Detecting declining and “extinct” frogs with eDNA

Carla Lopes and Kelly R. Zamudio

- **Brazil has the highest amphibian diversity in the world (> 1000 species)**
- **Half of these species are distributed in the Brazilian Atlantic forest**
- **Only 16% of its original cover**

- **Since the 1970s many amphibian populations declining or no longer registered (some > 80 years), mainly in highland streams**

Nevertheless, we were surprised by the length of time since most of the species of the former *Crossodactylus dispar* complex were last collected. Only *C. timbuhy* can still be found at known localities. Based on the specimens examined, *C. boulengeri* has not seen for more than fourty years; the other three species of the *C. dispar* complex seemed to disappear between the late 1960's and the early 1980's. *Batrachochytrium*

Drastic population reductions occurred for *Centrolenella eurygnatha, Ololygon perpusilla, Adenomera marmorata, Eleutherodactylus guentheri, E. parvus,* and *Hylodes phyllodes. Centrolenella eurygnatha* was commonly heard

observed. We have also noted the general decline of frog populations in 1979 at Teresópolis in the Organ Mountains. One species, *Thoropa petropolitana,* had been ex-

Forest Morphoclimatic Domain of southeast Brazil. In the Itatiaia region of the Serra da Mantiqueira, Elio Gouvêa (pers. comm.), the naturalist at the National Park, observed a marked decline of frog population sizes on the forest floor during 1979. One of us (Heyer) wished to

Due to life history aspects of certain species that make sampling effective, we are confident that five species have become extinct at Boracéia: *Crossodactylus dispar, Cycloramphus boraceiensis, C. semipalmatus, Hylodes asperus,* and *Thoropa miliaris. Crossodactylus dispar* is a diurnal

Although little effort has been dedicated to this subject to date, the number of reports of amphibian population declines and species extinctions in Southeastern Brazil is worthy of attention. Records exist for the highlands of Serra do Mar at Boracéia, in the state of São Paulo (Heyer *et al.* 1988, Bertoluci & Heyer 1995), Santa Teresa and Linhares, state of Espírito Santo (Weygoldt 1989, Papp & Papp 2000), Maciço da Tijuca and Teresópolis, state of Rio de Janeiro (Heyer *et al.* 1988, Weygoldt 1989, Izecksohn & Carvalho-e-Silva 2001), as well as for the Parque Nacional do Itatiaia, at Serra da Mantiqueira (Heyer *et al.* 1988, Guix *et al.* 1998, Pombal & Haddad 1999; Appendix). When compiled, the above-listed records yield reports for at least 31 species of Brazilian anurans (depending on taxonomic issues), involving representatives of five

- **Causes of disappearance are not known:**
  - Severe frosts or droughts
  - Habitat loss
  - Pollution and acid rain
  - Climate change
  - Ecological factors such as competition
  - Epidemic infections (*Bd*)
  - Invasive species
- **Some species detected after long disappearances**
  - Importance of long-term monitoring studies, adequate sampling effort, and sampling methods

Locality	Species	Last registered	Status	Range
Estação Biológica de Boracéia	*Crossodactylus dispar*	1977 (Pimenta et al. 2014)	Dis	NE
	Cycloramphus boraceiensis	1979 (Heyer et al. 1990)	LD	NE
	Cycloramphus semipalmatus	1979 (Heyer et al. 1990)	LD	NE
	Hylodes asper	1979 (Heyer et al. 1990)	LD	NE
	Phrynomedusa vanzolinii	1973 (Cruz 1991)	Dis	NE
	Thoropa taophora	1979 (Heyer et al. 1990)	LD	NE
	Vitreorana eurygnatha	1979 (Heyer et al. 1990)	LD	NE
Parque Nacional de Itatiaia	*Crossodactylus grandis*	1969 (Pimenta et al. 2014)	Dis	NE
	Crossodactylus werneri	1978 (Pimenta et al. 2014)	Dis	NE
	Holoaden bradei	1976 (Rocha et al. 2004)	Dis	En
	Hylodes glaber	1976 (Rocha et al. 2004)	Dis	En
	Hylodes ornatus	2012 (de Sá et al. 2015)	Dec	En
	Hylodes regius	2012 (CFBH Collection)	Dec	En
	Paratelmatobius lutzi	1978 (Pombal and Haddad 1999)	Dis	En
Parque Nacional da Serra da Bocaina	*Bokermannohyla claresignata*	1939 (Lutz and Lutz 1939)	Dis	NE
	Bokermannohyla clepsydra	1968 (Bokermann 1971)	Dis	En
	Megaelosia bocainensis	1968 (Giaretta et al. 1993)	Dis	En
Serra do Cipó	*Scinax pinima*	1987 (CFBH Collection)	Dis	En
Santa Teresa	*Allobates olfersioides*	1981 (Weygoldt 1989)	Dec	NE
	Crossodactylus gaudichaudii	1981 (Weygoldt 1989)	LD	NE
	Crossodactylus timbuhy	2005 (Pimenta et al. 2014)	Dec	NE
	Cycloramphus fuliginosus	1981 (Weygoldt 1989)	LD	NE
	Hylodes lateristrigatus	2009 (MNRJ Collection)	LD	NE
	Phasmahyla exilis	2009 (MNRJ Collection)	Dis	En
	Phrynomedusa marginata	1986 (MNRJ Collection)	Dis	NE
	Vitreorana eurygnatha	1981 (Weygoldt 1989)	LD	NE
Parque Nacional da Serra dos Órgãos	*Aplastodiscus musicus*	1995 (Berneck et al. 2016)	Dis	En
	Bokermannohyla claresignata	1954 (MNRJ Collection)	Dis	NE
	Phrynomedusa vanzolinii	1929 (Cruz 1991)	Dis	NE
	Thoropa petropolitana	1977 (Heyer and Crombie 1977)	Dis	En

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• compare to 5-year traditional monthly survey of the same streams

