

# Thesis

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**Molecular investigation of protozoa and bacteria of  
veterinary-medical importance from the faeces of captive  
reptiles**

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## 1. Introduction

Reptiles in captivity and as pets have greatly increased in popularity over recent years. With this greater demand for domestic reptiles brings a higher occurrence and diversity of parasites. While some reptiles sold as pets are bred in captivity, the majority are captured in the wild or are the offspring of wild-caught parents. Unfortunately, there is a high percentage of reptile pet owners who are unaware of the accurate management and husbandry practices to successfully keep domestic reptiles. For many species, the diet and housing requirements are unknown and in the wild, reptiles rarely come into contact with their own waste or uneaten food, which is a common occurrence in captivity. These factors result in the cold-blooded creatures having a suppressed immune system and therefore, a greater likelihood of infection. Many reptiles are asymptomatic carriers of parasites throughout their life. However, even if they are asymptomatic, captive and free-ranging reptiles can harbour and excrete a wide range of diseases that can also cause infections in humans. This zoonotic aspect makes this topic significant not only in veterinary medicine but also in human medicine.

Endoparasites are very common in both wild and captive reptiles, with many of them being only mildly pathogenic, however, with the stress of captivity and improper husbandry they can lead to serious disease and even death. Bacteria and protozoa are two vital endoparasites in the intestines of reptiles from a veterinary point of view, many of them are transferred via the faecal-oral route. The effects of these parasites in severe bacterial infestations can result in anorexia, regurgitation, stomach ulcers and intestinal obstructions. Signs of infection of protozoal infestation are loss of appetite and weight, vomiting, mucus-containing or bloody diarrhoea, and death.

In the present study, the National Reptile Centre of Ireland was selected for sampling. In this study, 98 reptiles and one amphibian were non-invasively sampled. These creatures came from 44 different species and four different orders (Testudines, Squamata, Crocodylia, Anura). Faecal samples were obtained from these animals in artificial enclosures with emphasis on avoiding soil contamination. Conventional PCRs were used to screen all DNA samples for eukaryotic DNA, protozoan parasite DNA (*Trichomonas* and *Trypanosoma* spp.), and bacterial DNA (from Anaplasmataceae, Rickettsiaceae and related families).

The aim of these analyses was to detect bacteria and protozoa in the faeces of a diverse range of captive reptiles of veterinary-medical significance to further improve our knowledge of reptile care and health.

## 2. Literature Review

### 2.1. Reptiles in general

Reptiles have descended from very ancient lineages over 200 million years ago (Shine, 2013). There have been 22,000 species and subspecies of reptiles discovered in over the last 260 years. The total taxa valid in 2019 was 13,361 containing 11,050 species and 2,311 subspecies excluding nominate subspecies (Uetz et al., 2019). However, this list of taxa is continuously updating with approximately two new species being described and logged every week on the Reptile Database. (Uetz & Stylianou, 2018). Reptiles belong to four different lineages, turtles with 300 species, tuatara which is 1 species, Squamata (Lizards and snakes) with 7750 species and crocodylians with 23 species (Shine, 2013). They are extremely resilient creatures living in habitats from mountaintops, deserts to a marine environment resulting in a wide range of adaptations allowing them to survive in these severe conditions (Taylor et al., 2021). Out of 364 collections, the majority with 87 were in Asia, 83 were found to be in Europe which included Russia and South America had 83. Others being in Central America, Africa, Middle East and Australasia (Uetz et al., 2019). Evolutionary biologists have difficulty with the fact that even though they are under the same taxa 'reptiles', they are not actually closely related to each other. They are not a monophyletic evolutionary group, although when you look at the phenetics you would think otherwise, for example, tuataras and crocodiles with their scales and distinct body shape look very similar to lizards. Thus, reptiles were defined from their external morphology but the actual main way in which they are similar is the way they regulate their body temperature (Shine, 2013).

Reptiles' diet might be an important factor in the infection of their gastrointestinal tract. Most reptiles are carnivores, particularly crocodiles and snakes. However, many lizards and chelonian species diets are supplemented with plant matter. Other species, especially tortoises have a primarily herbivore diet (Gilbert et al., 2019).

Reptiles are ectotherms (Taylor et al., 2021) meaning they regulate their internal temperature by depending on ambient thermal heterogeneity, for example, they move to sit in the sun for warmth and go to the shade to cool down (Shine, 2013) thus they are very sensitive to the environmental temperature resulting in it affecting reptiles drastically daily and seasonally (Taylor et al., 2021). These effects from the external temperature have been plotted as performance as a function of body temperature known as a "thermal performance curve"

stating that as temperature increases so does the performance until it reaches harmful levels and then it will decline (Huey & Berrigan, 2001). Human impact has resulted in a massive decline in populations of reptiles and increase in the spread of disease (Taylor et al., 2021) leading to a colossal loss of biodiversity (Zipkin et al., 2020).

The immune system of reptiles is very different to that of mammals and birds. Their immune system does not form germinal centres and do not contain Peyer's patches in the lumen of the intestinal tract. There is not much research of their adaptive immune response, but their innate immune system appears to respond against a wide range of pathogens very quickly as a non-specific first line of defence, seemingly a stronger response than that of mammals (Gilbert et al., 2019).

For many years, reptiles have been kept in captivity (Wilkinson, 2015) and they are becoming increasingly more popular as pets throughout the world (Wolf et al., 2014). Reptiles are a favoured host for a broad range of pathogens, such as helminths, protozoa and arthropod parasitic species (Mendoza-Roldan et al., 2020). The most important factor in captive reptiles' health is maintaining proper husbandry as this is the most common cause of illness. Factors such as temperature, lighting, humidity, nutrition and caging are vital to have correctly to reduce stress on the reptile for a thriving environment. Stress leads to an increase in disease as it causes immunosuppression (Wilkinson, 2015). Therefore, you must know about the variety of possible parasites that the species of reptile is subject to, in order to prevent a deterioration in their health. This is even more important for non-captive reptiles suffering from the harmful effects of climate change and habitat loss (San-martín-Órdenes et al., 2019).

## **2.2. The species tested**

### **2.2.1. Snakes**

Snakes are an important group of vertebrates (Streicher & Wiens, 2016). There are more than 3,400 different species of Serpentes throughout the world. Their habitats vary from arboreal, terrestrial, fossorial to aquatic and desert wastelands (Hsiang et al., 2015). Snakes, as legless predators, are unusual in their ability to paralyze and kill their prey by constriction, but the exact mechanism by which this pressure incapacitates and kills its prey is still unknown (Boback et al., 2015). Recent studies have shown that there is uncertainty among the different groups of snakes. There has been strong support to state that there are three different major monophyletic groups. The first group unites cylindrophiidae and uropeltidae,

the second groups boas, pythons and their relatives together and finally, the third group consists of bolyeriids (Round Island boas) with pythonids and their relatives (xenopeltidae and loxocemus) (Streicher & Wiens, 2016). For this study we will focus on eight different snake species which include the milk snake, boa constrictor, royal python, carpet python, yellow anaconda, gopher snake, burmese python and the california king snake.

The Milk Snake also known as *Lampropeltis triangulum* have a large variety of colour forms, mainly varying red, black and white stripes but are uniform in size and shape (Maresová et al., 2009). They prefer open habitats throughout (Shine & Fitzgerald, 1995) all of the seasons and their thermoregulation is more effective during Springtime (Row & Blouin-Demers, 2006). *Lampropeltis californiae*, the California Kingsnake, is one of the most popular captive snakes. They originate from North America (Santana-Hernández et al., 2021). Californian Kingsnakes are ophiophagus snakes. This means that they can eat prey snakes that are the same length as or longer than themselves. Corn snakes are their preferred diet which they subdue by constriction and swallow the head first. Within the first four days, corn snakes were roughly half digested and completely digested within 15 days (Jackson et al., 2004). Due to its small size, pleasant personality, hardy nature, and range of colours, ball pythons (*Python regius*) have become one of the most popular pet reptiles kept and bred in captivity. Hundreds of genetic mutations are combined to create new and unique phenotypes, resulting in approximately 6,000 colour combinations (Tillis et al., 2021). With the Morelia spilota Male-male combat involves a lot of biting and ritualized "wrestling" sessions, which resulted in a lot of bite scars in adult males. Males grow larger than females in this group, as predicted by sexual selection theory, whereas females grow larger than males in the previously studied southern population where males do not engage in physical combat for mating opportunities (Shine & Fitzgerald, 1995). *Pituophis melanoleucus*, gopher snake, is one of North America's most widely dispersed polytypic species complexes. *Pituophis melanoleucus* (sensu stricto), *P.catenifer*, and *P.ruthveni* are the three species that make up the melanoleucus complex (Rodríguez-Robles & De Jesús-Escobar, 2000). In the Florida Everglades, the Burmese python (*Python bivittatus*) is an invasive snake (Richard et al., 2019). Young Burmese pythons eat mostly tiny mammals, and food variety among snakes of this size is limited. At least 37 mammal and bird species have been recorded to be eaten by these snakes. By the fourth day, the snakes have completely consumed their victim, and bones, skin, and other soft elements are no longer discernible (Falk & Reed, 2015). Boa constrictor is a type of snake. These snakes are prevalent in periurban rainforest fragments in the Americas, where they have a wide distribution. Small animals, lizards, amphibians,

and birds are among the prey (Pizzatto et al., 2009; Cabral et al., 2019). Because of their non-venomous nature, they are overlooked as carriers of infections with zoonotic potential, such as fungus, viruses, and bacteria. (Rodamilans et al., 2020). Yellow anacondas (*Eunectes notaeus*) are big semiaquatic boid snakes that live in South American wetland habitats. They like moist, marshy environments and can be found primarily along rivers and floodplains, as well as their accompanying vegetative habitats (McCartney-Melstad et al., 2012).

### 2.2.2. Lizards

In our study we researched a wide variety of species of lizards. Gekkota, Scincomorpha, Autocharglossa and Iguania are the different suborders observed. Geckos are a large family of lizards that include over 1,700 species with a wide range of ecologies, behaviours and sizes (Kulyomina et al., 2019). They are primarily found in warm temperature locations and include several famous examples of island colonization across the three major oceans (Gil et al., 2020). The overall gecko body shape is adaptable to a variety of environments, from arboreal to terrestrial (Kulyomina et al., 2019). Geckos are nocturnal (Schmitz & Higham, 2018) and their dietary habits are assumed to be exclusively insectivorous (Gil et al., 2020). Geckos have a number of distinguishing features, such as adhesive digits, nocturnal activity, hard, calcareous eggshells, and they lack eyelids (Xiong et al., 2016). They have outstanding terrestrial locomotion abilities, allowing them to move through a wide range of terrains and surface orientations. To coordinate their limb motions, geckos use cyclical lateral bending of their flexible trunk and tail (Wang et al., 2020). The crested geckos, *Correlophus ciliatus*, are common in the pet trade. Crested geckos actively thermoregulate to maintain optimal performance temperatures, and their thermal biology is similar to that of other nocturnal or shade-dwelling species (Aparicio Ramirez et al., 2021).

