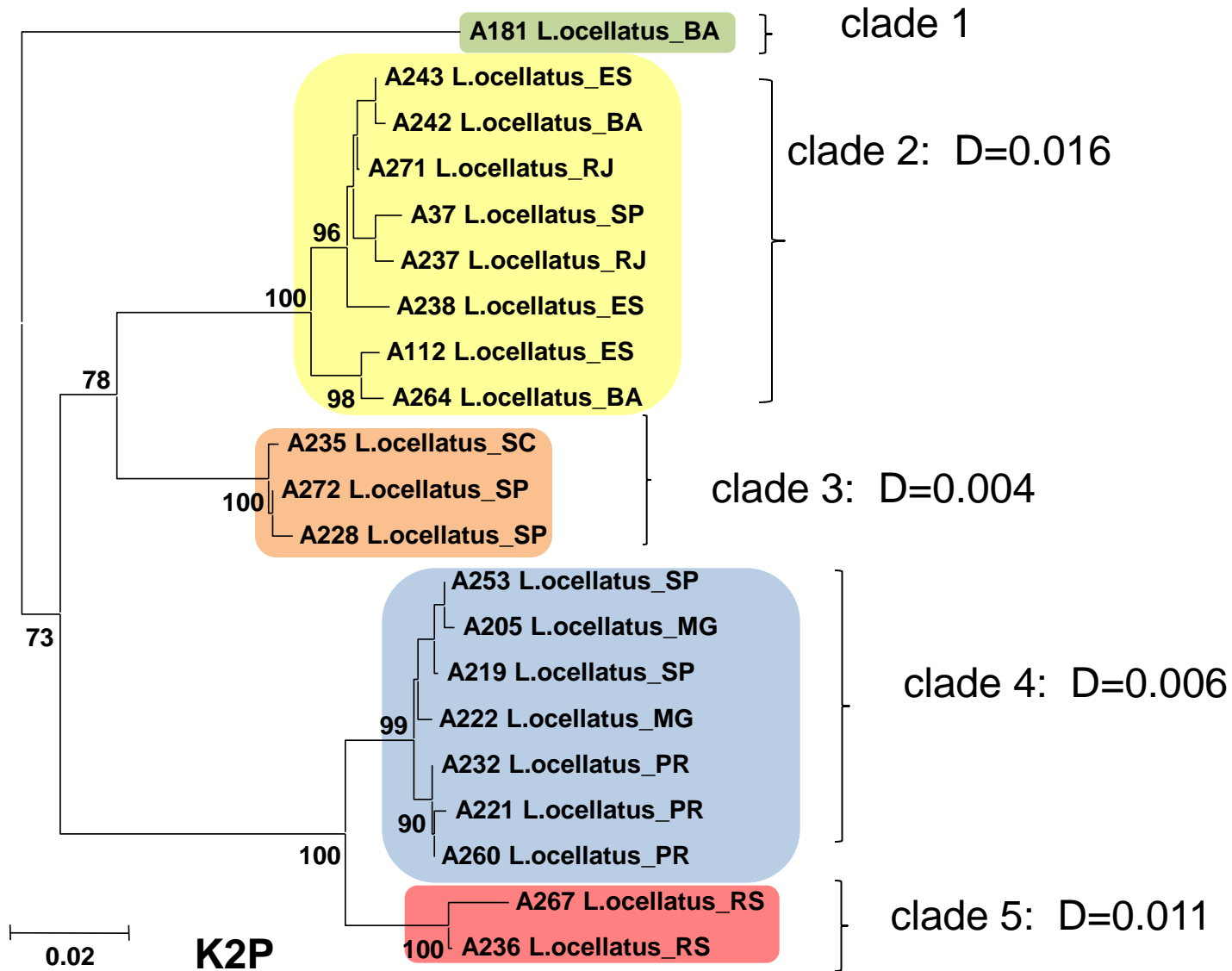


16S – NJ K2P



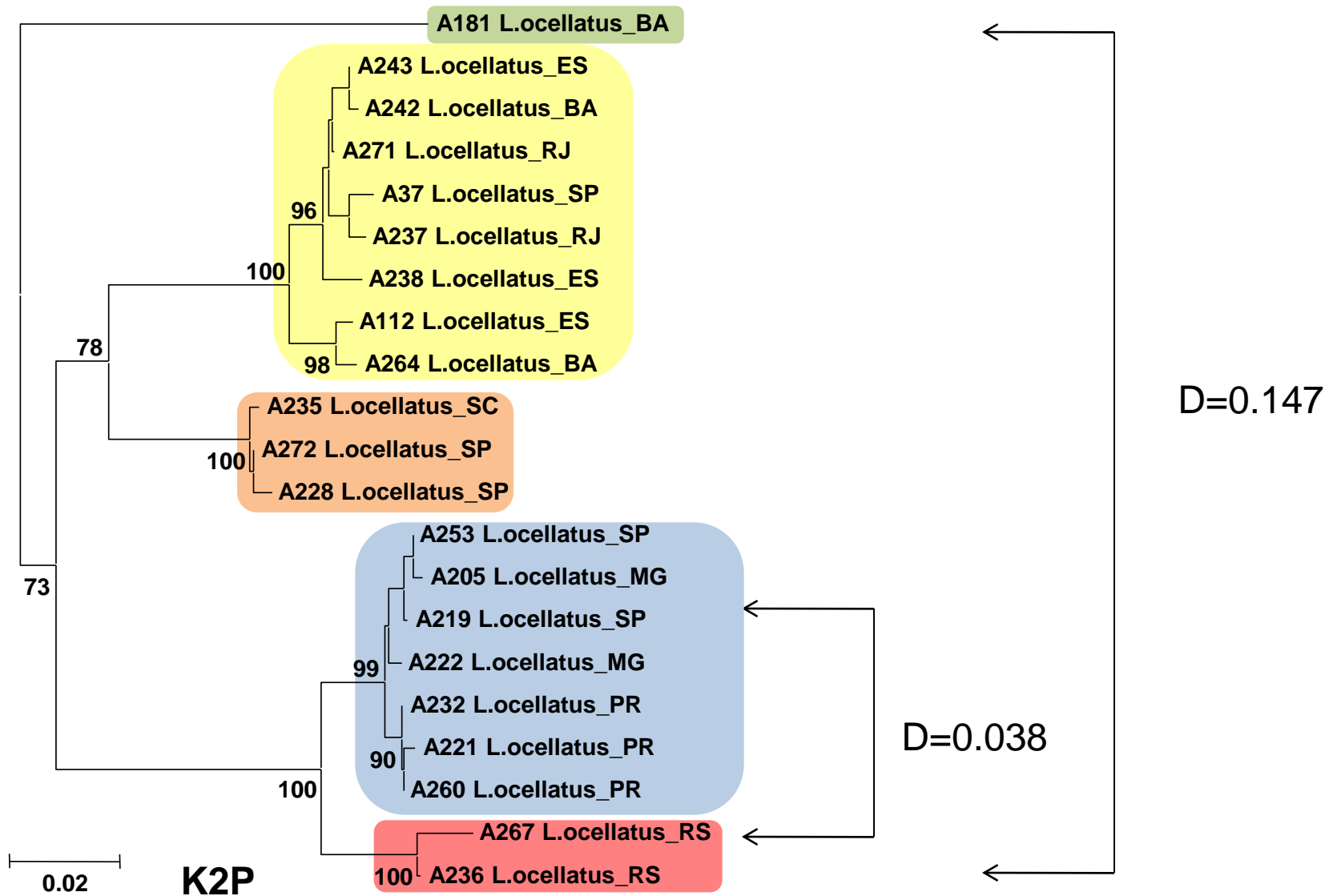


Leptodactylus ocellatus: intra-clade diversity



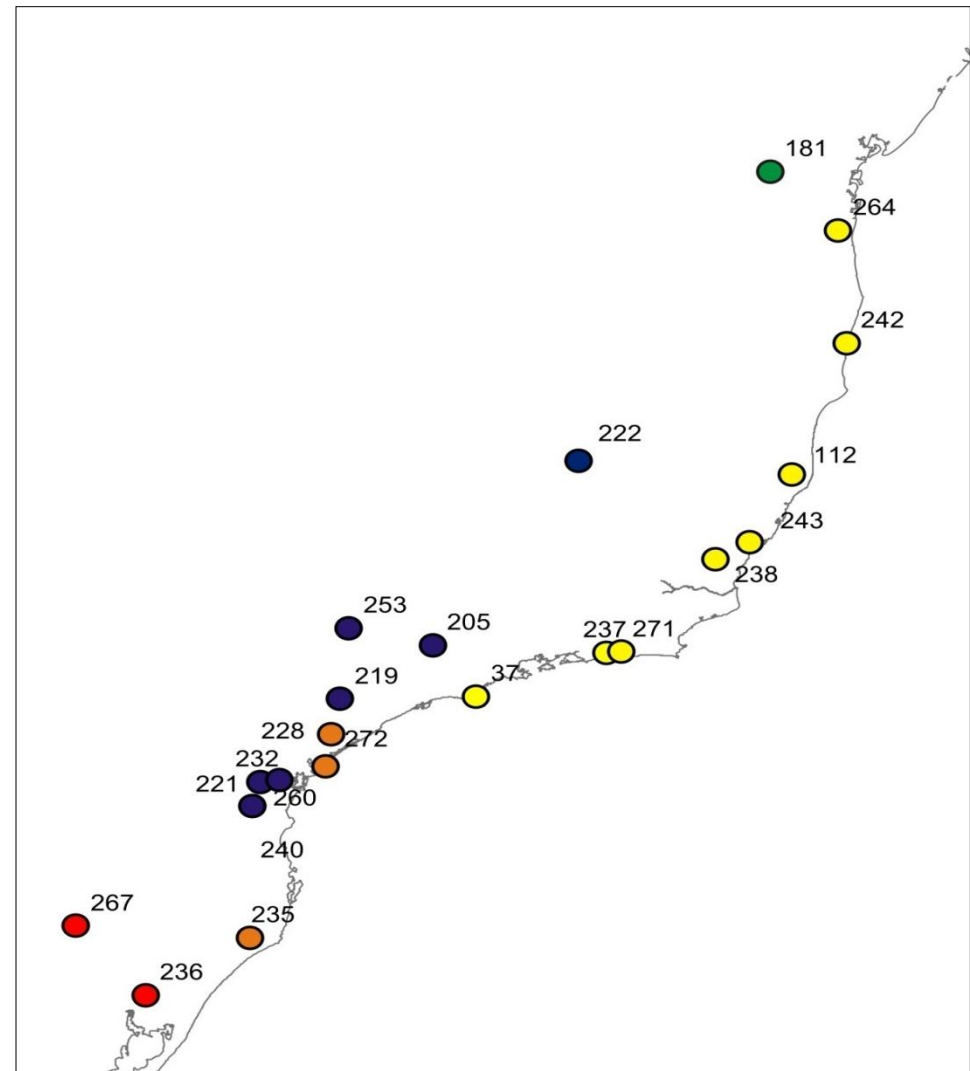
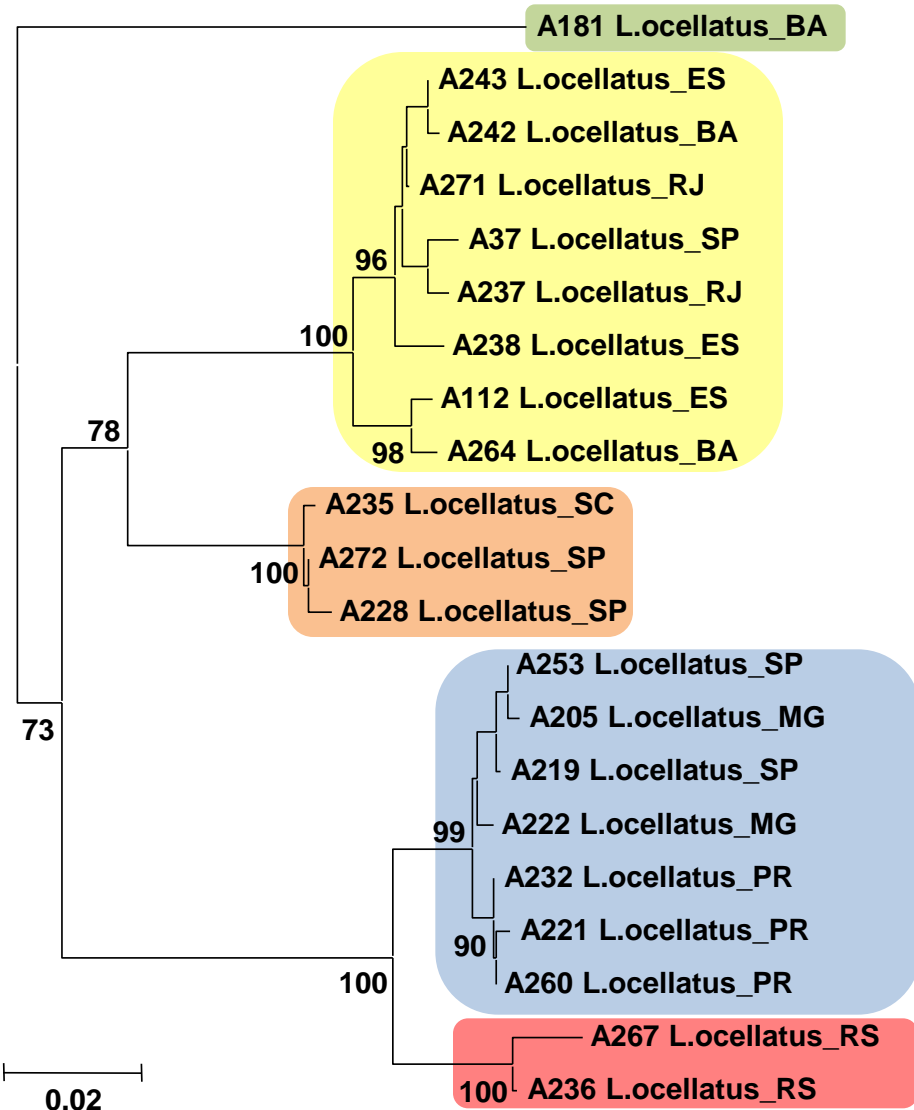