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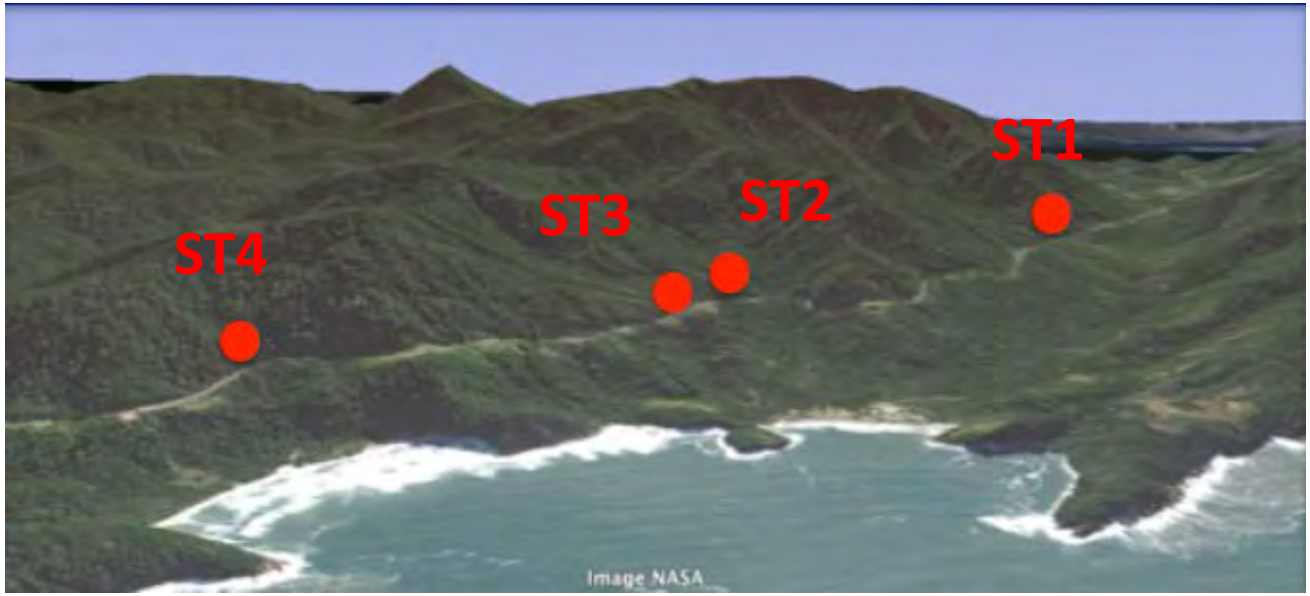

- **4 montane streams (ST1, ST2, ST3 e ST4)**
- **2 points in each stream (S1 e S2 – 100m de distância)**
- **2 different samples at each point - 20L and 60L**
- **12 PCR replicates for each eDNA sample**

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3 focal species:

- Easy to identify in the field
- Common in the sampled streams
- Complete life cycles in or very near streams (eggs, larvae, juvies, adults)

Community Reference database:

- 40/44 species known from the park included in the database
- EMBL 12S database for all anurans



eDNA detection probability relative to traditional survey methods?

eDNA analyses

Environmental samples (n=62)

Filtered water from Streams and one Pond

DNA Extraction

Total DNA

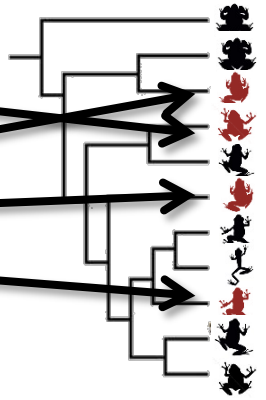
DNA Amplification (rRNA 12S)

Metabarcodes

ATCGCTAC
TCGCTACG
GCTACAGA
GGCTAGCC

Next-generation Sequencing

Species identification



Taxonomic identification

SPYGEN®

Sequence reference database

ATCGCTACCC
TCGCTACGTC
GCTACAGACA
GGCTAGCCCC
TATATCGTGG
TACCGCGTAG

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11 species detected with eDNA,

- **9 directly associated with streams at some life cycle stage**
- **2 species not frequently seen in streams – "occasional species"**

2 problems:

- taxonomic resolution (*Bokermannohyla* sp.)
- Rogue species: *Scinax ruber*

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Scinax ruber

- species from Amazon basin – not known from Picinguaba
- single unique sequence – 47 reads
- single PCR replicate from one sample

Contamination? How likely?

- strict protocols
- SpyGen is in France and never processed Amazonian samples
- never showed up in negative controls
- species breeds in open habitats, ponds, not in streams in dense canopy forest

2 'problems':
- taxonomic resolution (*Bokermannohyla* sp.)
- Rogue species: *Scinax ruber*