Since the early 1960s, the leopard gecko, *Eublepharis macularius*, has been reared and raised in captivity. This nocturnal and crepuscular gecko has become one of the most popular pet lizards (Oonincx et al., 2020). Wild leopard geckos are found in India, Iran, Nepal, Afghanistan and Pakistan. A yellow-and-black-spotted dorsal section, a greyish tail with white transverse stripes and black dots, a yellowish head with black dots, and a white/light cream ventral half of the body characterize the wild-type adult leopard gecko (Szydłowski et al., 2020).

Leaf-tailed geckos are a distinct group of carphodactylinae geckos found in eastern Australia's rainforests and rocky environments. *Phyllurus* (9 species), *Saltuarius* (6 species), and *Orraya* are the three genera recognized (1 species) (Hoskin & Couper, 2013).



We gathered data from a variety of Iguana species for our research. The most common squamate exotic pet reptile is the bearded dragon *Pogona vitticeps* (Pénzes et al., 2020). Bearded dragons change color in response to diverse backgrounds, and the intensity of illumination influences this color change. Bearded dragons hibernate by burying themselves under dirt or seeking shelter in fallen logs or tree stumps during the coldest months of the year (between May and September), when temperatures vary from 5 to 18 °C (Capraro et al., 2020). Throughout its vast geographical range, the species inhabits a variety of different surroundings covering a large part of central Australia (Cadena et al., 2017). The frilled dragon, *Chlamydosaurus kingii*, also originated from Australia. Their head and neck are surrounded by a huge disc of skin. This frill is generally curled back against the body, but it can flare out spectacularly to frighten predators and competitors away (Montandon et al., 2019). Another Australian agamid lizard is the water dragon, *Intellagama lesueurii*, that can grow to be over a metre long (Baird et al., 2014) which is a semi-aquatic squamate (Maclaine et al., 2018).

Green iguanas are arboreal lizards that are commonly kept as pets and in captivity (Macêdo et al., 2020). Given effective captive settings, this species thrives in captivity, and behavioral interactions appear to be very comparable to those found in natural populations (Phillips, 1990). Juvenile and neonate Green Iguanas eat young shoots, leaves, blooms, and fruits, as well as insects like grasshoppers whereas adults have been observed eating bird eggs and carrion in addition to these foods (Krysko et al., 2007). The black spiny-tailed iguana, *Ctenosaura pectinata*, is a Mesoamerican indigenous mammal with a range that stretches from Mexico to Panama. From sea level to 800 m, it has been found in both tropical dry and humid forest habitats, becoming one of the communities' food sources (Ríos et al., n.d.).

Varanids, popularly known as monitor lizards, are squamate reptiles that belong to the Anguimorpha suborder, which, along with iguanas and snakes, makes up the Toxicofera clade. There are currently 80 varanid species that have been identified (Iannucci et al., 2019). They are located in Asia, Australia and Africa. Varanids are a group of lepidosaurs having a similar body pattern and they are active foragers with great metabolic rates who use buccal pumping to counter ventilatory-locomotor restrictions (Cieri, 2018). They range from small in size to giant and include the largest limbed squamates today (Conrad et al., 2012). Bosc monitor, rough-necked monitor and asian water monitor are the species studied in this research.

*Heloderma suspectum*, the Gila monster and *Heloderma horridum*, the beaded lizard are found naturally in North and central America (Douglas et al., 2010). The beaded lizard is

one of the world's few known poisonous lizards which is also found in Mexico. Because of habitat damage and capture for the pet trade their populations are declining (Guadarrama et al., 2020). The Gila monster is also a venomous lizard that prefers a desert habitat (French et al., 2015).

The Black and white tegu, *Salvator merianae*, is from South America, and is now an invasive species in southern Florida. They are seasonally active and spend the dry or cold climates hibernating. Tegus have the highest body sizes (up to 5 kg) of any Teiidae member, and a diverse diet of fruits, invertebrates, small vertebrates, eggs, and carrion which may let them thrive outside of their natural habitat (Goetz et al., 2021).

Finally, the Blue-tongued skin, *Tiliqua Scincidae*, are known for their large blue melanin-pigmented tongues which they typically display when they feel threatened (Abramjan et al., 2015).

### 2.2.3. Tortoises

Chelonians consist of turtles and tortoises which have existed for about 220 million years. They have been an important part of the global ecosystem and have played a significant role in human culture (Stanford et al., 2020). Tortoises belong to the Testudines order and the Testudinidae family of land-dwelling reptiles (Coomansingh Springer et al., 2020). There are currently 335 species of chelonians in the taxonomic order Testudines (Zimmerman & Mitchell, 2017). Currently, more than half of the 360 live species and 482 total taxa (species and subspecies combined) are endangered (Stanford et al., 2020). Marine turtles are migratory animals that have a complicated life cycle. Adult migrations from feeding grounds to breeding sites are part of their life cycle, as are ontogenetic alterations that affect juvenile dispersal throughout a variety of maritime habitats (Álvarez-Varas et al., 2019). The phrases turtle and tortoise are still used interchangeably in many parts of the world. In North America, a turtle is any member of the family Testudinidae, regardless of whether they live on land or in water, however in the United Kingdom, tortoises are only members of the order Testudines that live on land (Coomansingh Springer et al., 2020). The chelonian shell is a remarkable evolutionary adaptation that allows chelonians to thrive in a variety of environments, including terrestrial, freshwater, and marine (Stanford et al., 2020). Tortoises are found in a variety of habitats and perform key ecosystem tasks like seed dispersion and burrowing. Resource availability, reproductive status, and local environmental circumstances have all been linked to tortoise movement in the past (Drabik-Hamshare & Downs, 2017). In this study we will concentrate on the leopard tortoise, pancake tortoise,

red footed tortoise, yellow footed tortoise, the roti island snake necked turtle and the alligator snapping turtle.

Leopard tortoises, *Stigmochelys pardalis*, are the most common and largest tortoise in Sub-Saharan Africa (Drabik-Hamshare & Downs, 2017). Within the Nama-Karoo biome, they are subjected to vast variations in environmental circumstances as well as the unpredictability of food and water supply. Leopard tortoises can change parameters such as transit rate, food intake, water loss, and urine osmolality to maintain body mass, water, and energy balance. This reveals that leopard tortoises in the wild can take advantage of unpredictably available food and water because of their digestive flexibility. (McMaster & Downs, 2008).

The Red-footed Tortoise, *Chelonoidis carbonaria*, has the most diverse variety of habitats among tortoise species, occurring in South American countries as well as nearly all Caribbean islands, including Grenada. They prefer a temperature range of 20 to 30 degrees Celsius throughout the year (Coomansingh Springer et al., 2020). They are primarily herbivores, eating foliage, fruits, and flowers, but they can also eat carrion and excrement meaning they have a coprophagic eating behaviour (Sharma et al., 2020). They are a solitary species who tend to only interact with conspecifics for mating opportunities (Wilkinson et al., 2010).

Yellow-footed tortoises, *Chelonoidis denticulatus* can be found across the Amazon Basin. The topographic gradient in this area affects soil type, vegetation composition, tree biomass, and tree falls. As a result, the topographic gradient may influence the distribution of nesting sites, food sources such as fruit, flowers, and fungi, and the existence of tree fall gaps, which yellow-footed tortoises frequently exploit for shelter and thermoregulation (Tavares et al., 2019).

The pancake tortoise, also known as *Malacochersus tornieri* is a soft shelled, small, dorsoventrally flattened chelonian with a patchy distribution located in the savannas of south-eastern and northern Kenya, as well as northern, eastern, and central Tanzania (Eustace et al., 2021).

Finally, the alligator snapping turtle is located in the southeast USA. They are part of the chelonian family and the scientific name is *Macrochelys temminckii* (Baker et al., 2019). The alligator snapping turtle is the largest turtle in North America and is known for its bite injuries (Johnson & Nielsen, 2016).

Habitat loss and degradation, over-collection of turtles and their eggs for food consumption and the worldwide pet trade, as well as over-collection for the trade in traditional remedies

derived from turtle parts, are the main challenges facing turtles and tortoises in the early twenty-first century (Stanford et al., 2020).

## 2.3. Possible Pathogens

### 2.3.1. Bacterial Pathogens of veterinary-medical significance in reptiles

In recent years there has been an increase in captive reptiles and with this, an increased record of zoonotic diseases. Salmonella can infect a diverse range of species including reptiles, mammals, birds and humans (Lou et al., 2019). Salmonella are gram-negative bacteria with six subspecies and over 2,600 serovars (Lou et al., 2019). They are facultative intracellular pathogenic bacteria species with the ability to invade dendritic, epithelial and macrophage cells (Siriken, 2013). Reptiles excrete salmonella through their faeces. This excretion is intermittent and thus, makes it difficult to detect salmonella if only one bacteriological exam is carried out. When several bacteriological exams are carried out on the samples, it is found that several Salmonella serovar are isolated from reptiles including *Salmonella enterica subspecies houtenae* and *Salmonella bongori* (Ebani, 2017). In a recent study, it was found that the most common subspecies which was present across all of the reptilian taxa is *Salmonella enterica subsp enterica* and a second common subspecies is *Salmonella enterica subsp diarizonae*. Although the latter was found mainly in snakes and a lot of these snakes were clinically ill. Dermatitis, anorexia and bony changes were the most common clinical signs found (Clancy et al., 2016). Another study in 2013, to test for *Salmonella enterica* consisting of 251 faecal samples from a variety of reptiles suffering from diarrhoea and 288 carcasses were dissected and examined for pathohistological results. There was a high percentage of salmonella-positive results with lizards at 35.5% positivity, 47.2% of the snakes and in the chelonians there was a 11.7% positive result (Sting et al., 2013). Research in Malaysia by Cheng, Wong and Dykes identified that captive lizard species have a higher percentage of carriage rate and shedding than wild free-living lizards with 25% of free-living lizards testing positive for Salmonella compared to 83.3% of captive lizards testing positive (Masila et al., 2020). Although *Salmonella* is widespread throughout the reptile species, most of the reptiles are asymptomatic carriers in their gastrointestinal mucosa (Bjelland et al., 2020). However, in some cases there has been pneumonia, septicaemia, hypovolemic shock, abscesses, osteomyelitis and death observed (Ebani, 2017). The research is vital as the most important reptile-associated zoonotic disease is *Salmonella*

(Lukac et al., 2015) which can cause severe fatalities in humans due to meningitis and septicaemia (Schröter et al., 2006).