Leptodactylus ocellatus: inter-clade diversity





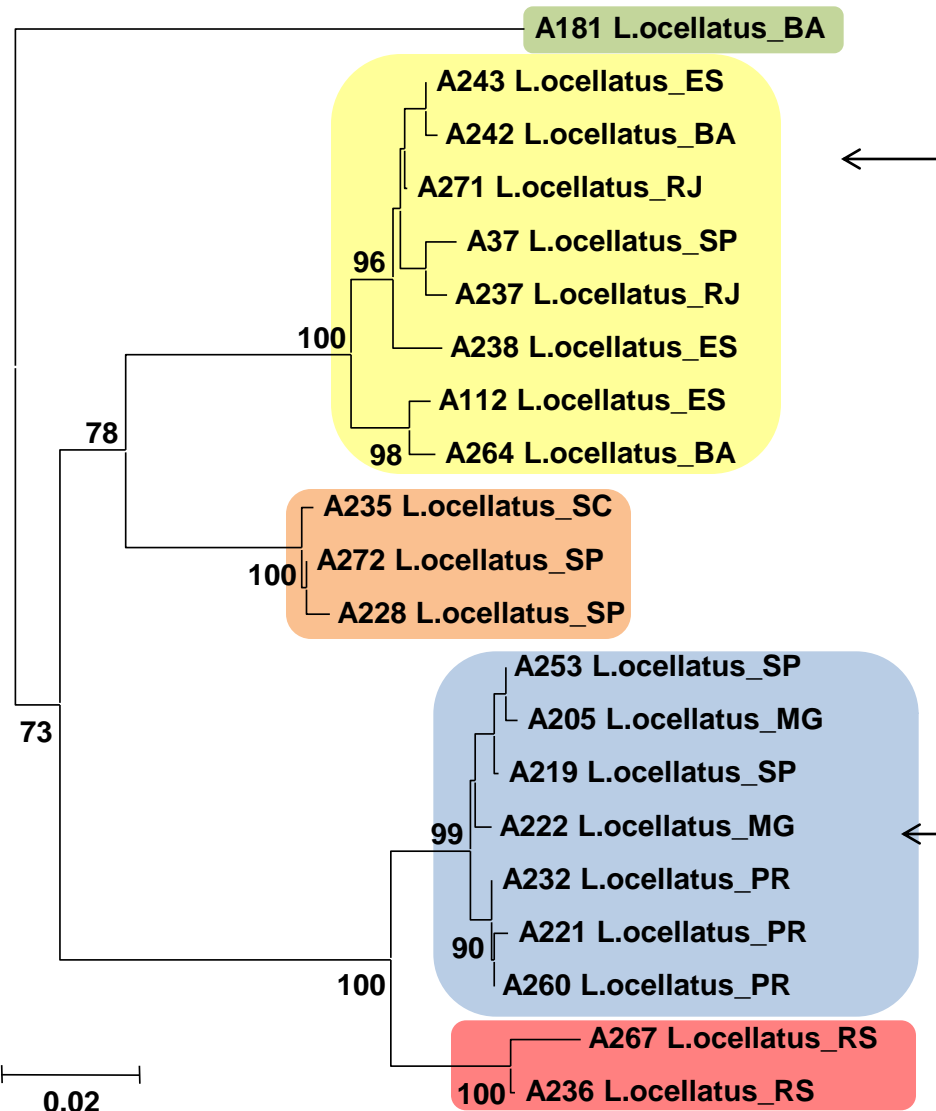
Leptodactylus ocellatus: geographic distribution



Strong correlated with geographical distribution



Candidate new species?