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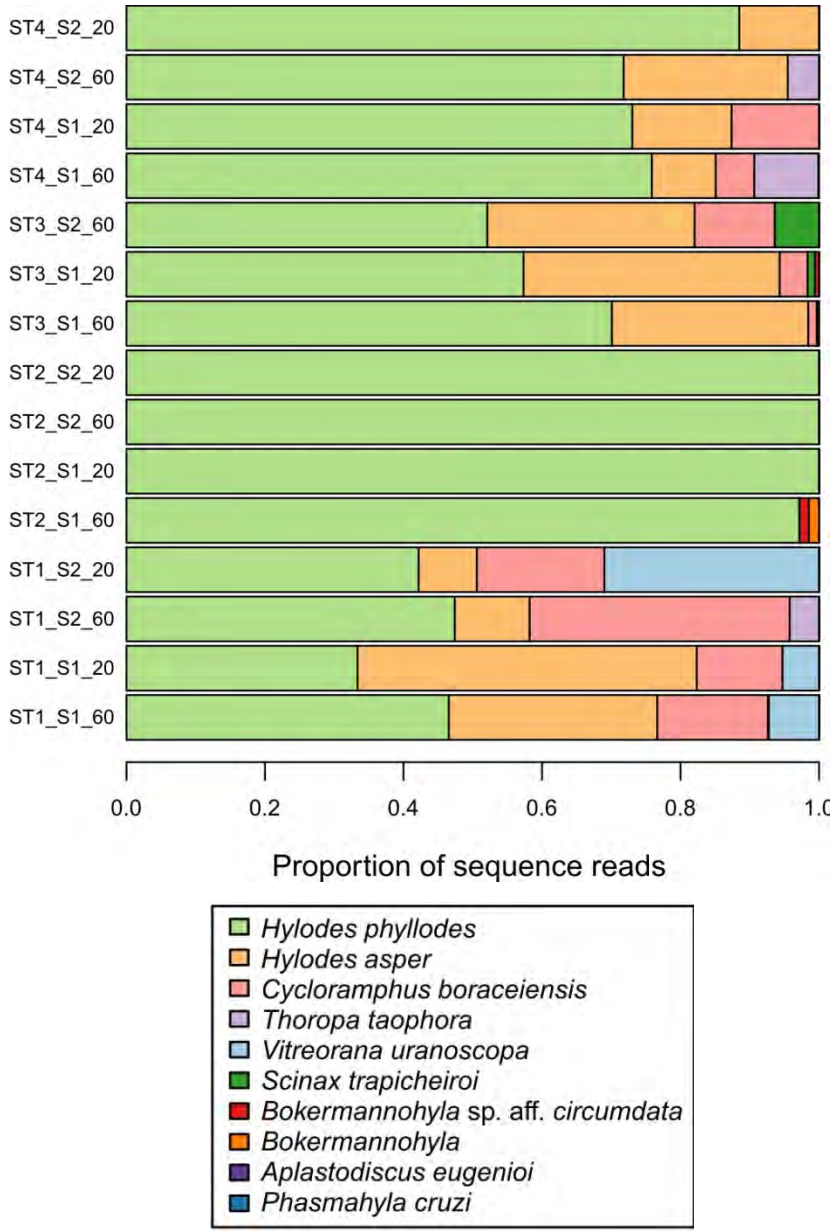

The good news:

More DNA retrieved from:

- More abundant species
- More aquatic species
- 3 focal species (*H. phyllodes*, *H. asper*, *C. boraceiensis*)
- Stream presence/absence matches the survey data

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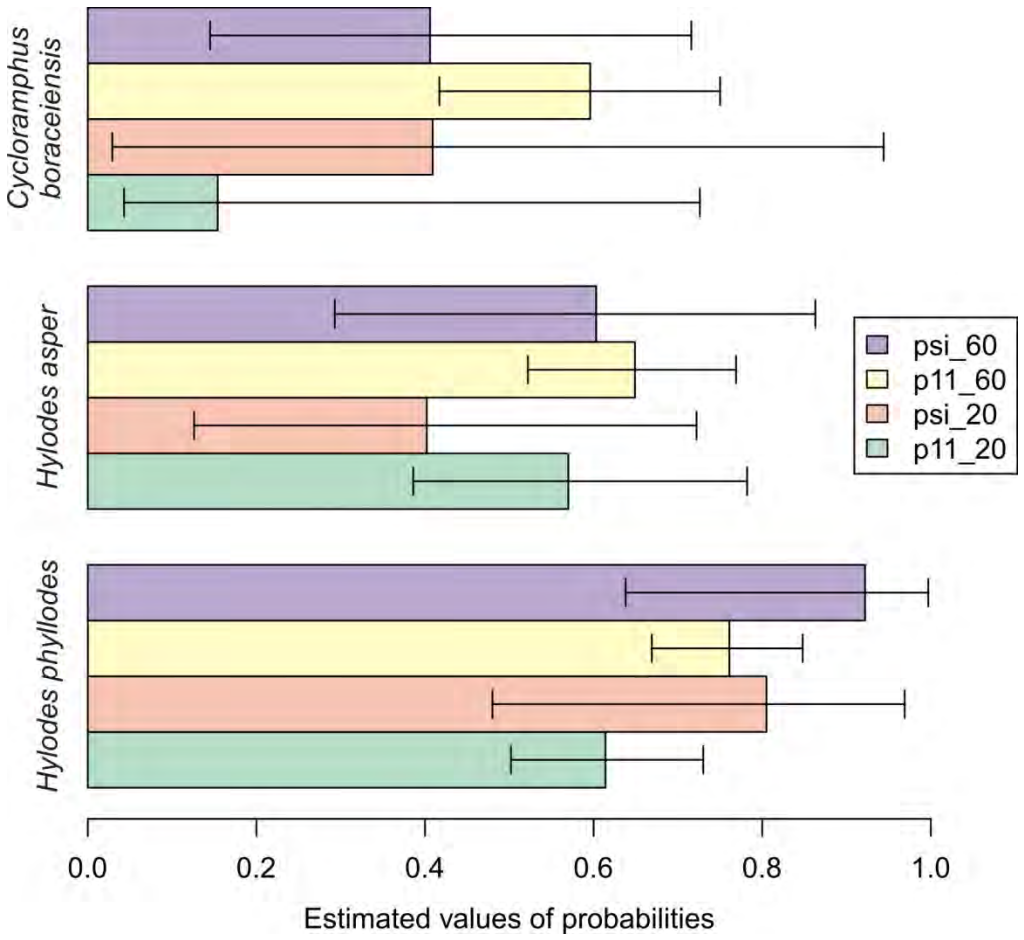

- **Higher detection probability with more water filtered**
- **Especially for low density species**
- **Importance of sampling multiple sites per stream**

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MOLECULAR ECOLOGY
RESOURCES

eDNA metabarcoding: a promising method for anuran surveys in highly diverse tropical forests