Another important reptile-associated zoonotic disease found in all reptilian taxa is *Epsilonproteobacteria* comprising *Campylobacter*, *Helicobacter* and *Arcobacter*. They have a wide host range including mammals, birds and reptiles (Gilbert et al., 2014). They are gram-negative, flagellated and rod-shaped bacteria. Most are microaerophilic with some members growing in anaerobic conditions and there are also certain members of *Arcobacter* that grow aerobically (Gilbert et al., 2019). They are primarily associated with the gastrointestinal tract (Gilbert et al., 2014). In most reptile species, *Epsilonproteobacteria* were present with no clinical symptoms. However, the highest percentage of *Epsilonproteobacteria* were found in herbivore reptiles, mainly Chelonians, who rely on intestinal microbiota for digestion but without adverse health effects (Gilbert et al., 2019). *Campylobacter* in Squamates has been found to be a source of transmission of campylobacteriosis, a gastrointestinal disease to humans. Studies over the last 20 years have shown that the most common *Campylobacter* species responsible are *C. fetus subsp. testudinum* and *C. fetus subsp. fetus* found most commonly in the green iguana - *Iguana iguana*, bearded dragons - *Pogona vitticeps*, blotched blue-tongued skink - *Tiliqua nigrolutea* and the western beaked gecko- *Rhynchoedura ornate* (Masila et al., 2020). However, although most Chelonians and lizards appeared clinically healthy, all snake species carrying *C.fetus subsp. testudinum* were ill or deceased (Gilbert et al., 2014). There have been records of fatalities in different species of snakes (Orozova et al., 2012) caused by the gram-negative, facultative anaerobic, rod-shaped bacteria *Aeromonas* which is widely distributed in aquatic ecosystems (Gonçalves Pessoa et al., 2019). In a study, Orozova et al., (2012) found that *Aeromonas hydrophila* was the causative agent for snake deaths. For example, a yellow anaconda (*Eunectes notaeus*), a corn snake (*Pantherophis guttatus guttatus*) and a jamaican boa (*Epicrates subflavus*), all captive snakes, died with haemorrhages in their intestines, spleen and lungs and also, containing petechial haemorrhages in their gums and mouth which are all signs of bacteraemia. All three snakes suffered from swollen abdomen and anus with their colons containing blood-tinged mucus. After obtaining pure culture growths of the internal organs, it was confirmed that *Aeromona hydrophila* was the causative agent. These deaths were linked to a sudden change in temperature caused by a broken temperature regulator dropping from 38-40°C to 18-20°C (Orozova et al., 2012).

Although Salmonella may be the most commonly found zoonotic bacteria in reptiles (Lukac et al., 2015) there are other bacteria detected in reptiles such as *Leptospira*, *Mycobacterium* and *Chlamydia* in cold-blooded animals (Rataj et al., 2011). Leptospirosis is one of the most common zoonotic diseases in the world caused by pathogenic spirochetes of the genus *Leptospira* (Bauso et al., 2020). Although leptospirosis is as widespread today as it is, there is still limited research on the zoonotic spread of leptospirosis, as well as chlamydiosis and mycobacteriosis from reptiles to humans (Ebani, 2017). It is mostly found in tropical regions where it is considered endemic (Pérez-Brígido et al., 2020). There is a large presence of cold-blooded animals in these tropical regions (Pérez-Brígido et al., 2020) and *Leptospira*'s preferred habitat is aquatic (Paz et al., 2019). They are transmitted by domestic and wild animals with rodents playing an essential part in the life cycle (Bauso et al., 2020). In Florida, there was a case reported by Feuer and Domash-Martinze (2011) of a human contracting leptospirosis after contact with an alligator (Rodamilans et al., 2020). Boa constrictors live in the fragments of rainforest in the peri-urban areas of Brazil in close contact with humans where there is a significant incidence of leptospirosis in humans, a study was carried out from blood samples of wild boa constrictors which confirmed the presence of *Leptospira interrogans*. There was a high percentage of positive cases which can be linked to the poor sanitation conditions and the diet of the snake, mainly being small rodents in these areas (Rodamilans et al., 2020). Leptospiral antibodies were confirmed in captive lizards, turtles and snakes by Lindtner-knific et al. (2013). The Caiman species also live in an ideal environment, large wetlands, to be involved in their transmission and so, a recent study investigated 45 blood samples of wild and captive caiman latirostris with positive antibody results (Bauso et al., 2020). It is believed that *Leptospira* spirochetes are excreted by these reptiles in their urine and thus, when the soil, water or food become infected, the bacteria can easily be transmitted to humans (Ebani, 2017). There have been various more studies to prove that reptilian species are hosts for *Leptospira*.

There are 14 species of *Chlamydia* of the family *Chlamydiaceae* described. They are gram-negative bacteria, obligatory (Zaręba-Marchewka et al., 2020) intracellular bacterial pathogens. They infect a broad range of species including both captive and wild herpetofauna (Taylor-Brown et al., 2015). There is not a full understanding of the route of transmission, but they may occur through direct contact or airborne aerosols containing dust or faecal particles or via fresh faeces in comparison to the routes of other chlamydial agents (Zaręba-Marchewka et al., 2020). There have been new species of *Chlamydia* identified within birds and reptiles, however, they still remain under the candidatus status. *Candidatus*

*C. corallus*, *Cand. C. ibidis* and *Cand. C. sanzinia* were found in snakes, and *Cand. C. testudinis* were identified in turtles. Chlamydiosis can be carried asymptotically in reptiles, however, it may also cause infection in internal organs such as the spleen, liver, heart and lung manifesting as granulomatous inflammation (Taylor-Brown et al., 2015). Chlamydia pneumonia is the most common agent found in humans and due to recent studies have proven that it is also the most prevailing chlamydia agent in reptiles (Zaręba-Marchewka et al., 2020). *C. pneumoniae*, together with *C. abortus* have been detected in a necropsy of a captive burmese python with chronic respiratory disease and severe diffuse suppurative pneumonia. Puff adders were unwell with clinical symptoms of mild respiratory disease and regurgitation and eventually death. At necropsy, it was found that they had pneumonia, myocarditis, hepatitis, splenitis, severe granulomatous pericarditis and enteritis and with collected tissue samples the two agents were identified. Again, the two chlamydia agents were identified together, with *Neochlamydia* species in a captive green sea turtle. The *Chelonia mydas* was suffering from lethargy, anorexia and reluctance to dive and under post mortem necrotizing myocarditis was found in the heart (Taylor-Brown et al., 2015). Other commonly reported species of *Chlamydia* in reptiles were *C. psittaci*, *C.p pecorum* and *C.c caviae* (Inchuai et al., 2021). Moorish turtles with pneumonia tested positive for an avian *C. psittaci* strain and Huckzermeyer et al. (1994) identified *C. psittaci* in Nile crocodiles (*Crocodylus niloticus*) with hepatitis and generalized oedema (Taylor-Brown et al., 2015). A study in 2021 found that the highest prevalence of *Chlamydia* was identified geographically in Australia and the highest rate in a reptilian species was in crocodiles. It is vital to be aware of reptilians' role in the life cycle of *Chlamydia* as it can cause disease and death not only in many species of reptiles but *C. pneumonia* and *C. psittaci* are a zoonotic pathogen (Inchuai et al., 2021).

*Mycobacterium* species belong to the family *Mycobacteriaceae*. They are gram-positive, non-motile, aerobic pleomorphic bacilli (Delghandi et al., 2020). Mycobacteriosis is a very important zoonotic disease and has a broad range of species acting as reservoirs including wildlife, companion animals and livestock (Ullmann et al., 2016). There are over 120 known species in the *Mycobacterium* genus such as *M. bovis*, *M.t tuberculosis* and non-tuberculous mycobacteria (NTM). They are responsible for many diseases in both animals and humans, the two most important are tuberculosis and leprosy causing high incidences of morbidity and mortality (Alcaide et al., 2017). *Mycobacterium* preferred habitat is in tropical and sub-tropical regions, however, they have been recorded in locations such as Norway, Chile and Canada where there is a colder climate (Alcaide et al., 2017). The transmission of

mycobacteriosis within reptiles is still not fully understood but it is believed that ingestion and/or cutaneous lesions are the main route. Non-specific signs such as lethargy, weight loss and anorexia can accompany the illness, with stomatitis, skin alterations and respiratory tract infections as the main lesions in reptiles with a chronic disease. It has been difficult to identify mycobacteriosis in reptiles due to it usually being a chronic disease, non-specific clinical symptoms and a difficult complex diagnosis (Ullmann et al., 2016). However, there have still been a large number of studies that have proven the presence of *Mycobacteria* in different varieties of reptilian species. *Mycobacterium chelonae*, a NTM, has been detected in tortoises and turtles many times mainly with clinical symptoms of cutaneous and oral mucosa lesions, plastral ulcerations, sepsis and disseminated intravascular coagulation. *M. chelonae* has also been detected in snakes and saurians with granulomatous lesions (Ebani, 2017). Another NTM species, *Mycobacterium haemophilum* has been detected in wild Juvenile leatherback turtles (*Dermochelys coriacea*) in Florida as the causative agent of disseminated mycobacteriosis (Donnelly et al., 2016). Another free-ranging loggerhead turtle in Italy was diagnosed with disseminated mycobacteriosis with tachypnea and reduced alertness (Nardini et al., 2014). *Mycobacterium kansaii*, *M. kumamotoneres*, *M. haemophilum*, *M. ulcerans*, *M. marinum* and *M. fortuitum* have all been detected in cold-blooded animals with typical disseminated granulomas. A captive freshwater crocodile was suffering from lethal pneumonia caused by *M. szulgai* (Ebani, 2017). Another study identified *Mycobacterium genevase* through immunological and molecular techniques (Ullmann et al., 2016) and identified in a python molurus and an exotic green snake, two captive snakes with clinical pneumonia, anorexia and lethargy (Ebani, 2017). Although there are many cases of mycobacterium in reptiles with clinical symptoms, most cases are asymptomatic. A study was carried out on clinically healthy pet reptiles where they collected the faecal samples and approximately 16% of the reptiles were shedding *M. chelonae*, *M. peregrinum* and *M. fortuitum* (Ebani, 2017).

*Rickettsia* species, *Ehrlichia* spp. and *Borrelia* spp. have been detected in reptile species as an increasing number of studies have found reptiles to be competent hosts for these bacteria (Miranda et al., 2020). The *Ehrlichia* and *Rickettsia* generas belong to the order *Rickettsiales*. They are both obligate intracellular bacteria (Andoh et al., 2015) with gram-negative qualities (Miranda et al., 2020). Ticks often transmit rickettsia and ehrlichia diseases (Andoh et al., 2015). *Rickettsia* are one of the most common microorganisms found in reptile ticks (Miranda et al., 2020). *Rickettsia*-carrying reptile-associated ticks can be zoonotic. A study found in Central Europe, *Ixodes ricinus* which carries *Borrelia*, *Anaplasma* and *Rickettsia*



can be found on certain lizards. Also, in Australia, *Rickettsia honei* which is the causative agent of Flinders Island spotted fever was carried by *Bothriocroton hydrosauri* ticks and these ticks feed on lizards and snakes. Lizards with ticks containing *Anaplasmatidae* and *Borrelia* were detected in Poland (Andoh et al., 2015). Research completed in Mexico identified 18 tick species on 42 different hosts of reptiles and amphibians in 36 countries. In this study, 23 *Rickettsia* taxa were detected. They also discovered *Rickettsia* sp. Strain *Colombianensi* and *Rickettsia amblyommatis* for the first time on ticks from a variety of reptiles in Mexico, Guerrero and Veracruz (Sánchez-Montes et al., 2019). Andoh et al., (2015) detected that *Ehrlichia* species were present at low rates and *Rickettsia* were identified at high rates in reptile-associated ticks (Andoh et al., 2015).