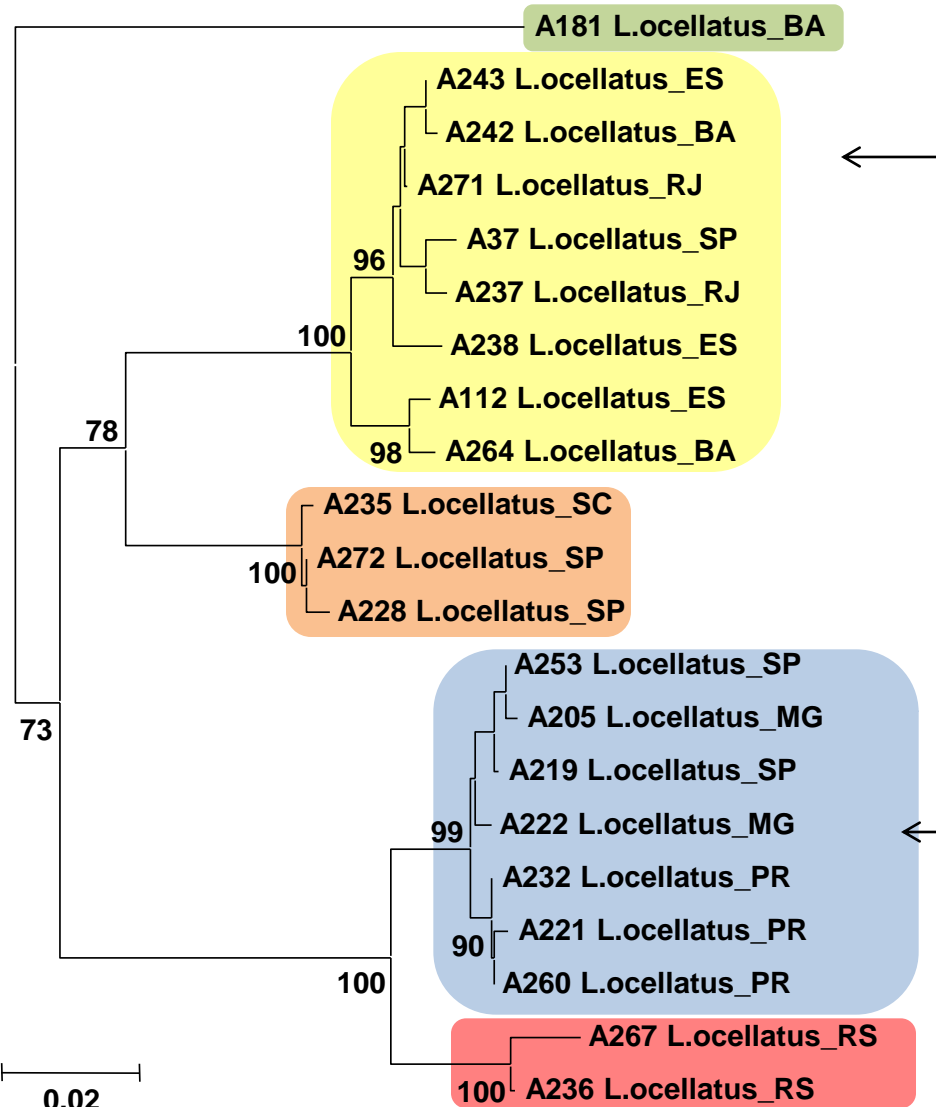


Bioacoustics and chromosomal differences
– coastal plain x plateau areas

(Silva et al. 2000)



Candidate new species?



Bioacoustics and chromosomal differences
– coastal plain x plateau areas

(Silva et al. 2000)

Diversity is underestimated

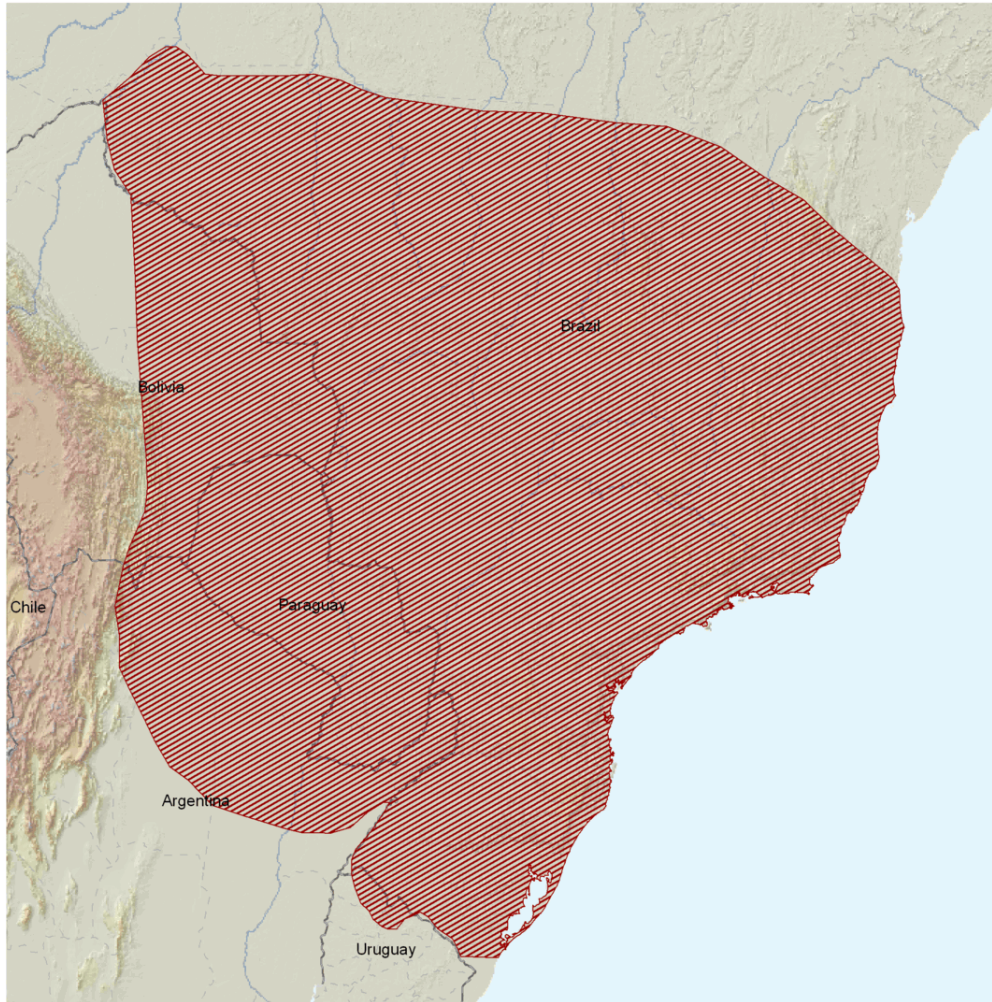


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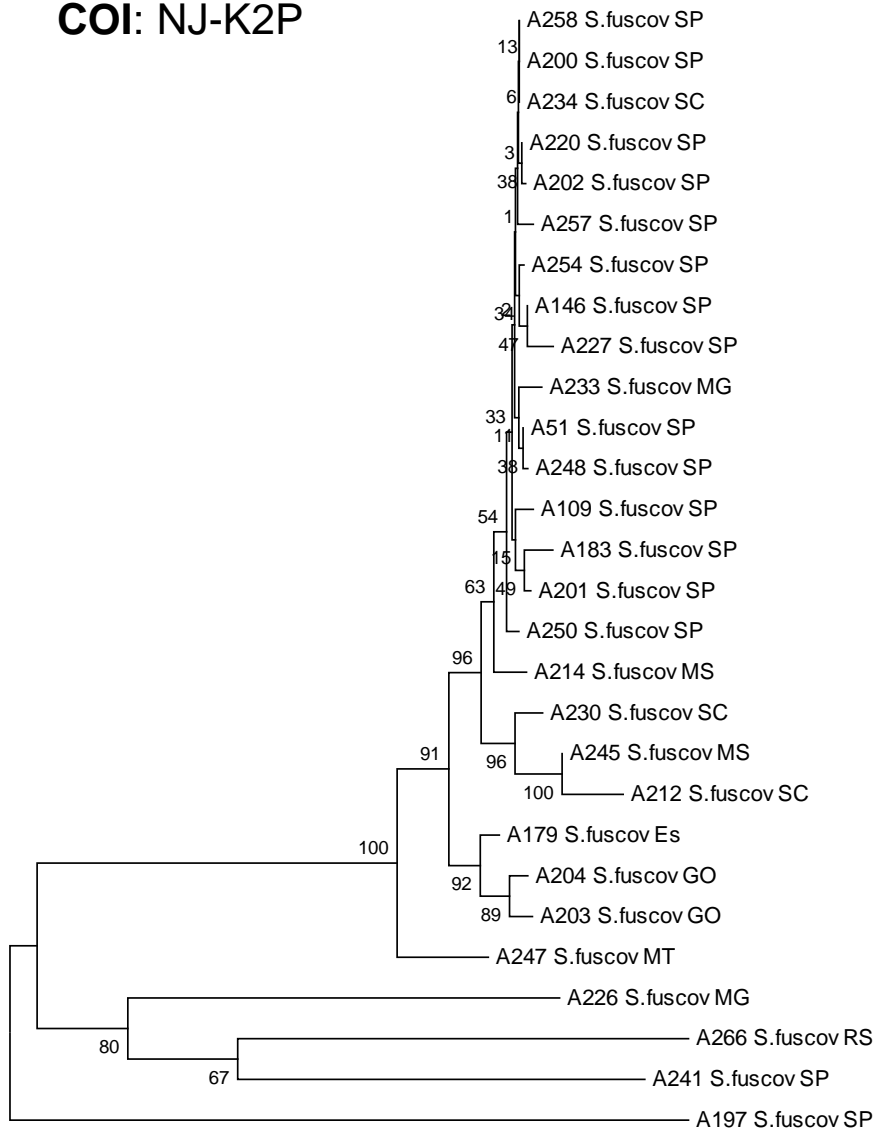
Scinax fuscovarius (Lutz, 1925)





