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# Detection of *Mycoplasma* spp. from snakes from five different families

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## Abstract

**Background** Mycoplasmas are an important cause of respiratory diseases in tortoises. In snakes, evidence of mycoplasma infections has been found almost exclusively in pythons. To better understand the occurrence of these bacteria in other snake species, samples submitted for routine testing for respiratory pathogens were also tested for mycoplasma by polymerase chain reaction (PCR). A total of 640 samples (mostly oral swabs) from snakes of 5 different families (Boidae *n* = 114, Colubridae *n* = 109, Elapidae *n* = 34, Pythonidae *n* = 301 and Viperidae *n* = 82) were included in the study. A genus-specific PCR (PCR1) developed for the detection of *Mycoplasma* [*Mycoplasmopsis*] *agassizii* and a pan-mycoplasma PCR (PCR2) were used. PCR products were sequenced for validation and phylogenetic analysis was performed. The sampled animals were from various owners and collections, all in human care at the time of sampling. Clinical background information was not provided.

**Results** Using PCR1, mycoplasmas were detected in 175 (175/640, 27%) samples (Boidae: 7/114, 6%; Colubridae: 3/109, 3%; Elapidae: 8/34, 24%; Pythonidae: 155/301, 51%; Viperidae: 2/82, 2%). A higher percentage of positive results were obtained using PCR2 (258/640, 40%; Boidae: 9/114, 8%; Colubridae: 25/109, 23%; Elapidae: 19/34, 56%; Pythonidae: 172/301, 57%; Viperidae: 33/82, 40%). The detected bacteria can be divided into at least 6 genetically diverse clusters representing different genera and species based on multiple sequence alignment and phylogenetic analysis.

**Conclusions** These results show that diverse mycoplasmas are found in pythons and other snakes. Further investigations are necessary to evaluate the role of various mycoplasmas in respiratory diseases in snakes.

**Keywords** Mycoplasma, Snake, Python, Elapid, Respiratory disease, Reptile

## Background

Reptiles are a highly diverse group of ectothermic vertebrates that belong to the Sauropsida lineage, which also includes birds. Non-avian reptiles are classified into four main orders: Crocodilia (crocodilians), Testudines (chelonians), Squamata (lizards and snakes), and Rhynchocephalia (tuataras). Among the extant reptiles, the Squamata represent the greatest diversity, with over 4,000 known species of snakes, distributed across 25 families [1, 2]. Like other vertebrate groups, reptiles face significant threats from a wide variety of sources, including climate change, habitat destruction, pollution, invasive species, and disease [3]. Additionally, reptiles are heavily traded internationally, and the European Union is a major hub of the international pet trade [4, 5]. Snakes, including pythons, boas, and colubrids, are particularly popular in this market [6]. In recent years, there has been growing interest in understanding infectious diseases and the microbiome of snakes, which has led to greater insights

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into their health and the potential risks associated with the reptile trade.

*Mycoplasma* spp. bacteria are characterized by small, wall-less cells and a limited genome making them the simplest free-living and self-replicating organisms [7]. Although they primarily live parasitically, mycoplasmas can act as commensals or as either primary or opportunistic pathogens in vertebrates, insects, and plants [7]. While the taxonomy of the genus *Mycoplasma* (*M.*) has been tentatively revised recently [8–11] we will adhere to the traditional names of genera and species, providing alternative names in brackets for clarity. In reptiles, mycoplasmas are generally considered opportunistic pathogens, but some mycoplasmas may play a major role in diseases of the upper respiratory tract in some reptiles. In chelonians in particular, mycoplasmas are recognized as a cause of upper respiratory tract disease (URTD), especially *M. agassizii* [*Mycoplasmopsis* agassizii] and *M. testudineum* [*Mycoplasmopsis testudinea*] in tortoises [12–17]. Related mycoplasmas have also recently been described in aquatic turtles, both in animals with respiratory disease and in clinically healthy animals [18–20]. Another mycoplasma species, *M.* [*Mycoplasmoides*] *testudinis*, was isolated from the cloaca of a Hermann's tortoise (*Testudo hermanni*) and is not considered a pathogen [21]. In the order Crocodilia, *M.* [*Mycoplasmopsis*] *crocodyli* and *M.* [*Mycoplasmopsis*] *alligatoris* have been reported in association with pneumonia, tracheitis, and polyarthritis [22–24]. There are only a few publications on mycoplasmas in lizards (order Squamata). *M.* [*Mycoplasmopsis*] *iguanae* and *M. insons* have been described in green iguanas (*Iguana iguana*). *M. iguanae* was suspected to have caused spinal disease in feral iguanas [25]. However, a subsequent study showed that *M. iguanae* is unlikely to be responsible for acute disease and that *M. insons* should be considered part of the normal flora in the respiratory tract of iguanas [25].

In snakes, the majority of mycoplasma detections have been in pythons. The first report of a mycoplasma was from a Burmese python (*Python bivittatus*) with proliferative lymphocytic tracheitis and pneumonia [26]. The 16S rRNA gene sequence of this mycoplasma showed a 90% similarity to *M. agassizii* [26]. A similar mycoplasma closely related to *M. testudineum* (95% identity in the 16S rRNA gene) was detected in a carpet python (*Morelia spilota*) with stomatitis [27] and related mycoplasmas have been detected in high percentages of pythons tested in Europe (60.2% of 271 pythons tested) [28]. Recently, similar mycoplasmas have also been detected in snakes in other families, including Boidae, Viperidae, and Elapidae. In a study in Brazil, a mycoplasma identical to the one from the carpet python was detected by culture and PCR in a ball python (*Python regius*), a boa constrictor (*Boa constrictor*) and a common lancehead (*Bothrops atrox*) [29]. Of the 26 snakes tested, nine had clinical signs including stomatitis, anorexia, dysecdysis, and weight loss [29]. In the only report of a mycoplasma detected in an elapid snake, PCR detected a mycoplasma in multiple tissues from a king cobra (*Ophiophagus hannah*) from a European zoological collection that presented with neurological signs and respiratory disease [30]. Genetically diverse mycoplasmas have also been detected in pythons. In a study screening pythons with respiratory disease for a variety of possible pathogens, mycoplasma-like sequences were detected in two pythons diagnosed with pneumonia at necropsy [31]. Further analysis of the 16S rRNA gene showed 95% sequence similarity to *M.* [*Mycoplasmopsis*] *caviae*, a commensal species from guinea pigs [32] and *M.* [*Mycoplasmopsis*] *fermentans*, a human-associated species with unclear pathogenicity [7, 33].