Apart from *Salmonella*, *Leptospirosis*, *Chlamydia*, *Mycobacteria*, *Rickettsia* and *Ehrlichia* bacteria species, reptiles can also excrete *Escherichia. coli*, *Campylobacter*, *Yersinia* and *Klebsiella* species, which are all enteric bacteria, in their faeces which may cause disease in humans. Herpetofauna can also be infected by *Streptococcus*, *Staphylococcus*, *Clostridium*, *Pasteurella* and *Pseudomonas* bacteria species (Ebani, 2017).

### 2.3.2. Protozoan Pathogens of veterinary-medical significance in reptiles

Protozoa is becoming an increasing gastrointestinal incidence worldwide. Coccidia are protozoan parasites (Robben & Sibley, 2004) which are eukaryotic unicellular microorganisms. Several protozoan parasites cause enteric disease and have an environmentally stable stage of an oocyst or cyst which allows them to infect other hosts through water or food-borne routes (Cama & Mathison, 2015). The most important species of protozoa include *Cryptosporidium* spp., *Giardia* spp., *Entamoeba* spp., *Dientamoeba fragilis*, *Cyclospora cayetanensis* and *Blastocystis* spp. (McHardy et al., 2014).

There has been a higher awareness of *Cryptosporidium* protozoa due to its zoonotic nature and therefore, public health concerns. Cryptosporidiosis infections are chronic and life threatening in reptiles (Santín, 2013), whereas, in other animals it is self-limiting in immunocompromised status. Cryptosporidiosis is a worldwide gastrointestinal disease present in mammals, birds, fish and reptiles particularly lizards and snakes (Dellarupe et al., 2016). It has been reported in at least 57 reptilian species (Xiao et al., 2004). The two main *Cryptosporidium* species found in reptiles are *C. saurophilum* and *C. serpentis* (Xiao et al., 2004) *C. saurophilum*, also known as *C. varnii* is found mainly in lizards as an intestinal parasite causing clinical symptoms such as weight loss, anorexia, abdominal swelling and mortality especially in juvenile lizards. *Cryptosporidium serpentis* is primarily detected in

snakes and it is a gastric parasite causing lethargy, anorexia, weight loss, mild body swelling and postprandial regurgitation. In lizards, *C. serpentis* is predominately asymptomatic (Dellarupe et al., 2016). A study was carried out in Beijing, China on eight species of snakes from 13 pet households where they collected 273 faecal samples and examined them by PCR. Nine of the thirteen snakes tested positive for *Cryptosporidium*. Ten of the samples contained *C. serpentis* and seven of them contained *C. varanii* (Zhang et al., 2020). Research carried out on leopard geckos in Argentina confirmed the presence of *Cryptosporidium* oocysts. These pet geckos were suffering from cachexia, anorexia and diarrhoea and the main causative agent was *C. varanii* (Dellarupe et al., 2016). Another study of a total of 123 samples, there were 48 snakes, 24 lizards and 3 tortoise positive *Cryptosporidium* samples. The species *C. serpentis*, *C. muris*, *C. parvum* bovine and mouse genotypes, *C. desert* monitor genotype, two new species in snakes, one new species in tortoises and one *C. serpentis*-like parasite in lizards were detected with *C. serpentis* and *C. desert* monitor genotype being the most frequent (Xiao et al., 2004). Certain species such as *C. muris*, *C. parvum* and *C. parvum* mouse genotypes are detected in reptiles from ingesting infected prey, for example mice (Dellarupe et al., 2016). Research in 2011 detected *Cryptosporidium* in one corn snake with diarrhoea and a dilated stomach, in two Sudan spiny-tailed lizards and a leopard gecko (Rataj et al., 2011).

The genus *Eimeria* is an obligate intracellular coccidia protozoa parasite and belongs to the phylum *Apicomplexa*. It has a broad range of hosts including mammals, fish, birds and reptiles causing enteric disease (Couso-Pérez et al., 2019). *Eimeria* is predominantly detected based on their oocyst's morphology. *Geochelone carbonaria* species appear to be commonly infected with coccidia as three of the seven animals tested were positive and *Isospora* oocysts were detected in the faeces of *G.d denticulata* (Lainson et al., 2008). *Eimeria* species were also identified in Western Australia in a King skinks faeces at a wildlife rehabilitation centre (Yang et al., 2013). Furthermore, *Eimeria* species have been detected in tokay geckos and day geckos while *Isospora* sp. has been detected in bearded dragons, green iguana and a plumed basilisk (Rataj et al., 2011).

Another protozoan parasite that infects reptiles is *Entamoeba invadens*. It rarely causes amoebiasis disease in crocodiles and chelonians. However, these species may serve as carriers and transmit these parasites to susceptible reptile species such as snakes and lizards (García et al., 2014). This pathogenic *Entamoeba* species can cause up to 100% mortality in snakes. In a study of a ball python found dead after suffering from short-term weight loss at necropsy, it was discovered the snake had severe necrotizing colitis and that there was a

huge number of entamoeba trophozoites present (Michaely et al., 2020). Amoebiasis causes non-specific clinical symptoms such as anorexia, lethargy, diarrhoea and can result in acute death (García et al., 2014). *Balantidium* is a ciliated protozoan parasite. It is zoonotic and has a preferred habitat of tropical and subtropical regions. It infects a wide range of hosts such as pigs, equine, ruminants and humans causing disease (Ahmed et al., 2020). However, species of *Balantidium* have also been detected in some reptilian species such as Sudan spiny tailed lizard, green iguana and in turtle species, mainly *Testudo hermanni* but also *Testudo graeca*, *Testudo marginata*, *Testudo horsfieldi* and *Geochelone elegans* (Rataj et al., 2011). Trichomonads are eukaryotic flagellated protists with a main life stage of a flagellated trophozoite. They are highly diverse with over 450 species including *Tritrichomonas foetus*, *Trichomonas gallinae*, *T. v vaginalis* (Smejkalova et al., 2014). They are found naturally within their host, mainly in the urogenital and digestive tracts. Snakes, lizards, chelonians and crocodiles are all susceptible to trichomonas. Transmission is via direct contact and intermediate carriers are not required (Vilela et al., 2003) Trichomonas have been detected in an *Eeryx johnii*, *Phelsuma dubia* and *Physignathus cocincinus* (Rataj et al., 2011).

### 2.3.3. Helminth Pathogens of veterinary-medical significance in reptiles

As discussed before, reptiles have a variety of infections, including protozoa, pentastomids, and arthropod parasite species, however, reptiles are also susceptible to a variety of helminths some of which are potentially harmful to humans (Mendoza-Roldan et al., 2020). Trematodes of the order *Diplostomida* are well-known as serious pathogens in humans, farm animals, and wild animals; members of the genus *Schistosoma* (*Schistosomatidae*) are responsible for human schistosomiasis, which affects more than 200 million people and animal schistosome infections in mammals and birds are of great veterinary importance. Their primary habitat is in tropical and sub-tropical regions (Horák et al., 2019). Another common helminth infecting reptiles is *Strongyluris*, a gastrointestinal parasite (Oda et al., 2020). The threadworm *Strongyloides stercoralis* is a soil-transmitted nematode that is one of the neglected tropical illnesses' most underestimated helminths (NTDs) (Fabian Schär, et al., 2014). In a parasitological study in Germany on 586 stool samples taken from 71 different captive snake species to detect gastrointestinal parasites. Of the faecal samples examined, 20 different parasite taxa were identified; the most common nematode species were *Ancylostomatidae Kalicephalus* spp. (3.3%), followed by *Strongyloides/Rhabdias* (2.6%) and infections with diverse pathogenic nematode genera were found in 14 (22.2%) of dissected snakes (Hallinger et al., 2020). Another study in Germany on Tortoise faeces

samples (n = 1005) from 19 different species kept as pets found that oxyurid nematodes (e.g. *Tachygonetria* spp.) were the most common parasites in faecal samples (43.18%), followed by ascarids (*Angusticaecum* spp.) (0.01%), *Hexamita* spp. (0.007%), *Balantidium* spp. (0.007 %), trichomonads (0.004%), *Strongyloides* spp (0.005 %) (Hallinger et al., 2018). Five nematodes from the genera *Ascaris*, *Capillaria*, *Strongylus*, *Dujardinascaris* and *Toxocara* were detected in the faeces of captive zoo-kept crocodile species. The eggs of *Ascaris lumbricoides* were found in the faeces of a *Crocodylus cataphractus*, *Capillaria* were detected from crocodiles located in Australia, Papua New Guinea and Irian Jaya, from the intestine of an Alligator in New York *Strongylus* sp. were acquired. While *Toxocara* and *Dujardinascaris* have been previously discussed in literature (Zhao et al., 2014). *Ophidascaris* is a genus of snake parasites that live in the intestines of different snake species. Over 30 *Ophidascaris* species have been discovered around the world (Zhou et al., 2021). Spargana are parasitic plerocercoid larvae of cestode tapeworms of the genus *Spirometra*, family *Diphyllobothriidae*, that feed on frogs, reptiles, birds, and mammals. The usage and consumption of amphibians and reptiles can spread this parasite disease to humans (Oda et al., 2016).

# The Study

## 3. Materials and methods

### 3.1. Origin of samples

In this study samples of 98 reptiles and one amphibian were collected at the National Reptile Zoo in Kilkenny, Ireland between March and July 2021. These captive animals represented 44 species and belonged to four orders (Testudines, Squamata, Crocodylia, Anura) (Table 1). From all of them faecal samples were collected in a non-invasive way (not directly taken from the cloaca, neither any swabs were made to avoid any sort of stress), fresh when found in artificial enclosures with an attempt to exclude soil contamination, and they were placed inside the pre labelled (HK1-99) Sarstedt tubes which were then stored in -20°C until sample processing.