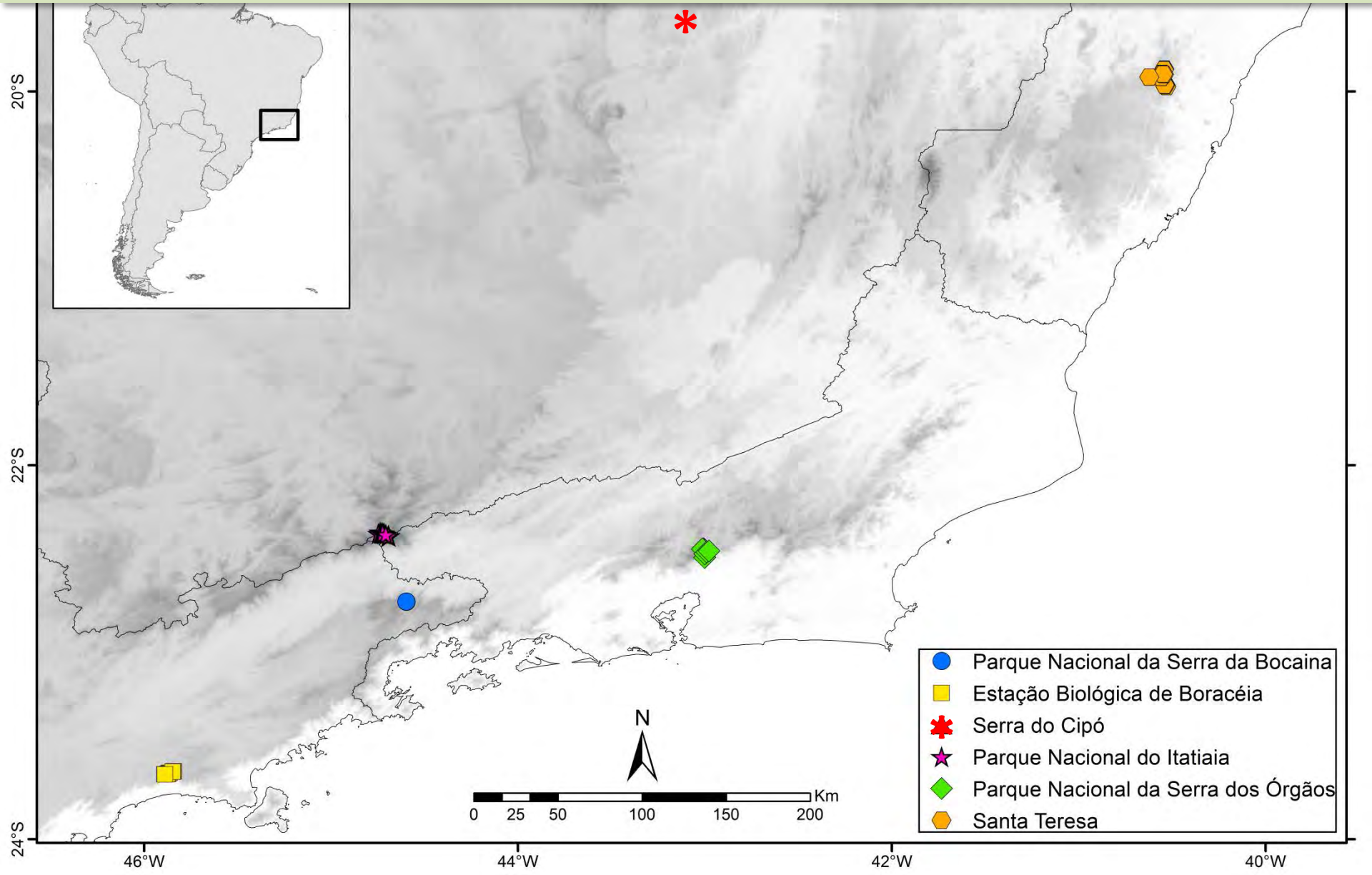
Carla M. Lopes[1*], Thais Sasso[2], Alice Valentini[3], Tony Dejean[3], Marcio Martins[2], Kelly R. Zamudio[4], Célio F. B. Haddad[1]

eDNA very sensitive and detected all species detected in field surveys

- Eggs and larvae in water contribute to the "DNA pool"
- eDNA can detect species that might be upstream

Locality	Species	Last registered	Status	Range
Estação Biológica de Boracéia	*Crossodactylus dispar*	1977 (Pimenta et al. 2014)	Dis	NE
	Cycloramphus boraceiensis	1979 (Heyer et al. 1990)	LD	NE
	Cycloramphus semipalmatus	1979 (Heyer et al. 1990)	LD	NE
	Hylodes asper	1979 (Heyer et al. 1990)	LD	NE
	Phrynomedusa vanzolinii	1973 (Cruz 1991)	Dis	NE
	Thoropa taophora	1979 (Heyer et al. 1990)	LD	NE
	Vitreorana eurygnatha	1979 (Heyer et al. 1990)	LD	NE
Parque Nacional de Itatiaia	*Crossodactylus grandis*	1969 (Pimenta et al. 2014)	Dis	NE
	Crossodactylus werneri	1978 (Pimenta et al. 2014)	Dis	NE
	Holoaden bradei	1976 (Rocha et al. 2004)	Dis	En
	Hylodes glaber	1976 (Rocha et al. 2004)	Dis	En
	Hylodes ornatus	2012 (de Sá et al. 2015)	Dec	En
	Hylodes regius	2012 (CFBH Collection)	Dec	En
	Paratelmatobius lutzi	1978 (Pombal and Haddad 1999)	Dis	En
Parque Nacional da Serra da Bocaina	*Bokermannohyla claresignata*	1939 (Lutz and Lutz 1939)	Dis	NE
	Bokermannohyla clepsydra	1968 (Bokermann 1971)	Dis	En
	Megaelosia bocainensis	1968 (Giaretta et al. 1993)	Dis	En
Serra do Cipó	*Scinax pinima*	1987 (CFBH Collection)	Dis	En
Santa Teresa	*Allobates olfersioides*	1981 (Weygoldt 1989)	Dec	NE
	Crossodactylus gaudichaudii	1981 (Weygoldt 1989)	LD	NE
	Crossodactylus timbuhy	2005 (Pimenta et al. 2014)	Dec	NE
	Cycloramphus fuliginosus	1981 (Weygoldt 1989)	LD	NE
	Hylodes lateristrigatus	2009 (MNRJ Collection)	LD	NE
	Phasmahyla exilis	2009 (MNRJ Collection)	Dis	En
	Phrynomedusa marginata	1986 (MNRJ Collection)	Dis	NE
	Vitreorana eurygnatha	1981 (Weygoldt 1989)	LD	NE
Parque Nacional da Serra dos Órgãos	*Aplastodiscus musicus*	1995 (Berneck et al. 2016)	Dis	En
	Bokermannohyla claresignata	1954 (MNRJ Collection)	Dis	NE
	Phrynomedusa vanzolinii	1929 (Cruz 1991)	Dis	NE
	Thoropa petropolitana	1977 (Heyer and Crombie 1977)	Dis	En