Scinax fuscovarius: tree

COI: NJ-K2P



0.02



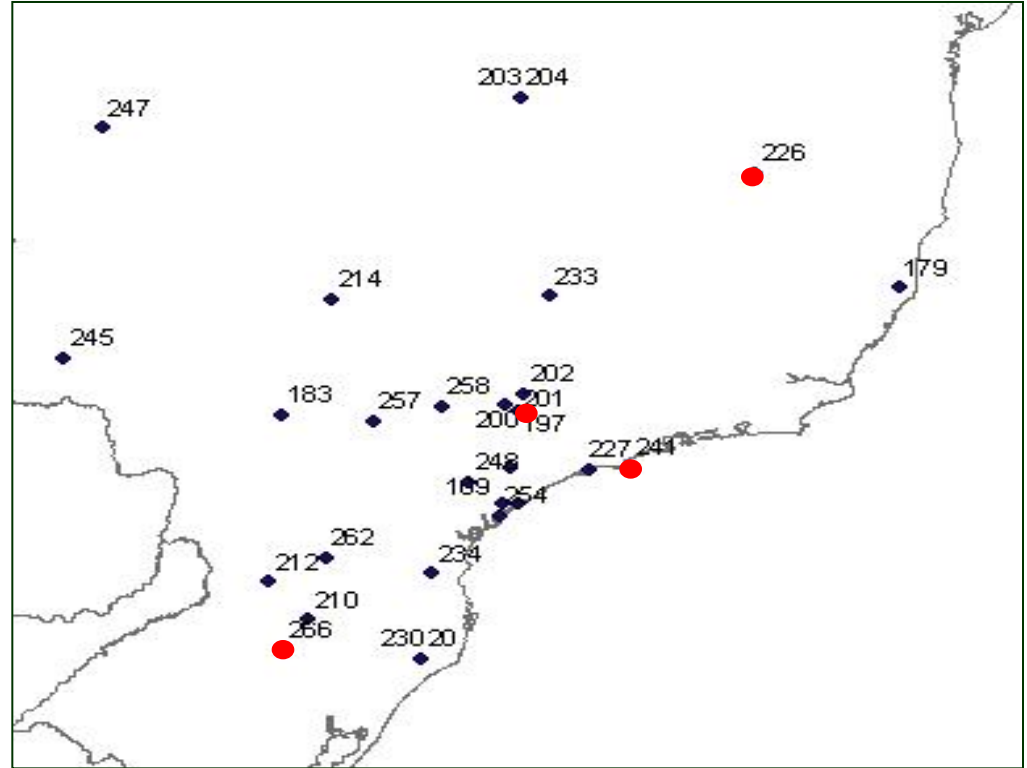
Scinax fuscovarius: geographic distribution

COI: NJ-K2P



Diversity

0 – 24%



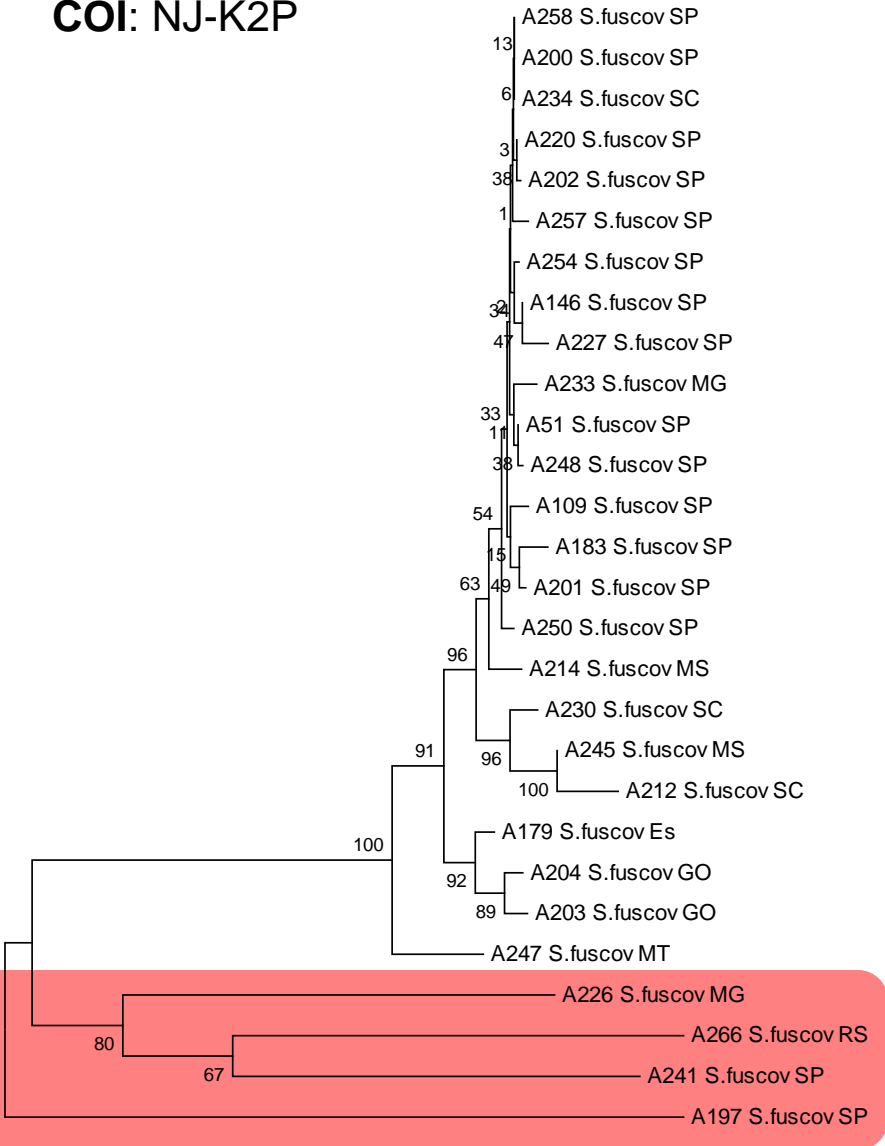
Not correlated with geographic distance

0.02



Scinax fuscovarius: collection

COI: NJ-K2P



Specimens collection:

Correction

- A197 *Scinax* sp.
- A226 *Sc. eurydice*
- A241 *Sc. cf. hayii*
- A266 *Scinax* sp.