Aside from the above-mentioned studies and individual reports, the occurrence of *Mycoplasma* spp. has been poorly investigated so far in different snake families, with no reports available on monitoring studies in wild snakes. To date, none of the mycoplasmas detected in snakes have been fully characterized or named.

This work was conducted to fill in this gap through testing for the presence of *Mycoplasma* spp. in a wide range of snake species belonging to the families Boidae, Colubridae, Elapidae, Pythonidae, and Viperidae, using two different PCRs targeting variable regions of the 16S rRNA to optimize the detection rate. The PCR products were sequenced in an attempt to identify the isolates at the species level.

## Results

### Detection levels in the different host families

Of the 640 snake specimens tested, 175 (27.3%) were positive in PCR1, originally developed for the detection of *M. agassizii*. The highest detection rate was in the family Pythonidae, followed by Elapidae while the percentages of positive samples were low for Boidae, Colubridae and Viperidae (Table 1). The detection rate was significantly higher in pythons than in snakes of the other families and higher in elapids than in boids, colubrids, or viperids (Table 1).

As expected, i) a higher number of positive samples were detected using PCR2 as it was developed to detect a wider range of mycoplasmas not limited to *M. agassizii* or related species and ii) all samples tested positive using PCR1 ($n = 175$ positive) were also tested positive using PCR2. The proportion of *Mycoplasma* spp. positive samples using PCR2 was 40.3% (258/640), with high detection rates in Pythonidae and Elapidae, followed by Viperidae and Colubridae, and the lowest detection rate in Boidae (Table 2). Of note, the

**Table 1** Mycoplasmal detection rates using PCR1 in each of the snake families included in the study and in the various sample types used. Statistical evaluation of differences in detection rates between families are shown. For sample types, only the two most common sample types, oral swabs and nasal lavages were compared. A $p < 0.05$ was considered significant, and $p$ values below this cut-off are bolded

| Family | **Boidae** | **Colubridae** | **Elapidae** | **Pythonidae** | **Viperidae** |
|---|---|---|---|---|---|
| No. *Mycoplasma* spp. positive samples (% positive) | 7/114 (6.1%) | 3/109 (2.8%) | 8/34 (23.5%) | 155/301 (51.5%) | 2/82 (2.4%) |
| **Boidae** | | | | | |
| **Colubridae** | $p = 0.369$ | | | | |
| **Elapidae** | $\mathbf{p = 0.009}$ | $\mathbf{p < 0.001}$ | | | |
| **Pythonidae** | $\mathbf{p < 0.001}$ | $\mathbf{p < 0.001}$ | $\mathbf{p = 0.002}$ | | |
| **Viperidae** | $p = 0.381$ | $p = 1$ | $\mathbf{p = 0.001}$ | $\mathbf{p < 0.001}$ | |
| Sample type | **Oral swabs** | **Nasal lavage** | **Lung lavage** | **Tissue** | |
| No. *Mycoplasma* spp. positive sample material (% positive) | 154/590 (26.1%) | 21/34 (61.8%) | 0/2 (0%) | 0/14 (0%) | |
| **Oral swabs** | | | | | |
| **Nasal lavage** | $\mathbf{p < 0.001}$ | | | | |
| **Lung lavage** | | | | | |
| **Tissue** | | | | | |

**Table 2** Mycoplasmal detection rates using PCR2 in each of the snake families included in the study and in the various sample types used. Statistical evaluation of differences in detection rates between families are shown. For sample types, only the two most common sample types, oral swabs and nasal lavages were compared. A $p < 0.05$ was considered significant, and $p$ values below this cut-off are bolded

| Family | **Boidae** | **Colubridae** | **Elapidae** | **Pythonidae** | **Viperidae** |
|---|---|---|---|---|---|
| No. *Mycoplasma* spp. positive samples (% positive) | 9/114 (7.9%) | 25/109 (22.9%) | 19/34 (55.9%) | 172/301 (57.1%) | 33/82 (40.2%) |
| **Boidae** | | | | | |
| **Colubridae** | $\mathbf{p = 0.003}$ | | | | |
| **Elapidae** | $\mathbf{p < 0.001}$ | $\mathbf{p < 0.001}$ | | | |
| **Pythonidae** | $\mathbf{p < 0.001}$ | $\mathbf{p < 0.001}$ | $p = 1$ | | |
| **Viperidae** | $\mathbf{p < 0.001}$ | $\mathbf{p = 0.011}$ | $p = 0.152$ | $\mathbf{p = 0.009}$ | |
| Sample type | **Oral swabs and tissue** | | **Nasal lavage** | **Lung lavage** | |
| No. *Mycoplasma* spp. positive sample material (% positive) | 235/590 (39.8 %) and 1/14 (7.1%) | | 22/34 (64.7%) | 0/2 (0%) | |
| **Oral swabs and tissue** | | | | | |
| **Nasal lavage** | $\mathbf{p = 0.004}$ | | | | |
| **Lung lavage** | | | | | |

prevalence detected using PCR2 was much higher in Colubridae, Elapidae, and Viperidae when compared to detection rates with PCR1 in these snake families, suggesting colonization by mycoplasma species not closely related to *M. agassizii*. These differences were statistically significant (Table 2).