Detecting declining or "extinct" species



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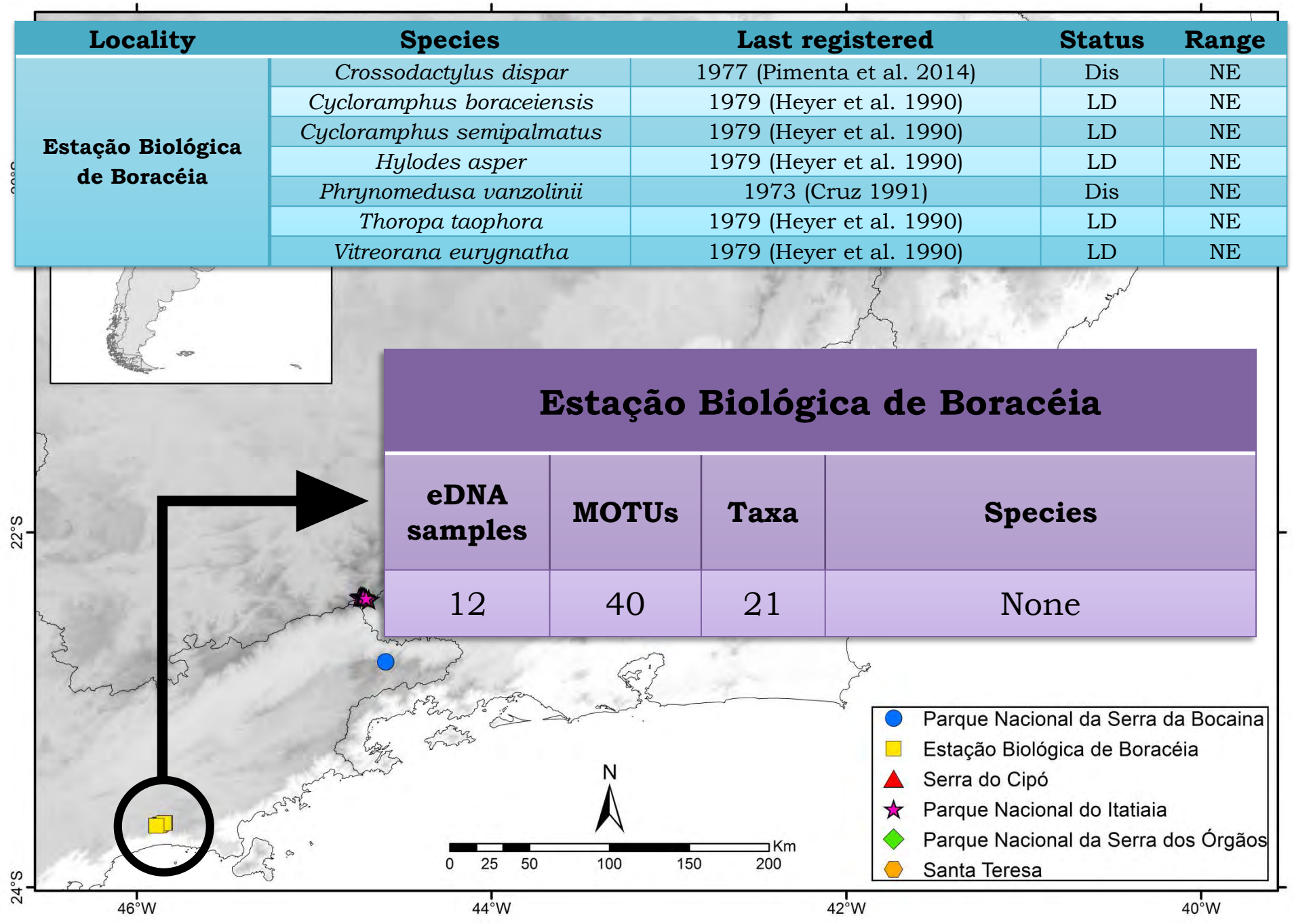
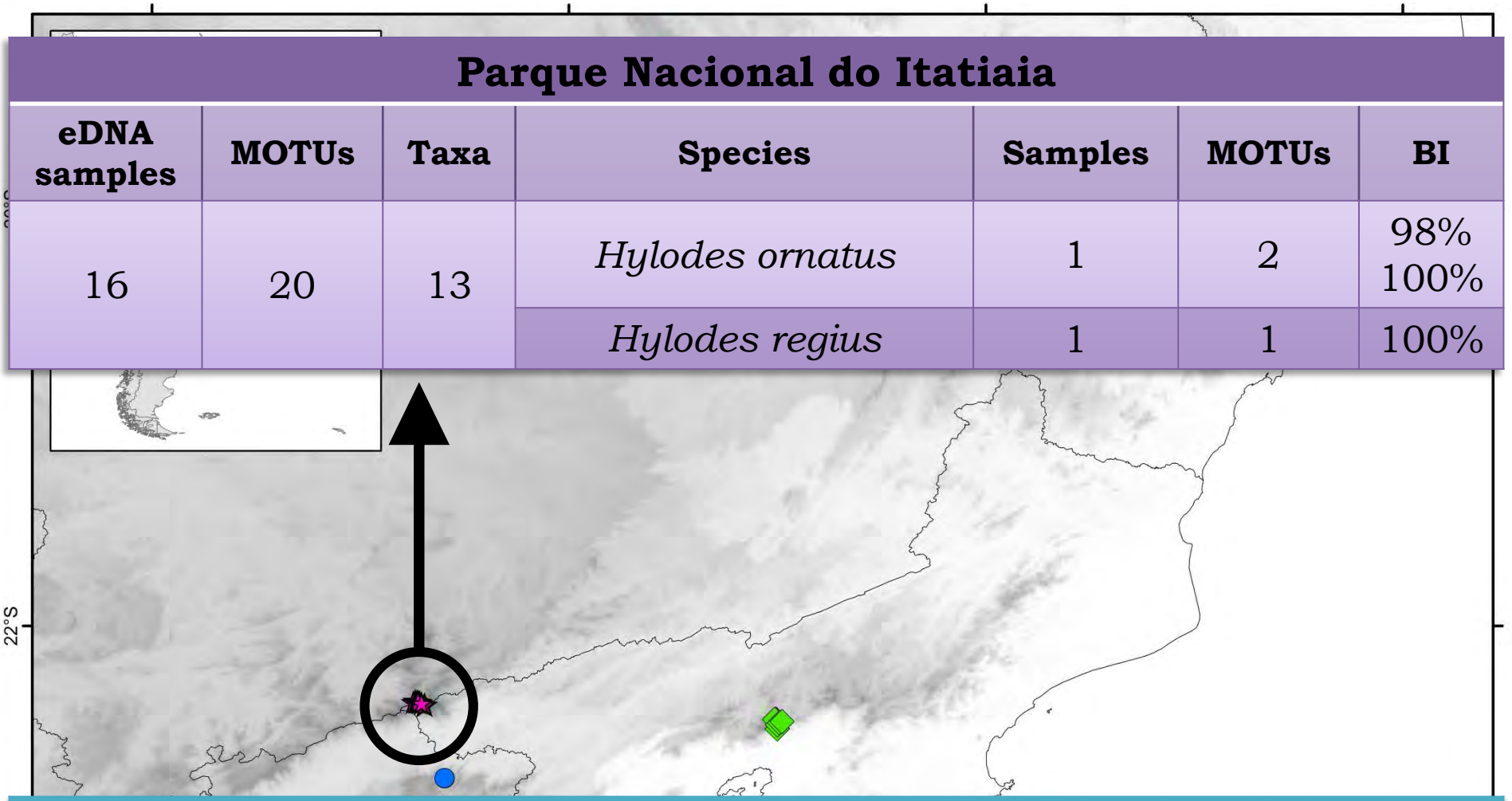
	Hylodes asper	1979 (Heyer et al. 1990)	LD	NE
	Phrynomedusa vanzolinii	1973 (Cruz 1991)	Dis	NE
	Thoropa taophora	1979 (Heyer et al. 1990)	LD	NE
	Vitreorana eurygnatha	1979 (Heyer et al. 1990)	LD	NE