| order      | suborder       | (super)family  | English name of species      | Latin name of species          | original number of samples              |
|------------|----------------|----------------|------------------------------|--------------------------------|---|
| TESTUDINES | Cryptodiara    | Chelydridae    | Alligator snapping turtle    | <i>Macrolemys temminckii</i>   | HK69                                    |
|            |                | Testudinidae   | Leopard tortoise             | <i>Stigmochelys pardalis</i>   | HK2, HK10, HK17, HK53                   |
|            |                |                | Red footed tortoise          | <i>Chelonoidis carbonaria</i>  | HK4, HK36, HK38, HK56, HK61, HK62, HK87 |
|            |                |                | Pancake tortoise             | <i>Malacochersus tornieri</i>  | HK7, HK96                               |
|            |                |                | Yellow footed Tortoise       | <i>Chelonoidis denticulata</i> | HK66, HK81                              |
|            | Pleurodira     | Chelidae       | Roti Island Snakeneck turtle | <i>Chelonia mccordi</i>        | HK32                                    |
| SQUAMATA   | Sauria         | Gekkonidae     | Leopard gecko                | <i>Eublepharis macularius</i>  | HK33, HK34, HK37, HK98, HK99            |
|            |                |                | Crested gecko                | <i>Rhacodactylus ciliatus</i>  | HK35, HK60, HK65, HK14                  |
|            |                |                | Henkel's leaf-tailed gecko   | <i>Uroplatus henkeli</i>       | HK85                                    |
|            | Scincomorpha   | (Scincoidea)   | Blue tongued skink           | <i>Tiliqua scincoides</i>      | HK15, HK8                               |
|            | Autarchoglossa | Helodermatidae | Gila monster                 | <i>Heloderma suspectum</i>     | HK23*, HK75*, HK97*                     |
|            |                |                | Beaded lizard                | <i>Heloderma horridum</i>      | HK23*, HK75*, HK97*                     |
|            |                | Gerrhosauridae | Sudan plated lizard          | <i>Gerrhosaurus major</i>      | HK3, HK71, HK76                         |
|            |                | Teiidae        | Black and white tegu         | <i>Salvator merianae</i>       | HK40, HK49, HK88*                       |
|            |                | Varandiae      | Bosc monitor                 | <i>Varanus exanthematicus</i>  | HK24, HK45                              |
|            |                |                | Rough-necked monitor         | <i>Varanus rudicollis</i>      | HK63                                    |

|         |           |        |                         |                                |                                       |
|---------|-----------|--------|-------------------------|--------------------------------|---------------------------------------|
|         |           |        | Asian water monitor     | <i>Varanus salvator</i>        | HK11                                  |
| Iguania | Iguanidae |        | Rhinoceros Iguana       | <i>Cyclura coronata</i>        | HK13, HK16, HK48, HK70,<br>HK79, HK89 |
|         |           |        | Fiji Banded Iguana      | <i>Brachylophus bulabula</i>   | HK19                                  |
|         |           |        | Spiny tailed iguana     | <i>Ctenosaura pectinata</i>    | HK22, HK77                            |
|         |           |        | Green iguana            | <i>Iguana iguana</i>           | HK52, HK5, HK72, HK88,<br>HK91        |
|         |           |        | Casquehead iguana       | <i>Laemactus serratus</i>      | HK68                                  |
|         |           |        | Desert Iguana           | <i>Dipsosaurus dorsalis</i>    | HK93                                  |
|         |           |        | Indian garden Lizard    | <i>Calotes versicolor</i>      | HK39, HK55, HK27                      |
|         | Agamidae  |        | Bearded dragon          | <i>Pogona vitticeps</i>        | HK9                                   |
|         |           |        | Frilled dragon          | <i>Chlamydosaurus kingii</i>   | HK6, HK8, HK25, HK42                  |
|         |           |        | Australian water dragon | <i>Intellagama lesururii</i>   | HK18, HK57                            |
|         |           |        | Striped water dragon    | <i>Tropicagama temporalis</i>  | HK54, HK50, HK44, HK59                |
|         |           |        | Asian water dragon      | <i>Physignathus cocincinus</i> | HK94                                  |
|         |           |        | Sulawesi sailfin lizard | <i>Hydrosaurus celebensis</i>  | HK73, HK90                            |
|         | Serpentes | Boidae |                         | Boa constrictor                | <i>Boa constrictor</i>                |
|         |           |        | Yellow anaconda         | <i>Eunectes notaeus</i>        | HK51                                  |

|                   |               |               |                        |                                 |                        |
|-------------------|---------------|---------------|------------------------|---------------------------------|------------------------|
|                   |               |               | Colombian rainbow boa  | <i>Epicrates maurus</i>         | HK92                   |
|                   |               | Pythonidae    | Royal python           | <i>Python reguis</i>            | HK21, HK41, HK64, HK74 |
|                   |               |               | Burmese python         | <i>Python bivittatus</i>        | HK26, HK67, HK80       |
|                   |               |               | Carpet python          | <i>Morelia spilota</i>          | HK28, HK43             |
|                   |               |               | Spotted python         | <i>Antaresia maculosa</i>       | HK84                   |
|                   |               | Colubridae    | Milk snake             | <i>Lampropeltis triangulum</i>  | HK1                    |
|                   |               |               | California kingsnake   | <i>Lampropeltis californiae</i> | HK20, HK29, HK78       |
|                   |               |               | Gopher snake           | <i>Pituophis catenifer</i>      | HK46, HK83             |
|                   |               | Elapidae      | Monocled cobra         | <i>Naja kaouthia</i>            | HK95                   |
|                   | Anguimorpha   | Anguidae      | Scheltopuski           | <i>Pseudopus apodus</i>         | HK82                   |
| <b>CROCODILIA</b> | Alligatorinae | Alligatoridae | Chinese alligator      | <i>Alligator sinensis</i>       | HK58                   |
| <b>ANURA</b>      |               | Pelodyadidae  | White-lipped tree frog | <i>Litoria infrafrenata</i>     | HK47                   |

\* Two species were housed together.

**Table 1:** Species that participated in this study.

| Target group            | Target gene              | Primer name    | Primer sequence (5'-3')        | Amplicon length (bp) | Thermocycling profile   | Reference             |
|-------------------------|--------------------------|----------------|--------------------------------|----------------------|---|-----------------------|
| <i>Trichomonas</i> spp. | 16S rRNA                 | 16SL           | TAC TTG GTT GAT CCT GCC        | 1550                 | 95 °C for 5 min; 35× (95 °C for 40 s; 50 °C for 30 s; 72 °C for 1 min); 72 °C for 7 min | Cepicka et al. (2005) |
|                         |                          | 16SR1          | TCA CCT ACC GTT ACC TTG        |                      |   |                       |
| <i>Trichomonas</i> spp. | alfa-tubulin (nestedPCR) | AtubA (outer)  | RGT NGG NAA YGC NTG YTG GGA    | 1200                 | 95 °C for 5 min; 35× (95 °C for 40 s; 55 °C for 30 s; 72 °C for 1 min); 72 °C for 7 min | Edgcomb et al. (2001) |
|                         |                          | AtubB (outer)  | CCA TNC CYT CNC CNA CRT ACC A  |                      |   | Cepicka et al. (2006) |
|                         |                          | AtubF1 (inner) | TAY TGY YWN GAR CAY GGN AT     | 1200                 | 95 °C for 5 min; 35× (95 °C for 40 s; 45 °C for 30 s; 72 °C for 1 min); 72 °C for 7 min |                       |
|                         |                          | AtubR1 (inner) | ACR AAN GCN CGY TTN GMR WAC AT |                      |   |                       |
| <i>Anaplasmataceae</i>  | 16S rRNA                 | EHR-16sD       | GGT ACC YAC AGA AGA AGT CC     | 350                  | 95 °C for 10 min; 40× (95 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s); 72 °C for 5 min | Brown et al. (2001)   |
|                         |                          | EHR-16sR       | TAG CAC TCA TCG TTT ACA GC     |                      |   |                       |
| <i>Rickettsia</i> spp.  | gltA                     | RpCs.877p      | GGG GGC CTG CTC ACG GCG G      | 380                  | 95 °C for 5 min; 40× (95 °C for 20 s; 48 °C for 30 s; 72 °C for 1 min); 72 °C for 5 min | Regnery et al. (1991) |
|                         |                          | RpCs.1258n     | ATT GCA AAA AGT ACA GTG AAC A  |                      |   |                       |



|                                     |             |                                       |                                      |     |  |   |
|-------------------------------------|-------------|---------------------------------------|--------------------------------------|-----|--|---|
| <i>Acanthamoeba</i> spp.            | 18S rRNA    | JDP1                                  | GGC CCA GAT CGT TTA CCG TGA<br>A     | 480 | 95 °C for 5 min; 35× (95 °C for 35 s; 56 °C for 45 s; 72 °C for 1 min); 72 °C for 7 min    | Niyiyati et al. (2016)                          |
|                                     |             | JDP2                                  | TCT CAC AAG CTG CTA GGG GAG<br>TCA   |     |  |   |
| <i>Trypanosoma</i> spp.             | ssu rDNA    | 609F                                  | CAC CCG CGG TAA TTC CAG C            | 900 | 95 °C for 5 min; 40× (94 °C for 40 s; 49 °C for 1.5 min; 72 °C for 1 min); 72 °C for 5 min | Da Silva et al. (2004)<br>Ramírez et al. (2012) |
|                                     |             | 706R                                  | CTG AGA CTG TAA CCT CAA              |     |  |   |
| <i>Gordonibacter</i> spp.           | 16S rRNA    | Gor16SF                               | AGC GGA ACC TCT AAC CCG A            | 580 | 95 °C for 5 min; 40× (95 °C for 30 s; 54 °C for 30 s; 72 °C for 45 s); 72 °C for 5 min     | in this study                                   |
|                                     |             | Gor16SR1                              | TGA ATG CTG GCA ACA TAT GG           |     |  |   |
|                                     |             | Gor16SR2                              | TCA ATG CTG GCA ACA TAC GA           |     |  |   |
| <i>Paraeggerthella</i> sp.          | 16S rRNA    | EHR-16sD                              | GGT ACC YAC AGA AGA AGT CC           | 580 | 95 °C for 5 min; 40× (95 °C for 30 s; 54 °C for 30 s; 72 °C for 45 s); 72 °C for 5 min     | Brown et al. (2001)<br>in this study            |
|                                     |             | Gor16SR1                              | TGA ATG CTG GCA ACA TAT GG           |     |  |   |
|                                     |             | Gor16SR2                              | TCA ATG CTG GCA ACA TAC GA           |     |  |   |
| <i>Barcoding PCR for eukaryotes</i> | COI         | LCO1490                               | GGT CAA CAA ATC ATA AAG ATA<br>TTG G | 710 | 95 °C for 5 min; 40× (94 °C for 40 s; 48 °C for 1 min; 72 °C for 1 min); 72 °C for 10 min  | Folmer et al. (1994)                            |
|                                     | HCO2198     | TAA ACT TCA GGG TGA CCA AAA<br>AAT CA |                                      |     |  |   |
| <i>Citrobacter</i> sp.              | urease gene | CugF                                  | TGA AGC TGA ACT ACC CGG AAT<br>C     | 454 | 95°C for 5 min; 35× (94°C for 40 s; 55°C for 40 s; 72°C for 1 min) 72°C for 7 min          | Chen et al. (2018)                              |
|                                     |             | CugR                                  | TGT CCA GGC TCA AAA CGT AC           |     |  |   |

**Table 2:** Primers and details for conventional PCR methods used in this study



**Figure 1:** *Cyclura coronata* in its place in the National Reptile Centre of Ireland.

### **3.2. DNA extraction, PCR and sequencing**

DNA was extracted using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with some modifications. For each faecal sample the following steps were performed. 180-220 mg stool was added to a 2 ml sterile Sarstedt tube contained 1ml InhibitEx Buffer. Thereafter it was vortexed continuously for 1 minute to get a thoroughly homogenized solution. The suspension was heated for 5 minutes at 70°C, then vortexed for fifteen seconds and centrifuged for 1 minute using the 14000 rpm (rotation per minute) speed as each time later. 200 µl of the supernatant was pipetted to a 1.5 ml microcentrifuge tube and 20 µl Proteinase K was added, then the solution was thoroughly mixed by pulse-vortexing. Before pipetting 200 µl of Buffer AL the solution was incubated at 56°C for sixty minutes. Then the lysate that already contained the Buffer AL was heated at 70°C for 10 minutes. The DNA was precipitated by adding ethanol (96%) in a volume of 200 µl. The solution was vortexed for fifteen seconds, centrifuged for a short time, and 600 µl from the lysate was carefully applied to the QIAamp spin column, then centrifuged for 1 minute. The column was placed in a new 2ml collection tube, and the tube containing the filtrate was discarded. All this was followed by the washing process during which 500 µl of Buffer AW1 was used twice, then 500 µl Buffer AW2 was added once to

provide the purity of DNA at each step by exchanging the collection tube. After washing with Buffer AW2 the collection tube containing the filtrate was discarded, the spin column was inserted into a new collection tube and centrifuge for 1 minute to remove residual ethanol. As a last step, the columns were transferred into a new 1.5 ml microcentrifuge tubes and 100 µl Buffer ATE was pipetted directly onto the QIAamp membrane and incubated for 1 minute at room temperature, then centrifuged for 1 minute to elute DNA. The DNA extracts were stored in -20°C until molecular analysis by conventional PCR.