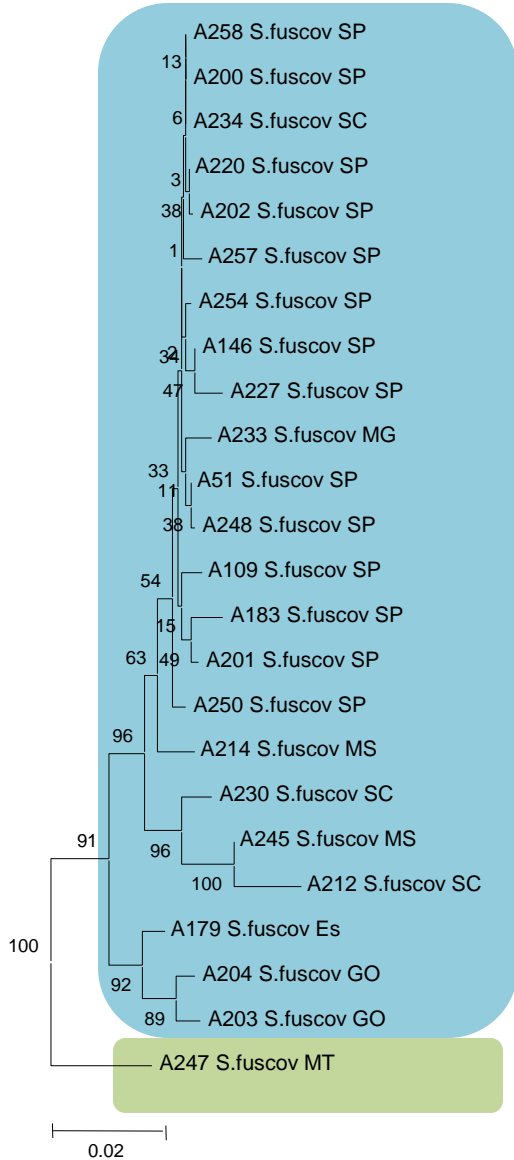


0.02



Scinax fuscovarius: synonymy

COI: NJ-K2P

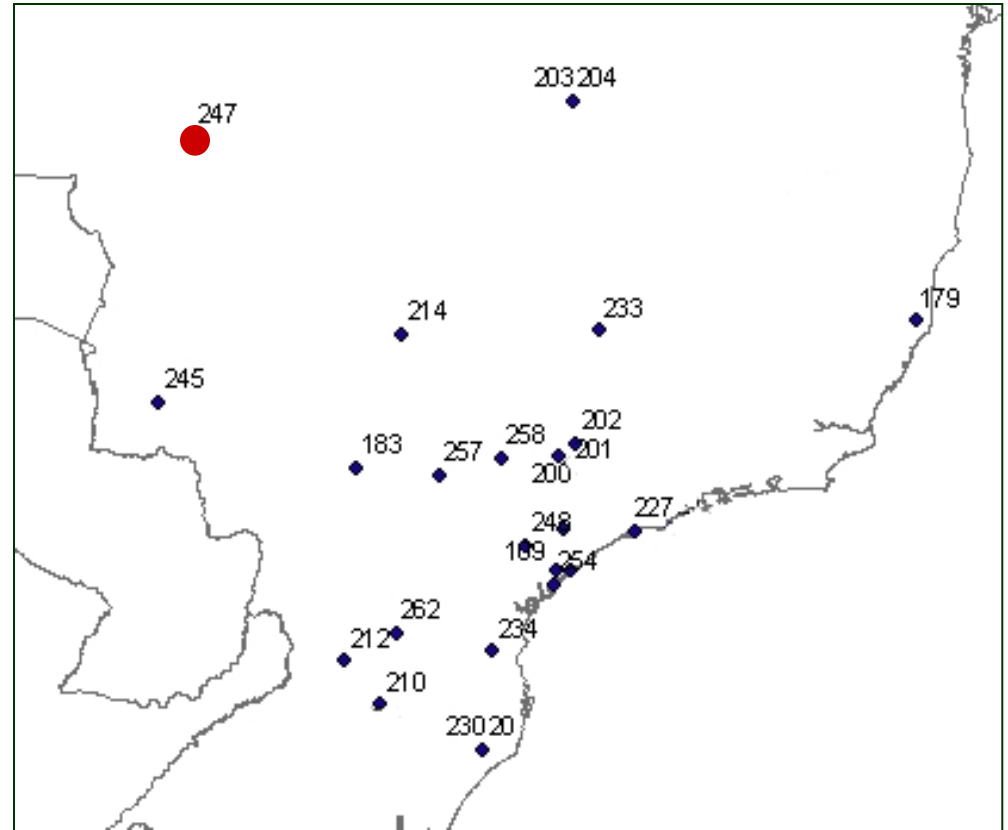
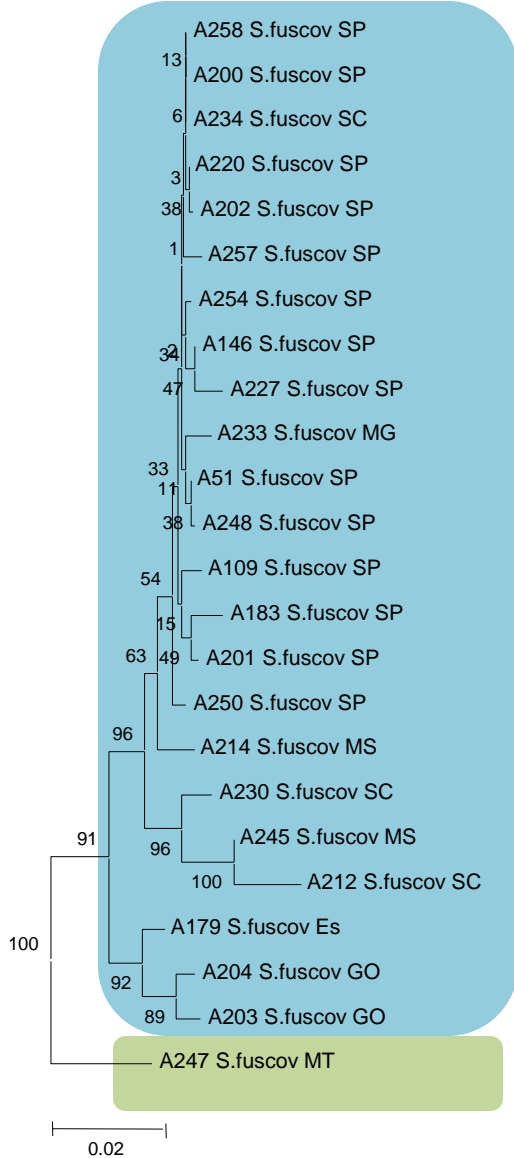


Diversity:
3.6 - 4.4%



Scinax fuscovarius: synonymy

COI: NJ-K2P

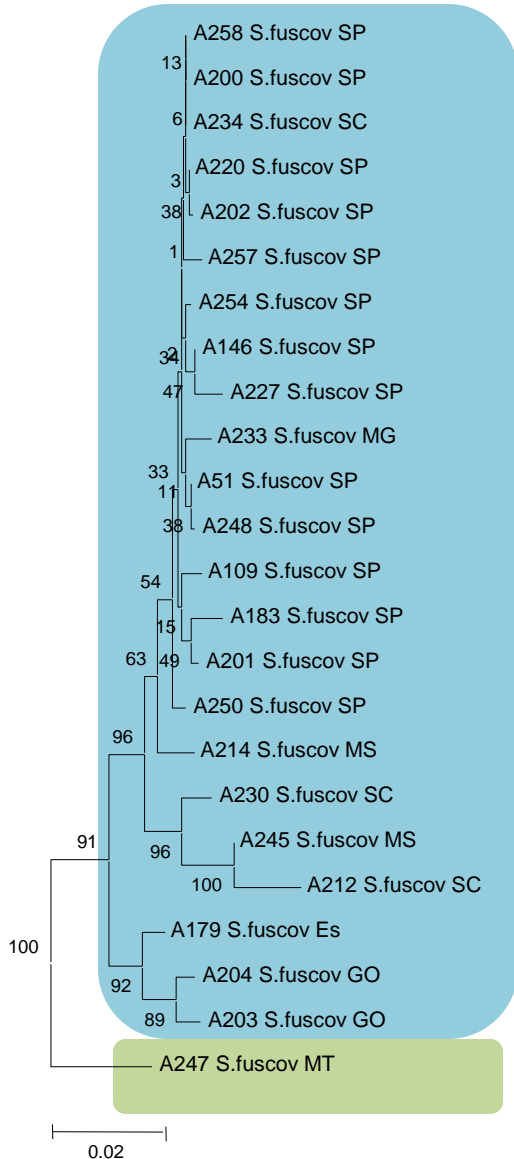


Diversity:
3.6 - 4.4%



Scinax fuscovarius: synonymy

COI: NJ-K2P



Scinax megapodia x *Scinax fuscovarius*

A247 - *Sc. megapodia*(?) distribution

Synonym revalidation?

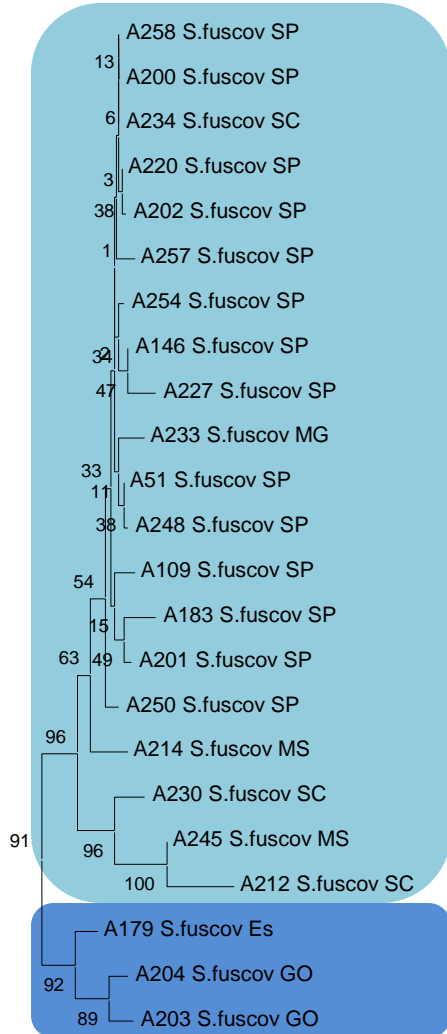
Look for other evidences

Diversity:
3.6 - 4.4%



Scinax fuscovarius: criptic diversity?