Estação Biológica de Boracéia

eDNA samples	MOTUs	Taxa	Species
12	40	21	None

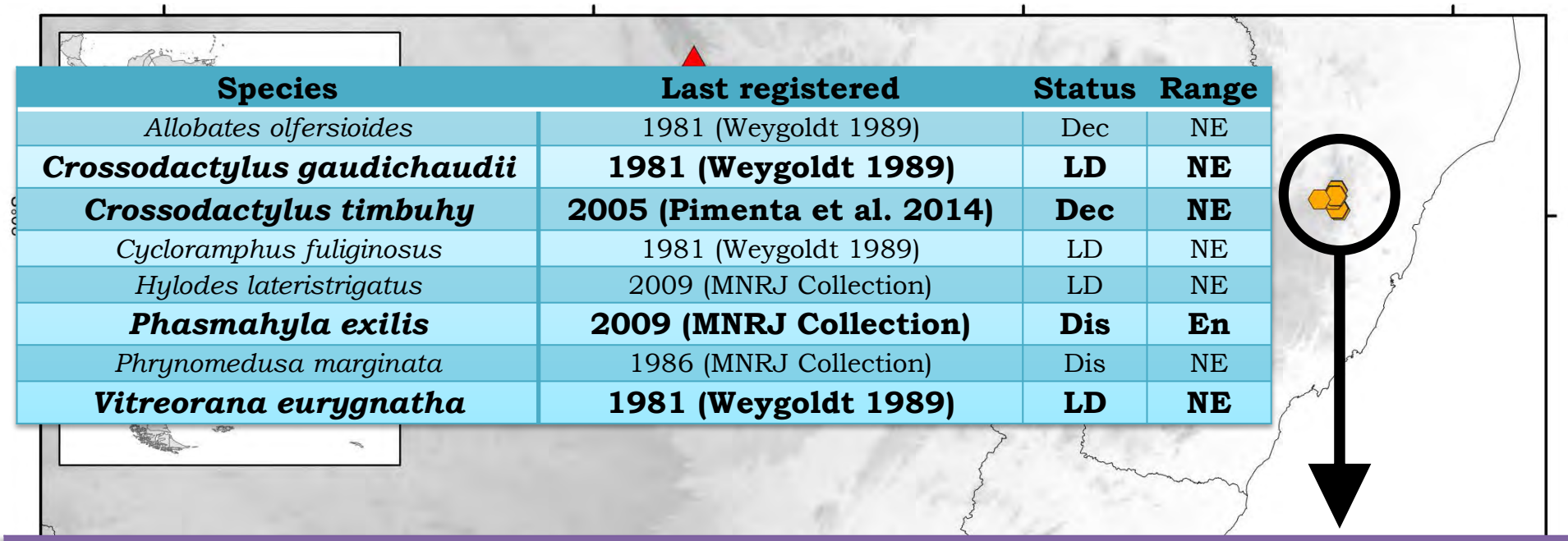




Parque Nacional do Itatiaia

eDNA samples	MOTUs	Taxa	Species	Samples	MOTUs	BI
16	20	13	*Hylodes ornatus*	1	2	98% 100%
			Hylodes regius	1	1	100%