Mycoplasmas were detected significantly more often in nasal lavages than in other sample types for both PCR1 and PCR2 ($p < 0.001$ and $p = 0.004$), independent of the host species (Table 1, Table 2).

### Putative identification of *Mycoplasma* species

A total of 249 PCR products distributed among every snake host family and a wide range of species tested were sequenced. These included 90 of 175 (51.4%) PCR1 products and 159 of 258 (61.6%) PCR2 products.

All sequences were confirmed to be mycoplasma sequences except for 3 PCR2 products that belong to the close order of Acholeplasmatales in the Mollicutes class (2/3) or to the Erysipelotrichia class (1/3). A subset of 57 (PCR1) and 106 (PCR2) sequences of sufficient length

(> 406 bp for PCR1 and >175 bp for PCR2) and quality were further analyzed. Similar sequences were combined into one representative consensus sequence, that was further trimmed and used for phylogenetic analysis. All sequences were grouped into clusters, with each cluster containing sequencing with a varying number of SNPs and represented by a consensus sequence (Fig. 1 and 2, additional file 1 and 2). The final length of the sequences used for phylogenetic tree building was 335 bp for PCR 1 and 175 bp for PCR2. All sequences have been deposited in GenBank and given the accession numbers PQ452173-PQ452205.

PCR1 sequences clustered with sequences from *M. agassizii* within the family *Metamycoplasmataceae* fam. nov. [11], previously referred to as the "Hominis" group (Table 3, Fig. 1). Reconstructed consensus sequences were split into two separate branches (cluster A with 54 sequences and cluster B with only 3 sequences) based on top hits in the leBIBI database and on clustering in the phylogenetic analyses (Fig 1). Each cluster included sequences with multiple SNPs (see additional file 1), and there were multiple differences in sequences between individual sequences in clusters A and B. However, there was only 1 SNP/335 nt that was consistently different between the two clusters. All of the analyzed sequences from PCR1 also clustered together with mycoplasmas previously described in snakes but not identified to the species level [27, 28]. Cluster A ($n = 54$) included representatives from all snake families, except Viperidae (2 sequences) that were exclusively found in cluster B ($n = 3$), together with one sequence from Boidae (Table 3, Fig 1).