For each PCR method, 5 µl of extracted DNA was added to 20 µl of reaction mixture containing 1.0 U HotStar Taq Plus DNA Polymerase (5 U/µl) (Qiagen, Hilden, Germany), 0.5 µl dNTP Mix (10 mM), 0.5 µl of each primer (50 µM), 2.5 µl of 10× Coral Load PCR buffer (15 mM MgCl<sub>2</sub> included) and 15.8 µl distilled water (DW). Except the reaction that targeted the *gltA* gene from *Rickettsia* sp., in which 2.5 µl template DNA was added to 22.5 µl reaction mixture containing 18.3 µl DW. As well as during another PCR that targeted 16S rRNA from *Gordonibacter* sp. one forward and two reverse primers were used at the same time in 0.5 µl and 0.25-0.25 µl (50 µM) amount. Further details of the PCRs are summarized in Table 2. In all PCRs sequence-verified positive controls were included. PCR products were electrophoresed in 1.5% agarose gel (100V, 55-60 min), stained with ethidium-bromide and visualized under ultra-violet light.

Purification and sequencing of the selected products were done by Biomi Ltd. (Gödöllő, Hungary). The obtained sequences were manually edited using the BioEdit program, then aligned with GenBank sequences by the nucleotide BLASTN program (<https://blast.ncbi.nlm.nih.gov>). All sequences retrieved from GenBank were trimmed to the same length prior to phylogenetic analysis. This dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analysis was conducted with the Maximum Likelihood method (Jukes–Cantor model) by using MEGA 7.0.

## 4. Results

### 4.1. Screening of *Trichomonas* and *Trypanosoma* species

All faecal DNA extracts were negative in the PCRs targeting *Trypanosoma* species, however, one (HK102) that came from a leopard gecko (*Eublepharis macularius*) proved to

be positive by the PCR targeting *Trichomonas* species, which one showed 93% (282/303 bp) identity to *Monocercomonas colubrorum*.

#### **4.2. Broad-range screening of eukaryotic DNA with the cox1 (barcoding gene) PCR**

Host DNA was detected in four samples. In two samples (HK21, HK66) the DNA of ball python (*Python regius*) was identified, with 100% sequence identity (643/643 bp and 624/624 bp, respectively) to only one entry in GenBank (accession number MN295676, from southern Togo). These two sequences showed 1-6 bp difference from other conspecific sequences in GenBank. In addition, from two samples (HK69, HK83) the DNA of Burmese python (*Python bivittatus*) was successfully amplified, showing 100% (598/598 bp) identity with three GenBank sequences of the same species (accession numbers: MH357848 from Florida, JX401109 and JX401106 from Kinmen Island). These two sequences showed 1-3 bp difference from other conspecific sequences in GenBank. Thus, this method was suitable to identify the host of origin in a limited number of cases, all from the genus *Python*.

From one faecal sample (HK28), the DNA of insect prey items was successfully amplified with this method. The corresponding sequence was 99.8% (634/635 bp) identical with that of two-spotted (or black) cricket (*Gryllus bimaculatus*) (under accession number KY646234, from India), on which the relevant Oriental (Indian) garden lizard (*Calotes versicolor*) fed.

Last but not least, the cox1 PCR detected an *Acanthamoeba* genotype in a yellow anaconda (*Eunectes notaeus*): it showed 100% (648/648 bp) identity to *Acanthamoeba* genotype T11 reported from an Austrian Zoo (accession number: MH124196). Therefore, this sample (HK53) was analysed further.

#### **4.3. *Acanthamoeba* genus-specific PCR targeting the 18S rRNA gene (ASA.S1 region in *Rns*)**

The above finding was confirmed by the PCR specific for the genus *Acanthamoeba* (Figure 2), and the species was identified with sequencing as *A. hatchetti*, showing 100% (407/407 bp) sequence identity to an isolate from beach water and hot springs water reported from Malaysia (accession numbers MN700304, MN700304 and MH790999, respectively). The phylogenetic positions of this species are shown in Figure 4. Furthermore, five other species'

positivity were detected by PCR, as hila monster/beaded lizard (*Heloderma suspectum/H. horridum* (HK23)), bosc monitor (*Varanus exanthemicus* (HK25)), frilled dragon (*Chlamydosaurus kingii* (HK43)), alligator snapping turtle (*Macroclemys temminckii* (HK71)), green iguana (*Iguana iguana* (HK94)).

#### **4.4. GltA gene PCR for Rickettsia and other Proteobacteria**

Concerning bacteria, the faecal sample (HK36) of a crested gecko (*Correlophus ciliatus*) contained the DNA of *Citrobacter freundii*. This sequence had 100% (310/310 bp) identity with two valid *Citrobacter* species, including several isolates of *Citrobacter freundii* (accession numbers: CP007557 from sink aerator, USA; AP022380 and AP022384 from hospital sewage, Japan).

#### **4.5. 16S rRNA gene PCR for Anaplasmataceae (Proteobacteria) and some Actinobacteria**

Three samples (HK25, HK29, HK69: from savannah monitor: *Varanus exanthemicus*, carpet python: *Morelia spilota*, and Burmese python: *Python bivittatus*, respectively) contained DNA from the genus *Gordonibacter*. All three sequences had 100% (284/284 bp) identity to each other and to the type strain of *Gordonibacter pamelaee* (accession number: MK544834, from Germany).

#### **4.6. PCRs for a longer part of the 16S rRNA gene of *Gordonibacter* and *Paraeggerthella***

A longer part of the 16S rRNA gene was successfully amplified from the samples positive for members of the family Eggerthellaceae (Figure 3). From *Varanus exanthemicus*, the longer sequence of *G. pamelaee* had 100% (497/497 bp) sequence identity with the type strain of this species (MK544834). From *Python bivittatus*, the species proved to be *G. faecihominis*, showing 100% (497/497 bp) sequence identity with its type strain (MK544836). In addition, the sample from *Morelia spilota* contained a *Gordonibacter* sp. which had only 99.2% sequence identity (493/497 bp) with the closest match, *G. urolithinfaciens* (MN326814). The phylogenetic positions of these three species are shown in Figure 5.

*Acanthamoeba* sp. 18S rRNA specific hotstart PCR reactions

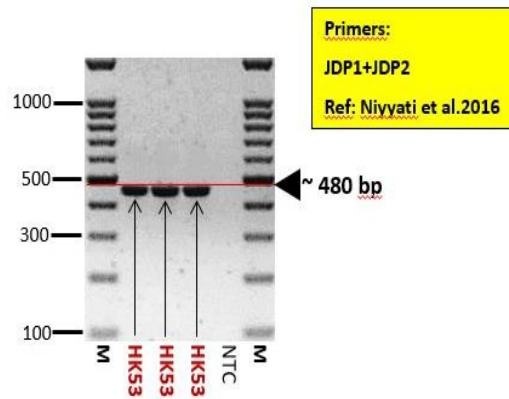


Figure 2: Agarose gel electrophoresis of the PCR products of *Eunectes notaeus*.