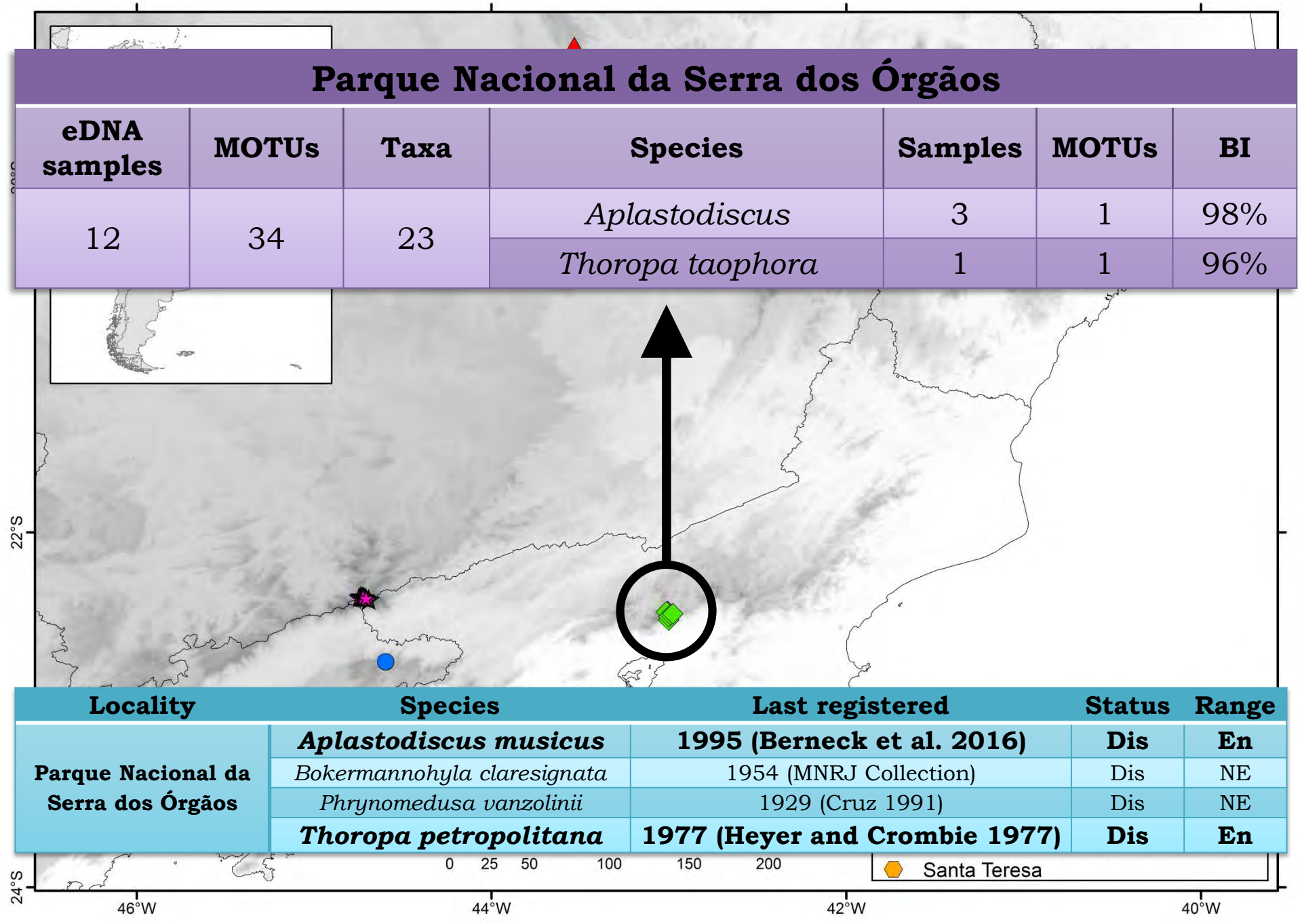
Locality	Species	Last registered	Status	Range
Parque Nacional de Itatiaia	*Crossodactylus grandis*	1969 (Pimenta et al. 2014)	Dis	NE
	Crossodactylus werneri	1978 (Pimenta et al. 2014)	Dis	NE
	Holoaden bradei	1976 (Rocha et al. 2004)	Dis	En
	Hylodes glaber	1976 (Rocha et al. 2004)	Dis	En
	Hylodes ornatus	**2012 (de Sá et al. 2015)**	**Dec**	**En**
	Hylodes regius	**2012 (CFBH Collection)**	**Dec**	**En**
	Paratelmatobius lutzi	1978 (Pombal and Haddad 1999)	Dis	En



Species	Last registered	Status	Range
Allobates olfersioides	1981 (Weygoldt 1989)	Dec	NE
Crossodactylus gaudichaudii	**1981 (Weygoldt 1989)**	**LD**	**NE**
Crossodactylus timbuhy	**2005 (Pimenta et al. 2014)**	**Dec**	**NE**
Cycloramphus fuliginosus	1981 (Weygoldt 1989)	LD	NE
Hylodes lateristrigatus	2009 (MNRJ Collection)	LD	NE
Phasmahyla exilis	**2009 (MNRJ Collection)**	**Dis**	**En**
Phrynomedusa marginata	1986 (MNRJ Collection)	Dis	NE
Vitreorana eurygnatha	**1981 (Weygoldt 1989)**	**LD**	**NE**