COI: NJ-K2P



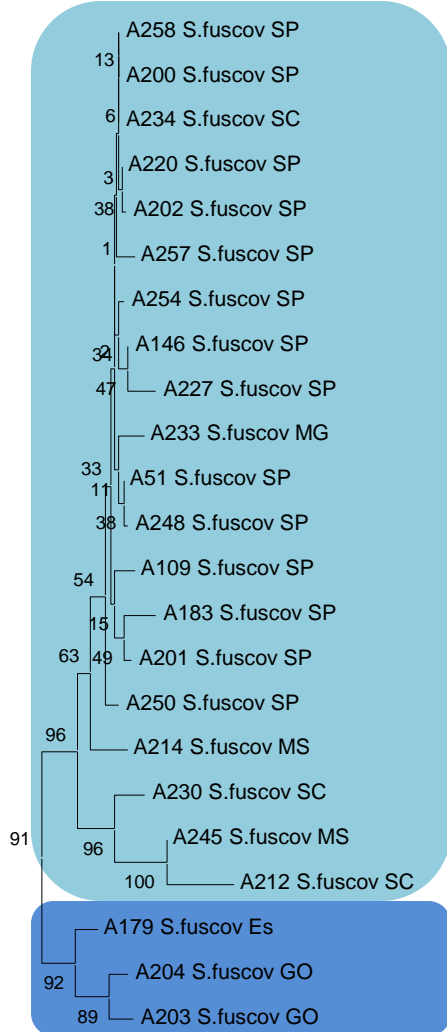
Diversity
~2.6%

0.02

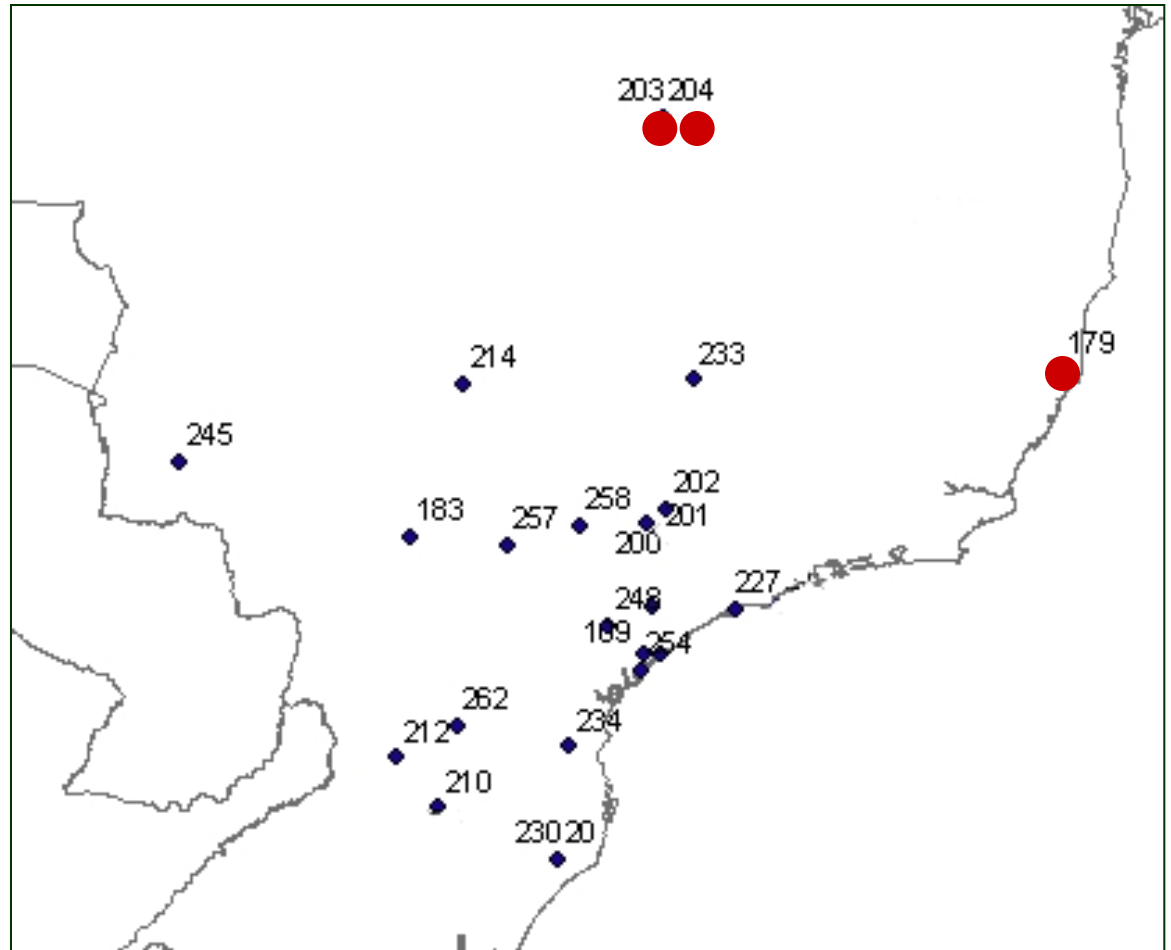


Scinax fuscovarius: cryptic diversity?

COI: NJ-K2P



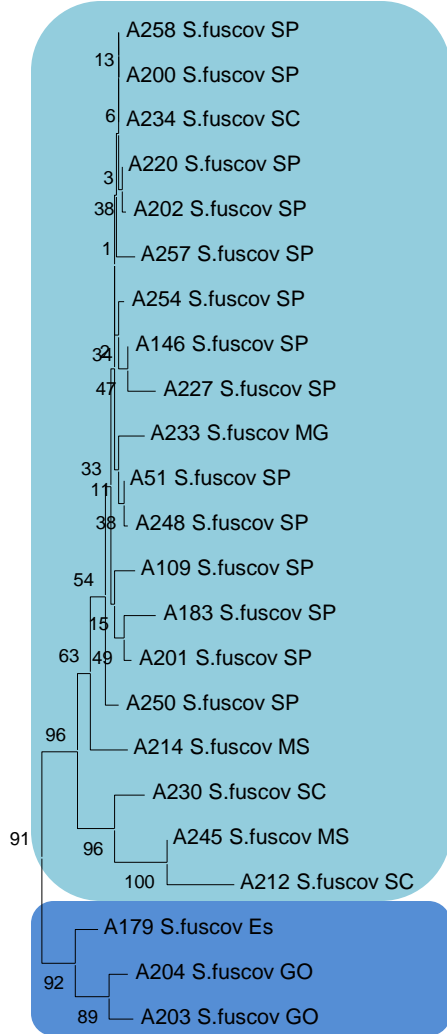
0.02



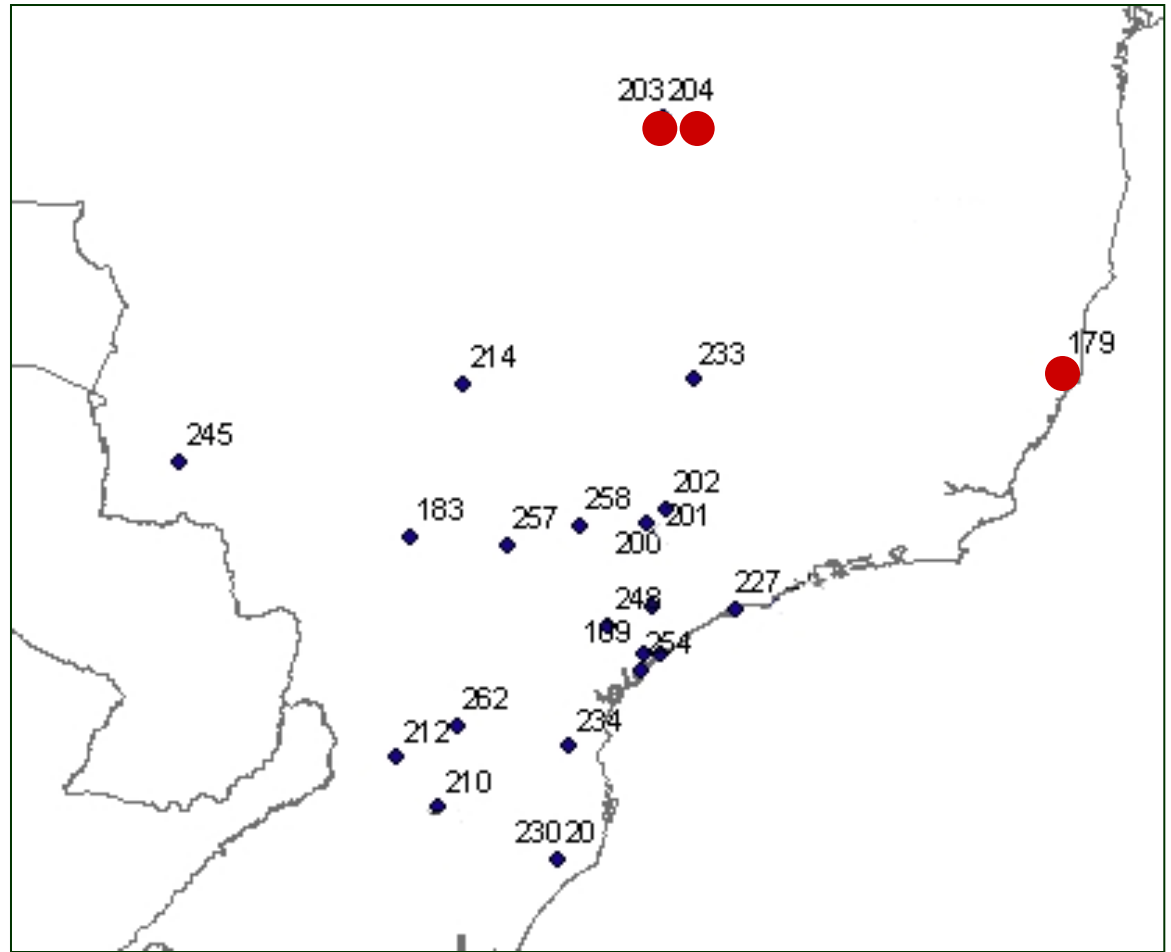


Scinax fuscovarius: cryptic diversity?

COI: NJ-K2P



0.02



Geographic distribution of diversity?
Look for other evidences



SUMARY AND CONCLUSIONS

1- Our primers for COI DNA Barcoding Anurans amplified all species sampled



SUMMARY AND CONCLUSIONS

- 1- Our primers for COI DNA Barcoding Anurans amplified all species sampled
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SUMMARY AND CONCLUSIONS

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- 3- Anuran diversity in Brazil is underestimated, and COI DNA barcodes may help flag potential new species
- 4- DNA barcodes may help to accelerate taxonomic revision (bioacoustics and morpho-anatomy)



SUMARY AND CONCLUSIONS

5 - DNA barcode may help identify misclassification in collection
(Biological collections certification)



SUMARY AND CONCLUSIONS

- 5 - DNA barcode may help identify misclassification in collection
(Biological collections certification)

- 6- DNA barcode may help resolve problems with synonymy



SUMARY AND CONCLUSIONS

- 5 - DNA barcode may help identify misclassification in collection
(Biological collections certification)
- 6- DNA barcode may help resolve problems with synonymy
- 7- The use of DNA barcode will help make a better estimative of amphibian species richness and may yield important findings for the knowledge and conservation of these species.



Phyllomedusa lemur –
Photo: ANAM

Obrigada

Thank you



MLL Fellowship

Prodoc nº 563.975/05-9



CFBH collection- UNESP



Laboratory projects UNICAMP

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GMYC

Pons et al 2006 – Syst.Biology 55: 595-609 – “single”

Fontanero et al 2007 – Plos Biology 5: 914-921 – “single”

Monaghan et al 2009 - Syst.Biology – advance access publ jul 2009 – “multiple”

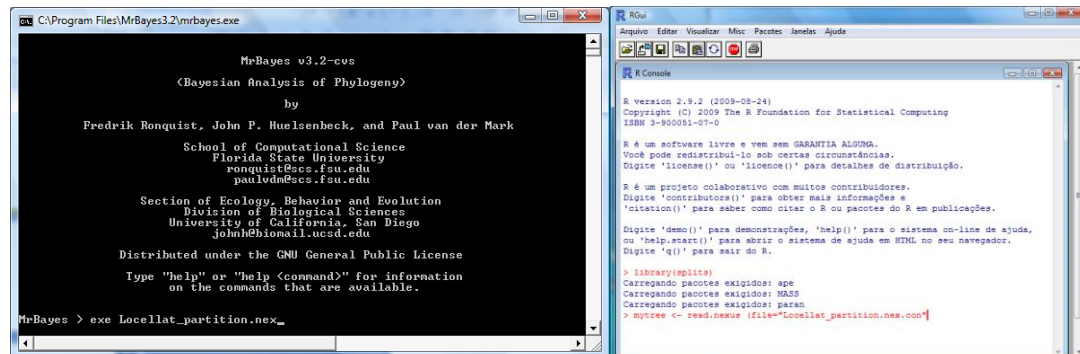
“General Mixed Yule-Coalescent model – GMYC”

Identificar limites de “espécies/grupos” baseado nos ramos de uma árvore que contém espécies e populações.