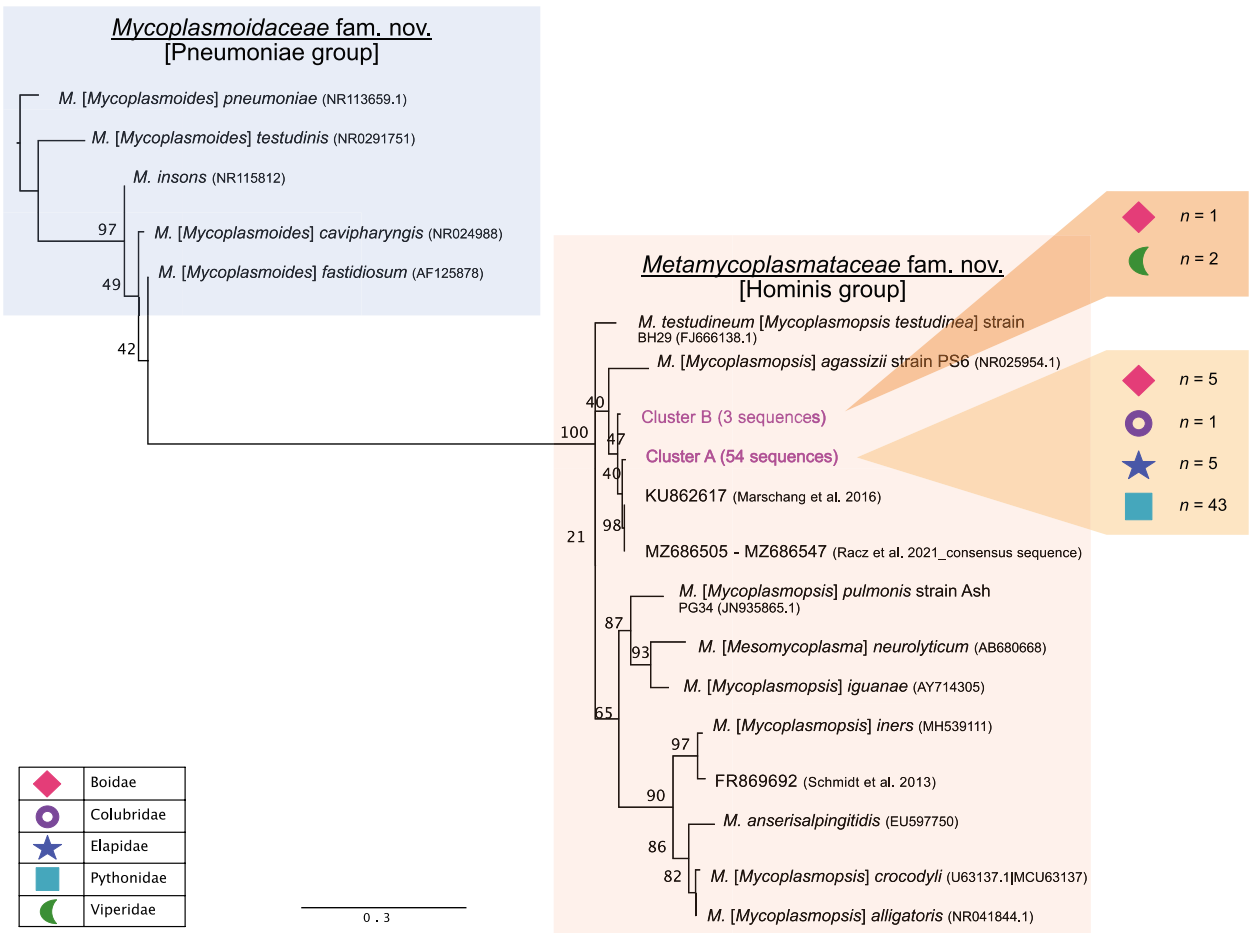


**Fig. 1** Phylogenetic tree based on sequences obtained using PCR1 [21]. A subset of sequences ($n = 57$) of the 175 positive tested snake samples were used for tree building. Sequences are grouped in clusters (named A, B, followed by the number of sequences represented in each cluster). Information on the host families (marked by different symbols) from which mycoplasmas in each cluster were found, together with the number of samples from each family included in the analysis can be found next to the respective clusters. The length of the sequences used was 335 bp. Names of previously described mycoplasmas are shown with species name followed by GenBank accession numbers. In cases in which the mycoplasmas have not been assigned to a species, the GenBank accession number and additional information is listed. Bootstrap support is shown on branches next to nodes in % of 1000 replicas

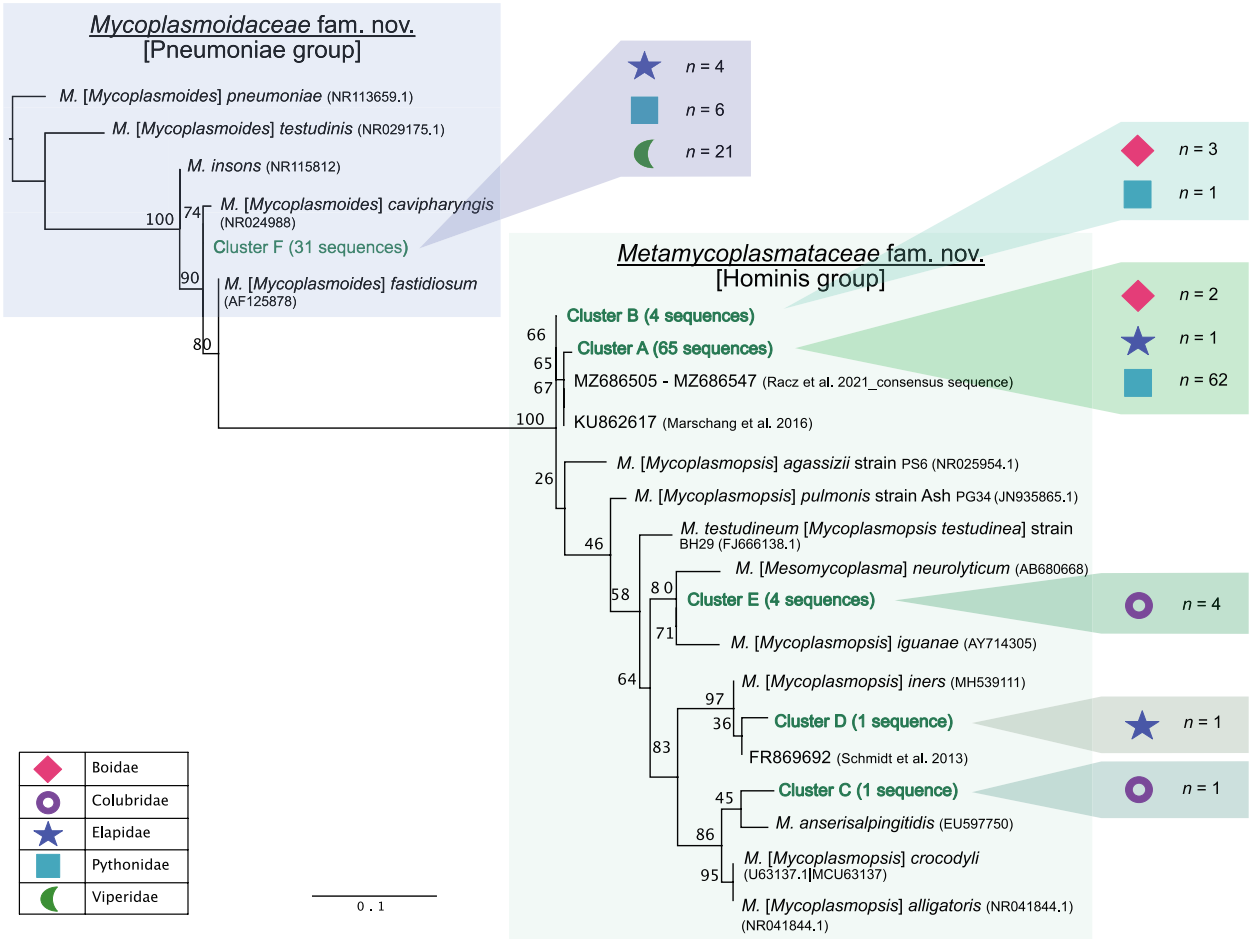


**Fig. 2** Phylogenetic tree based on sequences obtained using PCR2 [34]. A subset of sequences ($n = 106$) of the 258 positive tested snake samples were used for tree building. Sequences are grouped in clusters (named A, B, C, D, E and F, followed by the number of sequences represented in each cluster). Information on the host families (marked by different symbols) from which mycoplasmas in each cluster were found, together with the number of samples from each family included in the analysis can be found next to the respective clusters. The length of the sequences used was 175 bp. Names of previously described mycoplasmas are shown with species name followed by GenBank accession numbers. In cases in which the mycoplasmas have not been assigned to a species, the GenBank accession number and additional information is listed. Bootstrap support is shown on branches next to nodes in % of 1000 replicas