*Gordonibacter* 16S rRNA specific hotstart PCR reactions

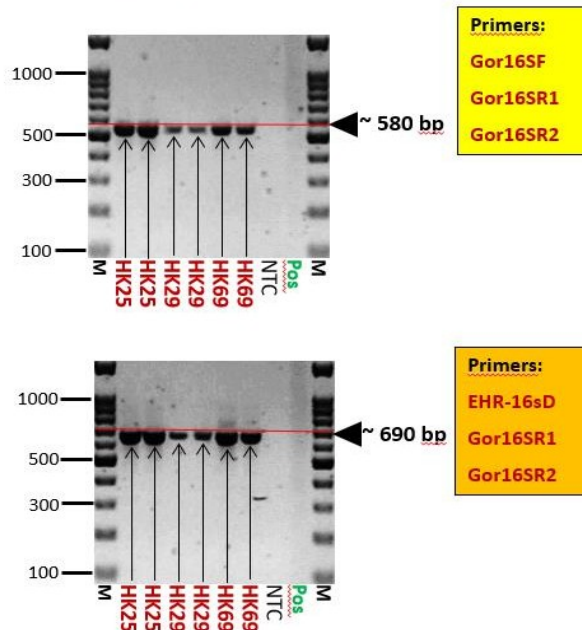
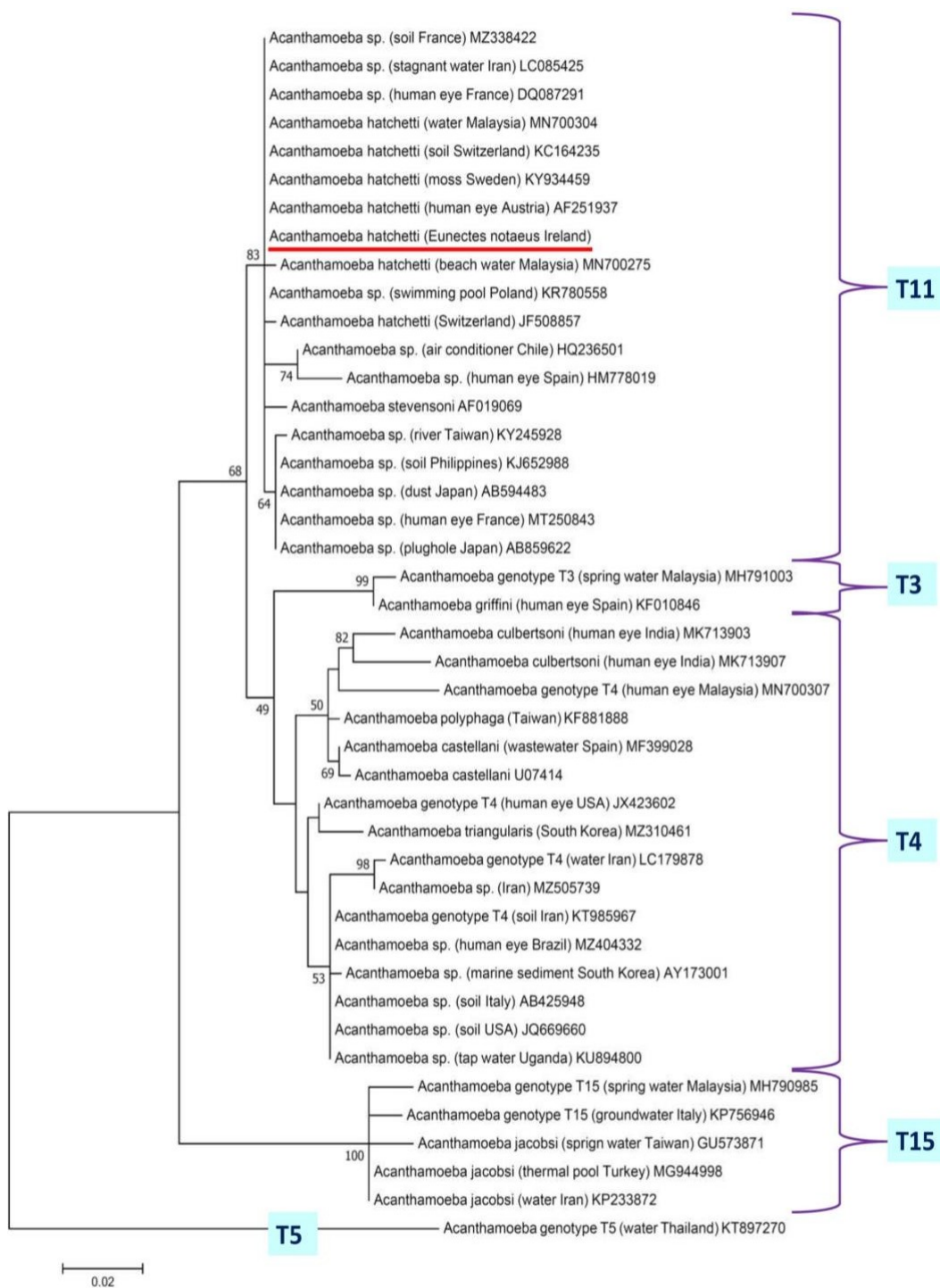
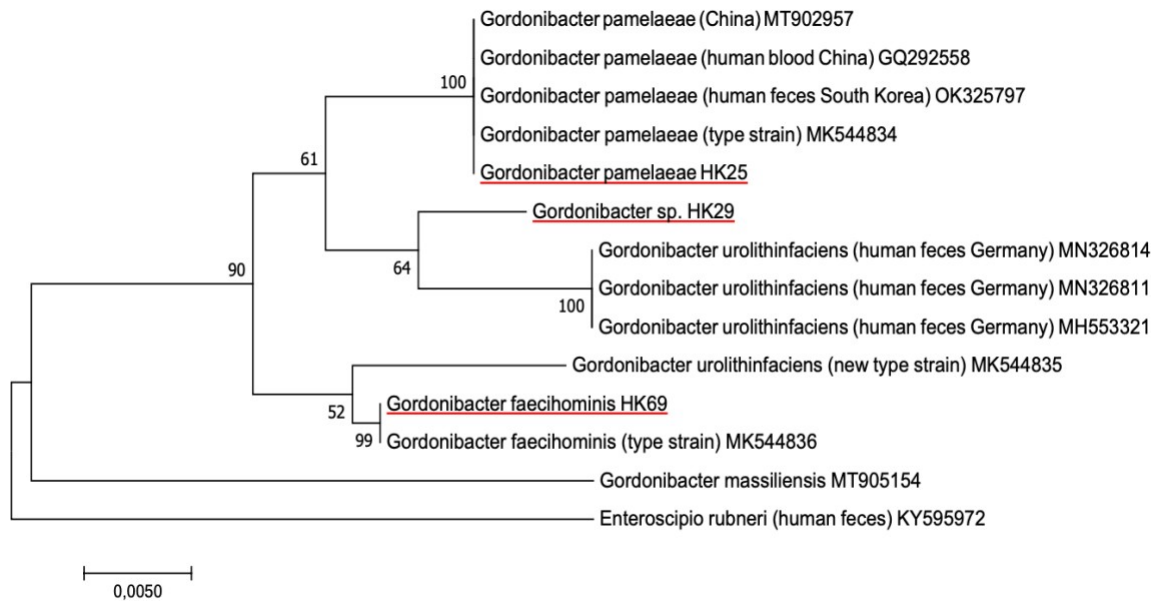


Figure 3: Agarose gel electrophoresis of the PCR products of *Varanus exanthematicus* and *Python bivittatus*.



**Figure 4.** Maximum Likelihood phylogenetic tree of *Acanthamoeba* 18S rRNA gene sequences from the morphological groups II-III, representing genotypes (T5, T15, T4, T3 and T11) that are more virulent as causative agents of human keratitis. The model K2P was used according to the selection by MEGA 7.0. Genus or species/isolate names are followed by the isolation source (whenever available), country of origin and GenBank accession numbers. The sequence from this study is underlined with red colour. The scale-bar indicates the number of substitutions per site



**Figure 5:** Maximum Likelihood phylogenetic tree of *Acanthamoeba* 18S rRNA gene sequences from the morphological groups II-III, representing genotypes (T5, T15, T4, T3 and T11) that are more virulent as causative agents of human keratitis. The model K2P was used according to the selection by MEGA 7.0. Genus or species/isolate names are followed by the isolation source (whenever available), country of origin and GenBank accession numbers. The sequence from this study is underlined with red colour. The scale-bar indicates the number of substitutions per site.

## 5. Discussion

By the fact that reptiles are increasingly kept as pet animals, the parasites and pathogens carried by them may be the source of infection for other animals or humans. Those reptiles which are sold as pets might not be born only in captivity but brought from the wild, thereby increasing the chances of carrying different pathogens (Rajat et al., 2011). In many cases it cannot be established whether the animals became infected during or before captivity. *Haemogregarina* sp., *Balantidium* sp., *Cryptosporidium* sp. and Trichomonadidae are frequently found protozoa in reptiles, e.g., in lizards and snakes. *Salmonella* sp., *Leptospira* sp., *Chlamydia* sp. and *Mycobacterium* sp. are also often diagnosed bacteria. Furthermore, tick-borne pathogens might be detected in reptiles (e.g.: *Rickettsia* spp., *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Coxiella burnetti*) thereby proving the important role of arthropod vectors (mainly in zoonotic concern) and highlighting the risk that is inherent in the common living space of animals and humans. In most cases, infected reptiles have only reservoir role since they do not show any symptoms (O'Donghue, 1997; Rajat et al., 2011; Dantas Torres et al., 2012; Mendoza Roldan et al., 2021).



To be able to test animals for parasites and pathogens, sampling is required which can be performed in an invasive or non-invasive way. Regarding reptiles and wildlife, it is worth using non-invasive sampling since it increases the safety for both the animal and the sampler. This method is advantageous to avoid capturing and handling the animal and thereby to minimize the stress to provide larger amount of sample (Pauli et al., 2010). Although cloacal or buccal swabbing is an easy and quick method, it is not recommended in reptiles with fragile bone structure (Miller et al., 2006; Jones et al., 2008). Collecting already excreted faeces is an easy non-invasive way to obtain appropriate amount of sample for DNA testing. In addition to quantity, quality can also set a limit, since the PCR inhibitors may be present in large amounts and the faecal DNA can be degraded (Taberlet et al., 1999; Walker et al., 2016). To study the DNA in the highest quality the faeces should be sampled freshly and promptly cooled or frozen in  $-20^{\circ}\text{C}$  (Nsubuga et al., 2004; Jones et al., 2008).

Beside detecting pathogens, samples can be used also for host species identification. Although in mammals the genetic species identification from faecal samples is a widely accepted method, it has hitherto been described only in a few studies (Jonas et al., 2008; Pearson et al., 2014; Statham et al., 2019). In point of fact, DNA barcoding is highly suitable for species identification and is of great importance in wildlife and endangered animals which are stress sensitive and difficult to access. A 658 bp segment of the mitochondrial gene cytochrome c oxidase subunit I (cox1, COI) is the most widely used genetic marker for animal barcoding (species identification). To amplify the DNA from faeces shorter amplicon might be needed, since the extraction of the DNA is more difficult (Jones et al., 2008; Walker et al., 2009). Furthermore, there are cases when 100-300 bp length segment (“DNA mini-barcode”) is amplified and eukaryotes might be also identified with that (Walker et al., 2009). In 2005 Kurose et al. examined feline faecal and muscle samples for species identification. In a more recently published study lizards were identified genetically from their field-collected scat (Pearson et al., 2014), similarly to what was achieved from tissue and scat samples by Statham et al. (2019). Although in our study, targeting 710 bp of the barcoding gene, lizards could not be genetically identified from their faeces, own DNAs of two ball python (*Python regius*) and two Burmese python (*Python bivittatus*) were successfully amplified.

However, besides host species identification, information can be obtained from the faeces on the feeding habits of wildlife (Kurose et al., 2005). Interestingly, from the faeces of insectivorous animals preys can be detected with barcoding (Walker et al. 2009). This might be necessary when the feeding behaviour of animals is unknown due to lack of

observation. Dietary investigations have been going for decades in which DNA metabarcoding is the most widely used approach. The latter process is a DNA barcoding which obtains high-throughput sequencing data about environmental samples, such as faecal samples (Ando et al., 2020). In 2015 Kartzinel et al. found DNA of several arthropods (mainly belonging to the order of Diptera, Lepidoptera, Blattodea, Hemiptera, Araneae, Coleoptera and Hymenoptera) in the faecal samples of an anole species (*Anolis sagrei*), however, crickets (from Orthoptera order) occurred with low prevalence in that study. Our investigation revealed the presence of the two-spotted cricket (*Gryllus bimaculatus*) in the faecal sample of Oriental (Indian) garden lizard (*Calotes versicolor*). It is worth noting that our result was relevant to the food of the reptiles studied, since animals in the National Reptile Centre are fed with cricket and locust every two days.