Processos coalescentes neutros (pops) X especiação

Ultrametric tree - MrBayes 3.2

Análise no R (split, ape, gee)



```
MrBayes v3.2-cvs
(Bayesian Analysis of Phylogeny)
by
Fredrik Ronquist, John P. Huelsenbeck, and Paul van der Mark
School of Computational Science
Florida State University
ronquist@scs.fsu.edu
paulvdn@scs.fsu.edu
Section of Ecology, Behavior and Evolution
Division of Biological Sciences
University of California, San Diego
johnh@biomail.ucsd.edu
Distributed under the GNU General Public License
Type "help" or "help <command>" for information
on the commands that are available.
MrBayes > exe Locellat_partition.nex_

R Console
R version 2.9.2 (2009-08-24)
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ISBN 3-900051-07-0
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ou 'help.start()' para abrir o sistema de ajuda em HTML no seu navegador.
Digite 'q()' para sair do R.
> library(splitr)
Carregando pacotes exigidos: ape
Carregando pacotes exigidos: MASS
Carregando pacotes exigidos: para
> source("C:/read.nexus (file=Locellat_partition.nex.com")
```

```
R Console
26 -0.001465 164.6877
27 -0.000912 164.6207
28 -0.000863 164.7831
29 -0.000759 164.9771

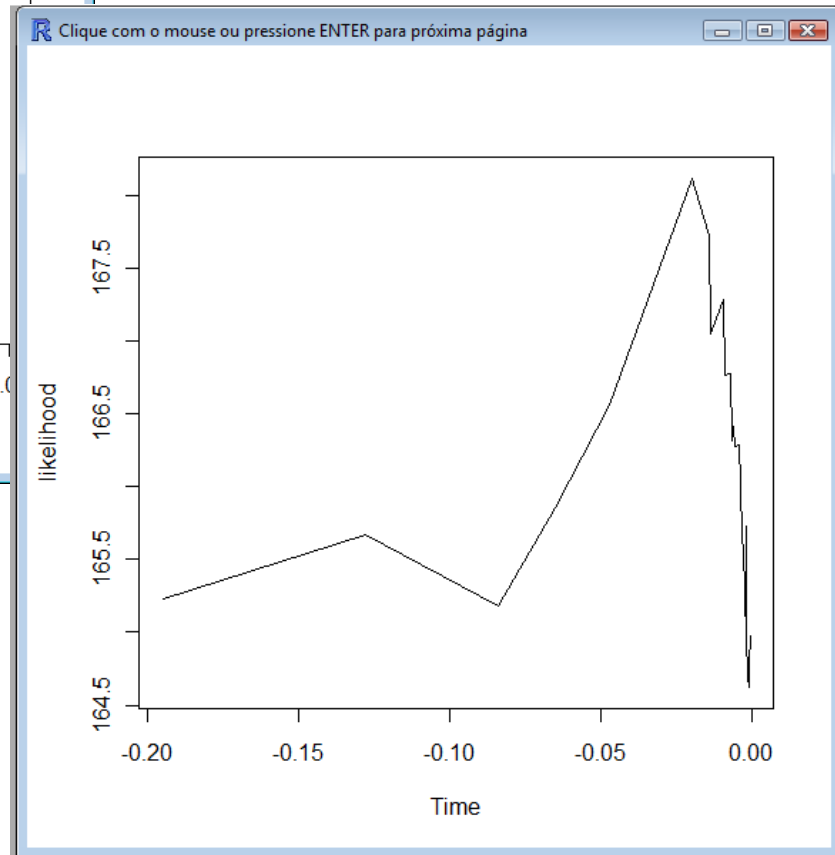
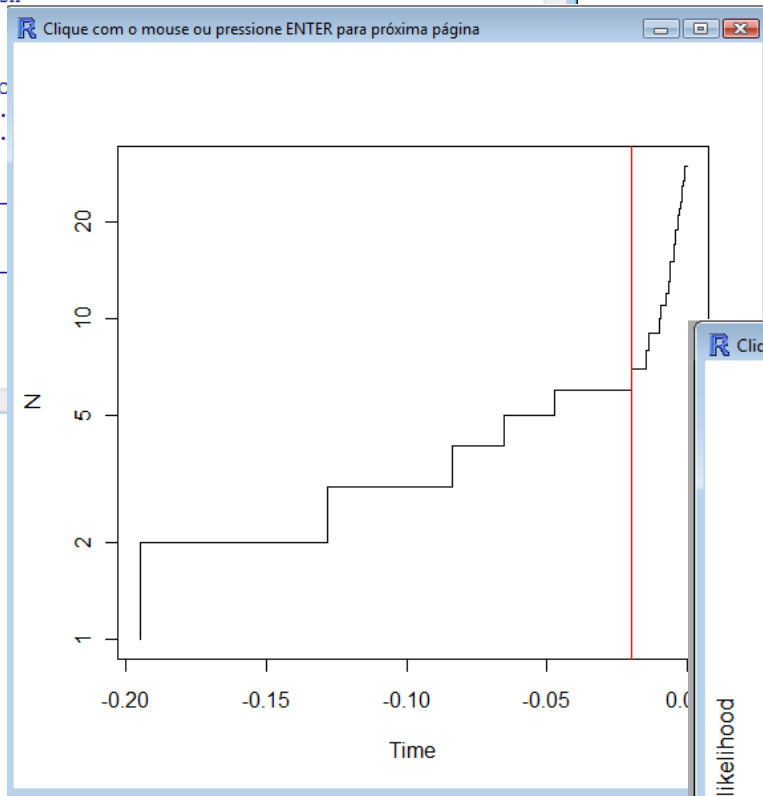
Thu Sep 24 07:04:50 2009
finish.
> summary(test1)
Result of GMYC species delimitation

method: single
likelihood of null model:
maximum likelihood of GMYC
likelihood ratio: 5.
result of LR test: 0.

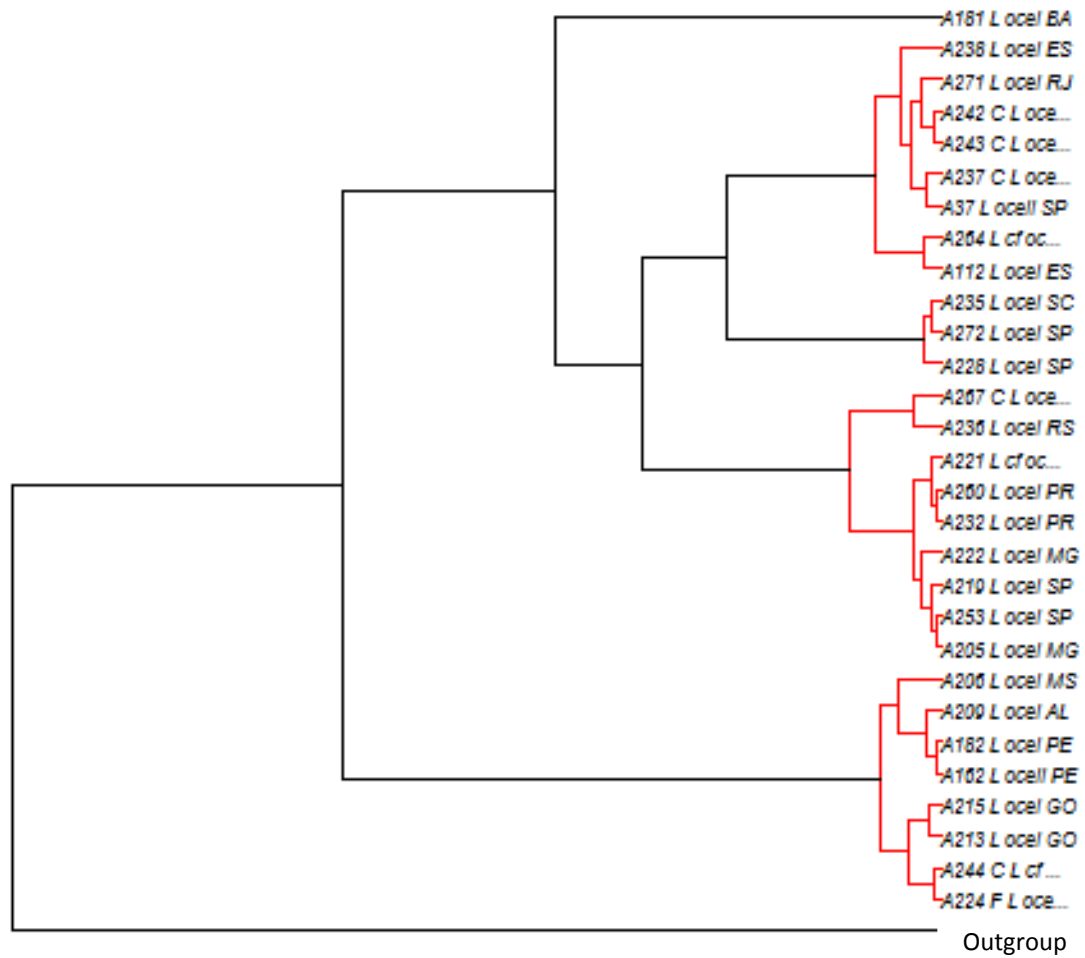
number of ML clusters: 4
confidence interval: 3-

number of ML entities: 6
confidence interval: 5-

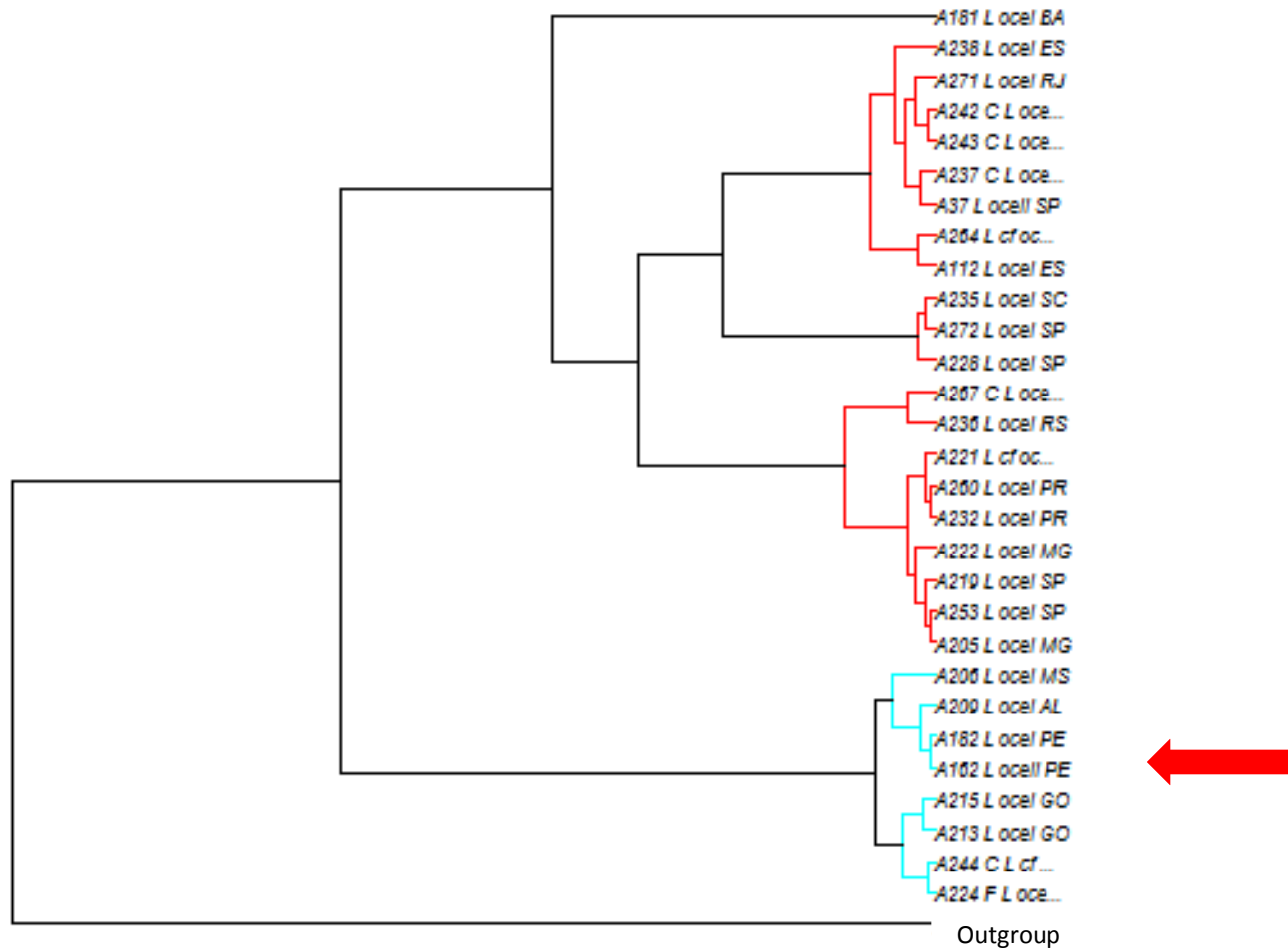
threshold time: -0.019892
> |
```