The sequences obtained using PCR2 were divided into 6 clusters (A, B, C, D, E and F, Table 4, Fig. 2, additional file 2) based on the results from the "leBIBI" database and phylogenetic analysis. Clusters A ($n = 65$) and B ($n = 4$) correspond to the clusters of the same name obtained using sequences from PCR1 and were close to *M. agassizii*. The other clusters from C to F ($n = 37$) were close to, respectively: cluster C to the avian species *M. anserisalpingitidis*; cluster D to *M.* [*Mycoplasmopsis*] *iners* and a mycoplasma strain described in two pythons [31] but not identified to the species level; cluster E to the iguana species *M. iguanae*; and cluster F to the equine species *M.* [*Mycoplasmoides*] *fastidiosum*. The mycoplasmas in clusters A through E were all found to be within the *Metamycoplasmataceae* fam. nov. [11], previously referred to as the "Hominis" group, while cluster F is most closely related to bacteria in the *Mycoplasmoidaceae* fam. nov. [11], which is associated with the "Pneumoniae" group (Fig. 2).

Mycoplasmas from clusters A, B and F were detected in different snake families, while mycoplasma from clusters C, D and E were each only found in a single snake family. Significant differences in detection rates of mycoplasmas from individual clusters were detected in the various host families. Clusters C and E were detected significantly more often than others in Colubridae ($p < 0.001$), clusters D and F were detected significantly more often than others in Elapidae ($p < 0.001$), mycoplasmas from clusters A and F were detected significantly more often than others in Pythonidae ($p < 0.001$), and cluster F was detected significantly more often than others in Viperidae ($p < 0.001$).

**Table 3** Mycoplasma sequences (PCR1, Brown et al. 1995 [21]) used for phylogenetic analyses grouped according to genetic clusters. The sequences used were grouped into clusters according to top hits using the database "leBIBI" [35] and host family and species

| Cluster (total sequences) | Database "leBIBI": Top hits | Snake family (total sequenced/total used for tree building) | Species (Common name, number of sequences from that species) |
|---|---|---|---|
| **A (54)** | URS00004D329C *Mycoplasmopsis agassizii*<br>URS0000076237 *Mycoplasmopsis testudinea* | Boidae (7/5) | *Acrantophis dumerili* (Dumeril's Boa, 1) |
| | | | *Boa constrictor constrictor* (Boa constrictor, 1) |
| | | | *Candoia paulsoni tasmai* (Solomon Island Ground Boa, 2) |
| | | | *Corallus caninus* (Emerald tree boa, 1) |
| | | Colubridae (3/1) | *Dispholidus typus* (Boomslang, 1) |
| | | Elapidae (5/5) | *Naja haje* (Egyptian Cobra, 1) |
| | | | *Naja kaouthia* (Monocled Cobra, 2) |
| | | | *Naja naja* (Common cobra, 1) |
| | | | *Naja nivea* (Cape Cobra, 1) |
| | | Pythonidae (73/43) | *Antaresia maculosa* (Spotted Python, 1) |
| | | | *Malayopython reticulatus* (Reticulated Python, 2) |
| | | | *Morelia azurea* (Northern green python, 1) |
| | | | *Morelia spilota* (Carpet Python, 3) |
| | | | *Morelia viridis* (Green tree python, 10) |
| | | | *Python regius* (Ball python, 26) |
| **B (3)** | URS0000076237 *Mycoplasmopsis testudinea*<br>URS00004D329C *Mycoplasmopsis agassizii* | Boidae (7/1) | *Boa constrictor constrictor* (Boa constrictor, 1) |
| | | Viperidae (2/2) | *Trimeresurus venustus* (Beautiful pitviper, 2) |

However, these detection rates were likely influenced by the biased samples used and should be interpreted with caution.

There was no significant correlation between sample material and cluster affiliation with either PCR1 or PCR2.

Of the fifty-one samples sequenced for both PCR1 and PCR2, 25 (49%) resulted in the exact same clusterization, in group A (24 sequences) or B (1 sequence). Of the remaining 26 samples, 2 resulted in crossed hits (cluster A using PCR1 but cluster B using PCR2 and vice versa). This discrepancy could be attributable to the differences in length between the PCR products or potential mixed infections, which may have led to variations in clustering results between the two assays. For 17 sequences, only products from either PCR1 or PCR2 could be used for phylogenetic analysis, so that a comparative analysis of the clusterization was not possible, while 7 sequences were of insufficient length and quality for further use in phylogenetic analyses and were not compared further.

## Discussion

Overall, the detection of mycoplasmas in up to 40% of the clinical respiratory specimens tested demonstrates a significant degree of colonization of the upper respiratory tract of snakes from various families by mycoplasmas. Snakes in the families Pythonidae and Elapidae were most frequently colonized (up to 56% detection rate) while mycoplasmas were least often detected in Boidae (8% detection rate). Mycoplasma species related to ones adapted to tortoises, i.e., *M. agassizii* or *M. testudineum*, were the most frequently found in Pythonidae, as expected based on similar results of a previous study [28]. In contrast, in Colubridae, Elapidae, and Viperidae, the majority of mycoplasma species detected were only distantly related to *M. agassizii*. Despite a lack of standardization of the sampling protocol, nasal lavages appear to be the best sample for detection of mycoplasmas in snakes. Nasal lavages are also often recommended for mycoplasma detection in tortoises, with a high sensitivity [13]. The majority of the samples tested were oral swabs. This could have led to an underestimation of mycoplasma prevalence. It is also possible that mycoplasmas from prey animals rather than from the snake were detected in some cases. To rule this out, all sequences were compared to the corresponding sequences from known mycoplasma species of the major target group "mice" [36] using the database "leBIBI"[35]. No close relation between the sequences from the snakes and those from known mouse mycoplasmas could be established. That, together with the phylogenetic evaluation of all sequences as distinct from previously described mycoplasmas from non-snake hosts leads us to conclude that the mycoplasmas detected in this study are all most likely of snake origin.