Santa Teresa

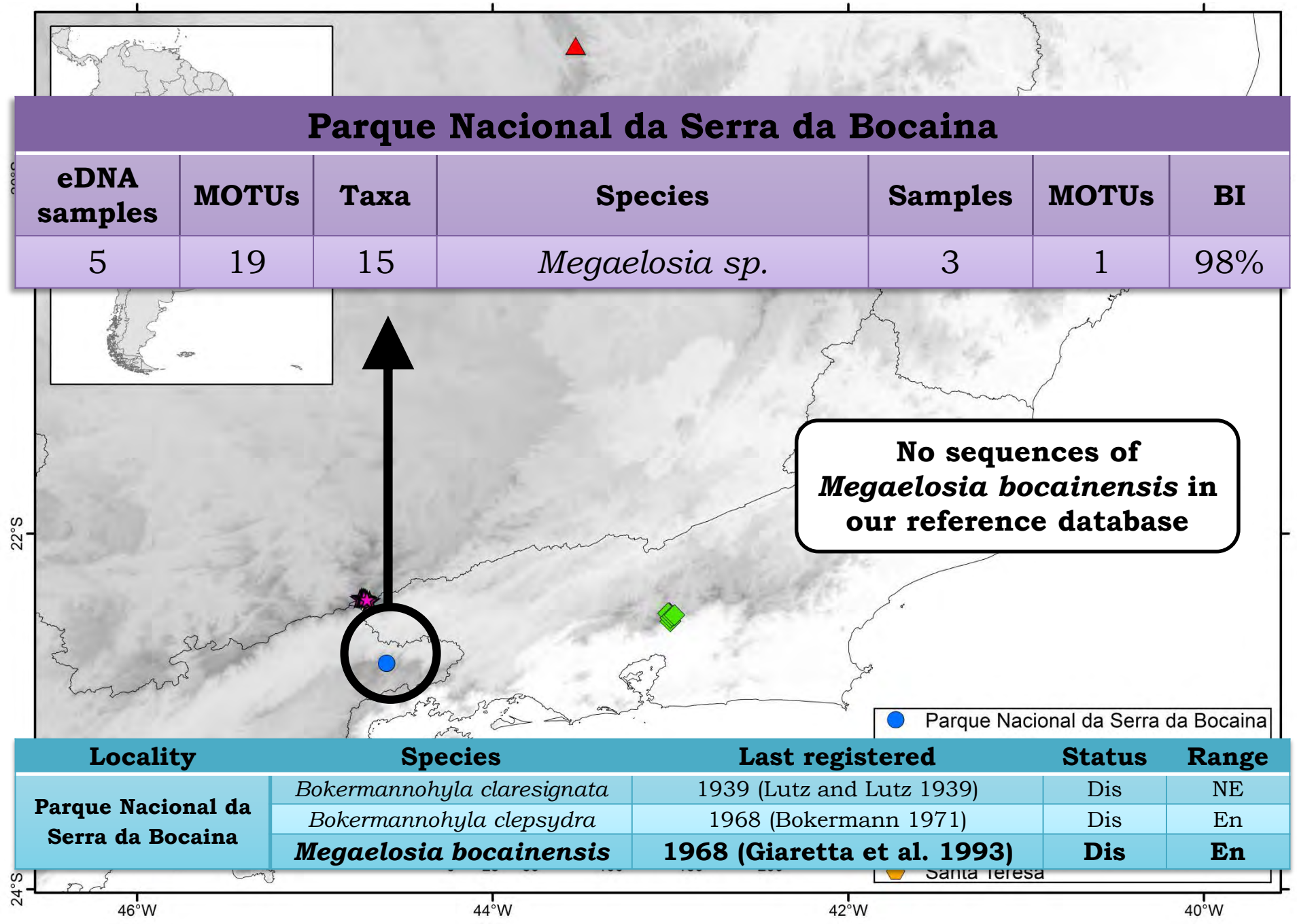
eDNA samples	MOTUs	Taxa	Species	Samples	MOTUs	BI
14	37	20	*Crossodactylus*	9 2	2	100% 96%
			Hylodes lateristrigatus	4 8 3 1 1	5	100% 100% 98% 96% 96%
			Vitreorana eurynatha	2	1	100%
			Phasmahyla exilis	2	1	100%



Parque Nacional da Serra dos Órgãos

eDNA samples	MOTUs	Taxa	Species	Samples	MOTUs	BI
12	34	23	*Aplastodiscus*	3	1	98%
			Thoropa taophora	1	1	96%

Locality	Species	Last registered	Status	Range
Parque Nacional da Serra dos Órgãos	***Aplastodiscus musicus***	**1995 (Berneck et al. 2016)**	**Dis**	**En**
	Bokermannohyla claresignata	1954 (MNRJ Collection)	Dis	NE
	Phrynomedusa vanzolinii	1929 (Cruz 1991)	Dis	NE
	Thoropa petropolitana	**1977 (Heyer and Crombie 1977)**	**Dis**	**En**



Parque Nacional da Serra da Bocaina

eDNA samples	MOTUs	Taxa	Species	Samples	MOTUs	BI
5	19	15	*Megaelosia sp.*	3	1	98%

Locality	Species	Last registered	Status	Range
Parque Nacional da Serra da Bocaina	*Bokermannohyla claresignata*	1939 (Lutz and Lutz 1939)	Dis	NE
	Bokermannohyla clepsydra	1968 (Bokermann 1971)	Dis	En
	Megaelosia bocainensis	**1968 (Giaretta et al. 1993)**	**Dis**	**En**

Serra do Cipó

eDNA samples	MOTUs	Taxa	Species (eDNA)	Species
3	12	8	None (eDNA)	1 *Scinax pinima*

Parque Nacional da Serra da Bocaina
Estação Biológica de Boracéia
Serra do Cipó
Santa Teresa

N

0 25 50 100 150 200

20°S
22°S
24°S
46°W
44°W
42°W
40°W

Locality	Species	Last registered	Status	Range
Serra do Cipó	*Scinax pinima*	1987 (CFBH Collection)	Dis	En

Conclusions

- **We detected with eDNA amphibian species no longer registered:**
  - **3 declining (*H. ornatus*, *H. regius*, *Crossodactylus sp.*)**
  - **2 local disappeared (*H. lateristrigatus*, *V. eurygnatha*)**
  - **2 disappeared (*M. bocainensis*, *P. exilis*)**
  - **2 require further investigations (*A. musicus*, *T. petropolitana*)**

- **Next: sequencing the 12S rRNA from museum species**

- **eDNA metabarcoding efficient in tropical running water courses, even when species are at low population densities**

- **These results reinforce the potential of this approach in monitoring and conservation studies, and to help overcome many of the challenges of traditional survey methods for species detection.**

Thanks!

Collaborators

Carla Lopes
Célio F. B. Haddad
Alice Valentini
Tony Dejean