“Single thresholds”: 6 unidades coalescentes



“Multiple thresholds”: 7 unidades coalescentes





Created a new account


BOLD Systems

http://www.boldsystems.org/views/login.php

BARCODE OF LIFE DATA SYSTEMS

Advancing species identification and discovery through the analysis of short, standardized gene regions

About BOLD Contact Us



Published Projects | Taxonomy Browser | Request an Account | Identify Specimen | Introductory Tutorial | Documentation | Citation

The Barcode of Life Data Systems (BOLD) is an online workbench that aids collection, management, analysis, and use of DNA barcodes. It consists of 3 components (MAS, IDS, and ECS) that each address the needs of various groups in the barcoding community.

MANAGEMENT & ANALYSIS

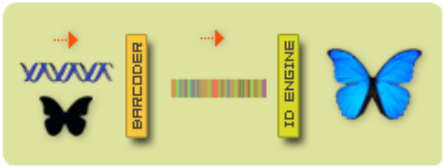
BOLD-MAS provides a repository for barcode records coupled with analytical tools. It serves as an online workbench for the DNA barcode community.

Username
Password

[Request a new user account](#)
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IDENTIFICATION ENGINE

BOLD-IDS provides a species identification tool that accepts DNA sequences from the barcode region and returns a taxonomic assignment to the species level when possible.



EXTERNAL CONNECTIVITY

BARCODE COUNTS

Formally Described Species With Barcodes	63,854
Total Barcode Records	699,647
Source	Breakdown
GenBank	94,119
Canadian Centre	516,208
Others	89,320

SYSTEM UPDATES

Sept-23-2008 - Merging Private Data with Public Projects (MAS)
The Project List page now displays all public projects as a distinct part of a user's access list. Users can view, merge, and analyze this data along with their own.

Aug-8-2008 - Performance Upgrade (MAS)
The BOLD data access modules have been upgraded to be more efficient and caching mechanisms have been integrated to improve user experience.

Apr-30-2008 - Primer Registry Interface (MAS)
A primer registry is now available from both the Project List page and the Project Console. The registry allows users to access and get information on all publicly available primers. A new form has been added to allow users to modify and maintain their own registered primers.

Mar-2-2008 - BOLD Integration with LIMS (MAS)
BOLD users that make use of our standard Laboratory Information Management System (LIMS) now have access to LIMS progress reports and audit trails directly from the specimen page of a record. Gel electrophoresis images are also available for each sample through this



Project Options

- Create New Project
- Merge Projects
- Search All Records
- View All Primers

Campaigns

- General Projects

<input type="checkbox"/>	Code	General Projects	Sequences	Specimens	Species	Species with Seq	Pub	Markers
<input type="checkbox"/>	DBABA	DNA Barcoding Anurans from Brazilian Atlantic forest	0	0	0	0		COI-5P/16S

Public Projects

<input type="checkbox"/>	Code	ACG Parasitoids	Sequences	Specimens	Species	Species with Seq	Pub	Markers
<input type="checkbox"/>	ASTAZ	ACG Generalist Tachinidae	712	2135	80	0	✓	COI-5P/ITS/28S-D2
<input type="checkbox"/>	ASMG	ACG Microgastrinae	1298	2575	323	6	✓	COI-5P/ITS
<input type="checkbox"/>	ASMIN	ACG Microgastrinae (Braconidae) - minimalist barcode	11	22	15	0	✓	COI-5P/ITS
<input type="checkbox"/>	HCIC	ACG Tachinidae (Belvosia)	246	736	32	0	✓	COI-5P/ITS/28S-D2
<input type="checkbox"/>	ASBE	ACG Tachinidae (Belvosia) II	249	670	28	0		COI-5P/ITS/28S-D2

<input type="checkbox"/>	Code	All Birds Barcoding Initiative	Sequences	Specimens	Species	Species with Seq	Pub	Markers
<input type="checkbox"/>	AAPR	ABBI additional public records	140	140	86	86		COI-5P
<input type="checkbox"/>	BARG	Birds of Argentina - Phase I	1594	1594	500	500	✓	COI-5P
<input type="checkbox"/>	ABNA	Birds of North America - Phase II	2572	2572	657	657	✓	COI-5P
<input type="checkbox"/>	TZBNA	Birds of North America	437	437	263	263	✓	COI-5P
<input type="checkbox"/>	BNACA	Birds of North America, Canadian geese	141	141	2	2	✓	COI-5P
<input type="checkbox"/>	BNABS	Birds of North America, Canadian passerines	120	120	38	38	✓	COI-5P
<input type="checkbox"/>	BNAUS	Birds of North America, General sequences	1874	1874	594	594	✓	COI-5P
<input type="checkbox"/>	BWA	Birds of Western Australia	75	78	31	31		COI-5P
<input type="checkbox"/>	KBBI	DNA Barcoding Korean Birds	255	255	102	102	✓	COI-5P
<input type="checkbox"/>	AROM	Royal Ontario Museum - Birds 1	352	352	79	79	✓	COI-5P
<input type="checkbox"/>	AROMB	Royal Ontario Museum-Birds2	128	128	10	10	✓	COI-5P
<input type="checkbox"/>	AROMC	Royal Ontario Museum - Birds 3 Rostratula	9	9	3	3	✓	COI-5P

“ DNA Barcoding Anuran from Brazilian of Atlantic forest”

Fellowship: Capes/CNPqProdoc nº 563.975/05-9



Order: Anura

15 families

76 species

Order: Gymnophiona

1 family

3 species

Order: Caudata

1 family

1 species

Total: 80 sp.