**Table 4** Mycoplasma sequences (PCR2, Kuppeveld et al. 1992 [34]) used for phylogenetic analyses grouped according to genetic clusters. The sequences used were grouped into clusters according to top hits using the database "leBIBI" [35] and host family and species

| Cluster (total sequences) | Database "leBIBI": Top hits | Snake family (total sequenced/total used for tree building) | Species (Common name, number of sequences from that species) |
|---|---|---|---|
| **A (65)** | URS00004D329C *Mycoplasmopsis agassizii*<br>URS0000076237 *Mycoplasmopsis testudinea* | Boidae (6/2) | *Boa constrictor constrictor* (Boa Constrictor, 1) |
| | | | *Corallus caninus* (Emerald Tree Boa, 1) |
| | | Elapidae (11/1) | *Ophiophagus hannah* (King Cobra, 1) |
| | | Pythonidae (93/62) | *Antaresia maculosa* (Spotted Python, 1) |
| | | | *Malayopython reticulatus* (Reticulated Python, 3) |
| | | | *Malayopython timoriensis* (Timor Python, 1) |
| | | | *Morelia spilota* (Carpet Python, 5) |
| | | | *Morelia viridis* (Green Tree Python, 10) |
| | | | *Python bivittatus* (Burmese Python, 1) |
| | | | *Python molurus* (Indian Rock Python, 2) |
| | | | *Python regius* (Ball Python, 38) |
| | | | *Simalia boeleni* (Boelen's-Python, 1) |
| **B (4)** | URS0000076237 *Mycoplasmopsis testudinea*<br>URS00004D329C *Mycoplasmopsis agassizii* | Boidae (6/3) | *Boa constrictor constrictor* (Boa Constrictor, 1) |
| | | | *Candoia paulsoni tasmai* (Solomon Island Ground Boa, 2) |
| | | Pythonidae (93/1) | *Morelia viridis* (Green Tree Python, 1) |
| **C (1)** | URS000017757F *Mycoplasma anserisalpingitidis*<br>URS00003A6282, URS000005A434 *Mycoplasmopsis crocodyli*<br>URS000015EBF3 *Mycoplasmopsis alligatoris* | Colubridae (16/1) | *Hierophis viridiflavus* (Green Whip Snake, 1) |
| **D (1)** | URS000028F9C7 *Mycoplasmopsis iners* | Elapidae (11/1) | *Hemachatus haemachatus* (Rinkhals, 1) |
| **E (4)** | URS00005FF178 *Mycoplasmopsis iguanae* | Colubridae (16/4) | *Heterodon nasicus* (Western Hognose Snake, 1) |
| | | | *Lampropeltis* sp. (Kingsnake, 1) |
| | | | *Pantherophis guttatus* (Red Cornsnake, 2) |
| **F (31)** | URS00004B2C12 *Mycoplasmoides fastidiosum*<br>URS00004A8904 *Mycoplasmoides cavipharyngis*<br>URS0000069699 *Mycoplasma insons* | Elapidae (11/4) | *Naja nigricollis* (Black-necked Spitting Cobra, 1) |
| | | | *Naja pallida* (African Cobra, 1) |
| | | | *Naja siamensis* (Indo-Chinese Spitting Cobra, 2) |
| | | Pythonidae (93/6) | *Aspidites ramsayi* (Woma-Python, 1) |
| | | | *Malayopython timoriensis* (Timor Python, 1) |
| | | | *Morelia viridis* (Green Tree Python, 2) |
| | | | *Python regius* (Ball Python, 2) |
| | | Viperidae (30/21) | *Atheris* sp. (Bush Viper, 1) |
| | | | *Bitis arietans* (Puff Adder, 2) |
| | | | *Bitis caudalis* (Horned Adder, 1) |
| | | | *Bothriechis schlegelii* (Eyelash Viper, 1) |
| | | | *Bothrops atrox* (Common Lancehead, 2) |
| | | | *Cerastes cerastes* (Desert Horned Viper, 3) |
| | | | *Craspedocephalus trigonocephalus* (Ceylon Pit Viper, 1) |
| | | | *Crotalus atrox* (Western Diamond-backed Rattlesnake, 1) |
| | | | *Crotalus durissus* (Cascabel Rattlesnake, 1) |
| | | | *Crotalus horridus* (Timber Rattlesnake, 1) |
| | | | *Crotalus ruber* (Red Diamond Rattlesnake, 1) |
| | | | *Crotalus* sp. (Rattlesnake, 1) |
| | | | *Crotalus stephensi* (Panamint Rattlesnake, 1) |
| | | | *Crotalus vegrandis* (Uracoan Rattlesnake, 1) |
| | | | *Crotalus viridis* (Western Rattlesnake, 1) |
| | | | *Pseudocerastes fieldi* (Field's Horned Viper, 1) |
| | | | *Vipera ammodytes* (Nose-horned Viper, 1) |

PCR1 was originally developed for use in the diagnosis of mycoplasma infections in tortoises but has also been previously used to detect mycoplasmas in snakes [21, 27, 28]. It is known to detect not only *M. agassizii*, for which it was designed, but also related mycoplasmas such as mycoplasmas of the *M. fermentans* group [21]. This PCR was the first to detect a mycoplasma with a 90% similarity to *M. agassizii* in a python [26]. Here, clusters A and B mycoplasmas, closely related to *M. agassizii*, were also close to mycoplasmas previously described in pythons in Europe as well as pythons, a boa and a viper from Brazil [27–29]. The finding of this group of mycoplasmas in such diverse species and in various parts of the world indicates both a wide host range and a high capacity for colonization. *M. agassizii* is a known cause of upper respiratory disease in tortoises [12–14, 17] and unidentified mycoplasmas close to cluster A reported previously were associated with respiratory disease in snakes [27–29], suggesting a virulence potential. This aspect was not explored in the present study due to the lack of clinical information related to the samples.

The mycoplasmas in cluster B were closely related to those in cluster A. Although both clusters contained sequences with higher variability, characterized by multiple SNPs (see additional file 1), the clusters themselves consistently differed by only a single SNP over the 335 nt region of the 16S rRNA analysed. Despite this minimal difference, both clusters A and B were treated as distinct clusters due to the overall variability within each cluster. Additionally, cluster B was found to be slightly more closely related to *M. testudine*um than *M. agassizii* according to the "leBIBI" site, contrary to their placement in the phylogenetic analyses (Fig 1 and 2). This discrepancy could be due to the fact that the original lengths of the sequences were used to determine the most closely related species using the leBIBI database, whereas the sequences were trimmed to one length for the phylogenetic analysis resulting in the loss of some information. *M. testudineum*, like *M. agassizii*, has been associated with upper respriatory tract disease in tortoises [15–17]. Whether clusters A and B represent different species or strains or simply variations within a single species has yet to be unraveled.

Of the other mycoplasma species found (clusters C to F), only the single sequence from an elapid in cluster D was related to previously described mycoplasmas from snakes. This sequence was closely related to those from mycoplasmas isolated from two pythons with pneumonia and closely related (95%) to *M. caviae* and *M. fermentans* [31] (Fig. 2).

While the results of the sequence analyses clearly demonstrate the presence of genetically diverse mycoplasmas in snakes, the extent of that diversity, and which of the detected mycoplasmas represent new species requires further study. The specificity of individual mycoplasmas for specific host species or families is not known. The pet trade has been hypothesized to have played a role in transmission of pathogens between species and in increased pathogen diversity in multiple cases [37–39]. Transmission of mycoplasmas between snakes from various families originating from very different geographic regions is likely facilitated by the pet trade and husbandry practices [4, 28].

While this study included a wide range of host species in different snake families, the diversity of mycoplasmas detected means that in some cases, only a small number of sequences was available from individual host species and individual clusters. Another limitation of this study is the lack of detailed clinical background information on the sampled animals. Some had respiratory disorders, but others might have been clinically healthy animals sampled for quarantine testing or general health checks. Storage and freezing and thawing of DNA samples could also have influenced the results. DNA degradation could have led to reduced detection rates or changes in the diversity of the detectable mycoplasmas. To mitigate these effects, freeze-thaw cycles of the respective DNA samples were minimized. Inhibition and extraction controls were employed as additional measures to mitigate this, and analyses of samples was carried out over the entire period in which samples were collected, so that various samples were tested at different points in time.

## Conclusion

There is an urgent need to be able to grow snake mycoplasmas in vitro in order to have enough materials to run whole genome sequencing analysis and define if necessary new species. Here, the identification of the closest mycoplasma species is a first step to define which medium will be best to grow the different strains detected in snakes. This study provides an initial overview of the possible diversity of mycoplasmas in snakes as well as indicating how common these bacteria are in a wide variety of snakes. It should serve as a basis for encouraging mycoplasma diagnosis in snakes as well as examining the relationship between mycoplasma detection and clinical signs.

## Methods

### Sampling

Between January 2020 and December 2022, samples from snakes submitted to a commercial veterinary laboratory (Laboklin GmbH & Co. KG, Bad Kissingen, Germany) for routine diagnostic testing for respiratory pathogens were screened for the presence of mycoplasmas. Samples were

not specifically collected for the purpose of this study. Instead, leftover DNA from completed respiratory pathogen testing was used. Inclusion criteria were identification of the animal at least to genus level and inclusion of up to 300 samples from Pythons, as well as approximately 100 samples from snakes of other families to ensure a diverse representation of species.

In total, 640 samples from Boidae ($n = 114$), Colubridae ($n = 109$), Elapidae ($n = 34$), Pythonidae ($n = 301$), and Viperidae ($n = 82$) were tested (additional file 3). These samples originated from reptile rescue centers, zoological gardens, or private collections in Austria, Germany, the Netherlands, Switzerland, Slovakia, and Spain. All snakes were under veterinary care at the time of sampling. Clinical background information was not available for most animals and was therefore not included in the analyses. Most of the samples were oral swabs ($n = 590$) as well as nasal lavages ($n = 34$), tissues ($n = 14$), and lung lavages ($n = 2$). Nasal lavages were submitted primarily from pythons and boas, tissue samples mostly from viperids and elapids, and the two lung lavages were obtained from one python and one viper.

#### DNA extraction and PCR analysis

DNA was extracted from clinical specimens within 1 day after arrival in the laboratory. In brief, swabs were soaked in 750 µL of lysis buffer (Roche Diagnostics, Mannheim, Germany) supplemented by 75 µL proteinase K (500 µg/ml) (Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and incubated for 20 min at 65 °C. Tissue samples (10 µg) were resuspended in 500 µL of the same lysis buffer before being mechanically disrupted 40 sec at 6500 rpm in "MagNA Lyser Green Bead"-tubes (Roche Diagnostics, Mannheim, Germany) and then further incubated for 1 h at 65 °C to activate proteinase K. Two hundred µL of the lysis supernatant were used to extract DNA using a commercially available kit (MagNA Pure 96 DNA and Viral NA Small Volume Kit, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. After processing, the final volume of 100 µL of the purified DNA samples were stored at 4 °C for 1 week and tested for respiratory pathogens as requested by the submitting veterinarian, followed by storage at −18 °C. Testing for mycoplasma as detailed below was carried out continuously during the 3-year study period, and freeze-thaw cycles were minimized to reduce possible effects of DNA degradation on detection.

Samples were screened for the presence of mycoplasmal DNA using two PCRs. PCR1 targeted the V3 variable region of the 16S rRNA gene from *M. agassizii* as previously described using the primers Myc-affor1 (5'-CCTATATTATGACGGTACTG-3') and Myc-agrev1 (5'-TGCACCATCTGTCACTCTGTTAACCTC-3') and the original conditions [21]. PCR2 targeted a mycoplasma genus-specific sequence between the V6 and V7 16S rRNA regions, using primers GPO-3 (5'-GGGAGCAAA CAGGATTAGATACCCT-3') and MGSO (5'-TGCACC ATCTGTCACTCTGTTAACCTC-3') [34]. PCR2 was run with a modification of the original cycle: 94 °C for 2 min, 10 "touch down" cycles at 94 °C for 60 s, 65 °C for 60 s (−1 °C per cycle) and 72 °C for 60 s, followed by primer annealing and polymerization: 30 cycles at 94 °C for 60 s, 55 °C for 60 s and 72 °C for 60 s, then a final extension step at 72 °C for 7 min. Total volume used was 20 µl including 5 µl DNA. PCRs 1 and 2 were run using a commercial buffer (FastStart Essential DNA Probes Master, Roche Diagnostics GmbH, Mannheim, Germany) and the Biometra Tone platform (Analytik Jena GmbH & Co.KG, Jena, Germany). Each PCR run included a negative and a positive control as well as an extraction control (DNA or RNA Process Control Detection Kit, Roche Diagnostics GmbH, Mannheim, Germany) in each sample to assess nucleic acid extraction efficiency and PCR inhibition. The expected sizes of the PCR products, i.e., 576 bp for PCR1 and 270 bp for PCR2, were verified by capillary electrophoresis using a QIAxcel Advanced system (QiagenQIAGEN GmbH, Hilden, Germany).

#### Sequence analysis

To verify the specificity of the reactions and attempt species identification, a subset of PCR products was subjected to Sanger sequencing. Due to financial limitations, not all PCR products were sequenced, but at least half of the positive samples per snake family were randomly chosen for sequencing (PCR1: $n = 90$; PCR2: $n = 159$). Briefly, PCR products were purified using a MinElute purification kit (Qiagen, Hilden 40724, Germany) according to the manufacturer's instructions and sequenced on both strands using a Big-Dye Terminator v3.1 cycle sequencing kit (Life Technologies, Bochum 44799, Germany). Results were analyzed with an ABI 3130 sequencer (Applied Biosystems, Thermo Fisher Scientific, 64293 Darmstadt, Germany). Sequences were edited using Geneious Prime® 2024.0.2 [40] primer sequences were removed and the forward and reverse strands were assembled into a consensus sequence.

Identification of *Mycoplasma* to species level based on these partial 16S rRNA sequences was done using BLASTN [41] analysis against NCBI databases [42, 43] and the leBIBIQBPP online tool [35].

Phylogenetic analysis was carried out with Geneious Prime® 2024.0.7 [40], with MUSCLE 5.1 [44] for multiple alignment and RAxML [45] for maximum likelihood estimation (with GTR GAMMA as the nucleotide model, default settings and a bootstrap analysis of 1000 repeats). Samples were clustered according to their phylogenetic

assignment and the first hits in the leBIBI database. Within each cluster a consensus sequence was created for further phylogenetic analyses. The sequences were compared with those selected based on the closest hits in the leBIBI database, as well as with previously described mycoplasma species in reptiles and other well-known mycoplasma species.

### Statistical analysis

The Pearson chi-squared test was used with a type I error α of 0.05 to test the independence of compared detection rates using SPSS (Statistical Package for Social Science, Version 29.0.0.0 IBM®). Fisher exact test was used for small sample sizes tested ($n < 30$). Bonferroni correction was used to control Pearson chi-squared tests computed with multiple values. Yates's correction for continuity was used when at least one cell of the table had an expected count smaller than 5.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04487-4.

Additional file 1 (.pdf): Alignment of the sequences assigned to the respective clusters. Sequences obtained using PCR1 [21]. Consensus sequences and sequence alignments of each cluster used for phylogenetic tree building. Sequences are named with the host family followed by an individual identification number of a representative sequence and in parentheses the total number of samples from which identical sequences were obtained.

Additional file 2 (.pdf): Alignment of the sequences assigned to the respective clusters. Sequences obtained using PCR2 [35]. Consensus sequences and sequence alignments of each cluster used for phylogenetic tree building. Sequences are named with the host family followed by an individual identification number of a representative sequence and in parentheses the total number of samples from which identical sequences were obtained.

Additional file 3 (.pdf): Tabulated additional information on the snake samples examined. Information on all examined snakes grouped according to family, followed by species and number of animals of each species that were tested positive for mycoplasmas using two different PCRs, PCR1 according to Brown et al., 1995 [21] and PCR2 according to van Kuppeveld et al., 1992 [35].

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**Authors' contributions**
R.E.M., M.P., and M.M.F. planned the study; E.M. supplied the resources; M.M.F. carried out the analyses; M.M.F., F.T., F.S., and R.E.M. analyzed the data; M.M.F., R.E.M., and F.T. wrote the main manuscript; M.M.F., F.S., and F.T. prepared figures 1 and 2. All authors reviewed the manuscript.

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**Data availability**
Sequence data generated in this study have been deposited in GenBank with the accession numbers PQ452173-PQ452205. Other data is provided within the manuscript and in the supplementary files.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
Authors M.M.F., F.S., E.M., and R.E.M. are employed by a commercial veterinary laboratory.

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## Publisher's Note