In addition to the above, DNA extracts from faeces can be used for different purposes such as detecting parasites and pathogens. Trichomonads are widespread parasites in both humans and animals. Although D.W.W. Kannangara in 1970 already described that *Trichomonas* species are frequently found protozoa in reptiles of Cheylon (*Vipera russeli*, *Calotes versicolor*), it has not since been clarified which *Trichomonas* species may be present in each reptile. Trichomonads are considered to be part of the normal intestinal flora of reptiles, however these flagellated protozoa can proliferate due to suppressed immune system of the animal causing loss of appetite, diarrhoea and weight loss. In some cases, establishment in the gallbladder results in cholangitis. This is supported by the fact that in the faecal sample of a viper (*Bothrops jararaca*) with diarrhoea large amount of *Trichomonas* species were detected microscopically (Vilela et al., 2002), and in a black throat monitor lizard (*Varanus albigularis ionidesi*) a coinfection with *Trichomonas* and *Cryptosporidium* sp. caused diarrhoea, salivation, vomiting, anorexia and lethargy (Corriveau and Thompson, 2013). In our study, one *Trichomonas* PCR positive sample of a leopard gecko (*Eublepharis macularius*) showed 93% identity to *Monocercomonas colubrorum* (from Trichomonadida order) which is considered a common species in snakes and lizards causing moderate depression, less activity and weight loss (Zwart et al., 1984; Moskowitz, 1951). However, this leopard gecko might be an asymptomatic carrier, since to the best of our knowledge it showed no signs of infection. Furthermore, we also investigated the samples for other flagellates, namely *Trypanosoma* species, since stercorarian trypanosomes (*T. cruzi*) have been observed in lizards that were ingested an infected kissing bug (*Dipetalogaster maximus*) (Teixeira et al. 1987). In different circumstances, lizards could become infected by vectorial transmission of *T. cruzi* (San Juan et al., 2020). In

addition, other arthropods might be the vector of *Trypanosoma* species, as in a study sand flies might have been the vectors of snakes' trypanosomes (Viola et al., 2008). We had assumed that the reptiles in the National Reptile Centre could have become infected by consuming intermediate host in a similar way as in the previous case, since every two days they received cricket and locust. Our findings did not prove the assumption that trypanosomes passed with the faeces of the captive reptiles sampled here.

*Acanthamoeba* species are widespread opportunistic protists which can be found in the environment, including soil, water reservoirs and hospitals (Cooper et al., 2021). They are of a great importance in human health since they can cause granulomatous encephalitis, skin lesions in immunocompromised patients and keratitis (*Acanthamoeba Keratitis*) in people wearing contact lenses (Siddiqui et al., 2012; Marciano-Cabral et al., 2020). In addition, these species have also veterinary significance, since their presence is proven in animals, as dogs, cats, pigs, horses, rabbits, birds, amphibians and reptiles (Siddiqui et al., 2012). They also have clinical significance, since conjunctival swabs of dogs, cats and birds contained these protozoa, however, only dogs showed signs of keratitis and conjunctivitis (Cooper et al., 2021). We confirmed with our test the presence of *A. hatchetti* in yellow anaconda (*Eunectes notaeus*) which protist is known to have clinicopathological significance in both humans and animals. In domestic animals so far only *Acanthamoeba* caused encephalitis was known, however, *A. hatchetti* was detected in a horse with severe placentitis (Begg et al., 2014). Although *Acanthamoeba* species were found in freshwater fishes and reptiles gut content (Franke and Mackiewicz, 1982; Sesma et al., 1989), to the best of our knowledge, this is the first time when *A. hatchetti* is detected in faecal sample of reptiles. This result could be also important in terms of human health since reptiles are frequently kept as pet animals.

Beside the presence of protozoa, bacteria are also common in reptiles. *Citrobacter* spp. are Gram-negative bacteria belonging to Enterobacteriaceae family and known in the normal gut flora of several vertebrate classes. But these bacteria are also considered as opportunistic pathogens. Although their place of occurrence is soil and water, they can be also detected in the intestinal tract of humans (Chen et al., 2018; Oberhettinger et al., 2020). In a study *Citrobacter* sp. was the most prevalent bacterium in reptiles (Graves et al., 1988). During our investigations in the faecal sample of a crested gecko (*Correlophus ciliatus*) *C. freundii* was identified. *Citrobacter freundii* was considered an important food-borne and hospital-acquired bacterium which had caused diarrhoea and urinary tract infection. It can also cause neonatal meningitis and abscess formation. In addition, the mortality and

morbidity of meningitis caused by *Citrobacter* spp. is extremely high. These pathogens were detected in faecal samples of humans and *C. freundii* in oral and cloacal samples of Burmese pythons (*Python molurus bivittatus*) (Jho et al., 2011; Chen et al., 2018, Oberhettinger et al 2020). Based on these, the zoonotic transmission of *C. freundii* between reptiles and humans cannot be ruled out.

Members of the genus *Gordonibacter* are Gram-positive bacteria which belong to the recently erected family Eggerthellaceae (Gupta et al., 2013). *Gordonibacter* spp. are an integral part of the human gut microbiota, transforming diet components into bioavailable metabolites known as urolithins (Selma et al., 2014; Selma et al., 2016). Urolithins show anti-inflammatory, antioxidant, anticarcinogenic, antimicrobial, cardioprotective, and neuroprotective effects in vitro and in animal models (Espín et al., 2013). To the best of our knowledge, *Gordonibacter* spp. were hitherto almost exclusively been identified in the human gut flora. However, in a recent study unidentified members of this genus were shown to be part of the gut microbiota of snakes in China (Zhang et al., 2019). While *G. pamelaee* is regarded as an indispensable species in the human gut flora, it is also able to cause bacteraemia in men (Woo et al., 2010). Furthermore, *Paraeggerthella* spp. are also Gram-positive anaerobic bacteria which are residents of normal intestinal flora but they can cause bacteraemia in human clinical cases (Lau et al., 2004; Würdeman et al., 2009). In the future it is necessary to examine whether the same is true in reptiles. Nevertheless, our findings provide the first evidence on the occurrence of human-associated Eggerthellaceae, identified on the species level, in several reptilian species.

## 6. Conclusions

The above findings of opportunistic pathogens highlight the importance to monitor protozoa and bacteria in the faeces of pet reptiles, most importantly from the point of view of other animals or humans living nearby. Furthermore, these data might even have epidemiological relevance in natural ecosystems, e.g., when raw juice is made for human consumption from fruits that may have become contaminated with the faeces of arboreal reptiles.

## 7. Summary

Reptiles are frequently kept as pet animals. Adding to parasites and bacteria causing disease in reptiles themselves, they are considered as important reservoirs of pathogens with veterinary-medical significance. The aim of this study was to contribute to our knowledge on reptiles in the latter context.

During this study, 98 reptiles and one amphibian were sampled in a non-invasive way at the National Reptile Zoo in Ireland. These animals represented 44 species and belonged to four orders (Testudines, Squamata, Crocodylia, Anura). From these animals, faecal samples were collected in artificial enclosures, with an attempt to exclude soil contamination, into 2 ml sterile Sarstedt tubes. DNA was extracted from the central part of the sample, whenever possible, to minimize the chances of environmental contamination, using the Qiagen Fast Stool Mini Kit. All DNA samples were screened with conventional PCRs to detect broad range eukaryotic DNA (based on amplifying the cytochrome c oxidase subunit I, i.e., the *cox1* gene), protozoan parasite DNA (of *Trichomonas* and *Trypanosoma* spp.), as well as bacterial DNA (from Anaplasmataceae, Rickettsiaceae and related families).

All samples were negative in the tests for *Trypanosoma* species, however, one sample that came from a leopard gecko (*Eublepharis macularius*) proved to be positive by the PCR targeting *Trichomonas* species. The *cox1* gene PCR detected host DNA (i.e., was suitable to identify the host of origin) in four samples. From one faecal sample, the DNA of insect prey items was successfully amplified with this method. In addition, the *cox1* PCR detected an important opportunistic *Acanthamoeba* genotype in a yellow anaconda (*Eunectes notaeus*). This finding was confirmed by a PCR targeting the 18S rRNA gene (ASA.S1 region in *Rns*) of the genus *Acanthamoeba*, and the species was identified with sequencing as *A. hatchetti*. This species is known to have clinicopathological significance in both humans and animals. Concerning bacteria, the faecal sample of a crested gecko (*Correlophus ciliatus*) contained the DNA of *Citrobacter freundii*. The presence of *Citrobacter* spp. is known in the normal gut flora of several vertebrate classes, but these bacteria are also considered as opportunistic pathogens. More importantly, three faecal samples (from savannah monitor: *Varanus exanthematicus*, carpet python: *Morelia spilota*, Burmese python: *Python bivittatus*) contained DNA from the genus *Gordonibacter* which, to the best of our knowledge, was hitherto only identified in the human gut flora. At least *G. pamelaee* is also able to cause bacteraemia in men. The above findings of opportunistic pathogens highlight the importance to monitor protozoa and bacteria in the faeces of pet reptiles, most importantly from the point of view of other animals or humans living nearby. Furthermore, these data might even have

epidemiological relevance in natural ecosystems, e.g., when raw juice is made for human consumption from fruits that may have become contaminated with the faeces of arboreal reptiles.

## 8. Összefoglaló

A hüllőket ma már közkedvelt társállatként tartják számon. A megbetegedésüket okozó parazitákon és baktériumokon kívül állat-egészségügyi szempontból jelentős kórokozók fontos rezervoárjai is lehetnek. Vizsgálatunk az utóbbival kapcsolatos ismereteinket hivatott bővíteni.

A kutatás során 98 hüllőből és egy kétéltűből történt nem invazív bélsármintavétel az írországi Nemzeti Hüllő Állatkertben. A vizsgálatban részt vevő 44 faj rendszertanilag négy rendbe sorolható (Testudines, Squamata, Crocodilia, Anura). A hüllők bélsármintáinak gyűjtése mesterséges kifutókban, ill. terráriumokban történt 2ml úrtartalmú mintavételi csövekbe (Sarstedt), megkísérelve a talajjal történő kontamináció kizárását. A DNS kivonást Qiagen Fast Stool Mini Kit-tel végeztük. Minden egyes mintát konvencionális PCR-rel vizsgáltunk a következő célcsoportokra: eukarióták széles köre (a citokróm c oxidáz alegység, azaz *cox1* gén alapján), valamint az egysejtű paraziták két neme (*Trichomonas* és *Trypanosoma* fajok) és egyes baktériumok (Anaplasmataceae, Rickettsiaceae és rendszertanilag közeli családok).

A minták negatívnak bizonyultak a *Trypanosoma*-fajokra irányuló vizsgálatok során. Azonban egy leopárd gekkó (*Eublepharis macularius*) mintája pozitív lett a *Trichomonas* fajokra irányuló PCR vizsgálattal. A *cox1* gén négy minta esetén igazolta a gazda DNS-t (azaz alkalmas volt a gazda fajának azonosítására). Ugyanezen módszer alkalmazásával sikeresen felerősíthető volt a táplálékforrás DNS-e egy bélsárminta esetében. Ezen felül, a *cox1* PCR sárga anakondában (*Eunectes notaeus*) egy fontos opportunistá *Acanthamoeba* genotípust mutatott ki. Ezt megerősítettük az *Acanthamoeba* genus 18S rRNS génjét (*Rns* ASA.S1 régióját) célzó PCR-rel is. A szekvenálás *A. hatchetti* fajt azonosított, amelynek ismert a klinikopatológiai jelentősége embereknél és állatoknál egyaránt.

A baktériumokat tekintve, egy új-kaledóniai vitorlásgekkó (*Correlophus ciliatus*) bélsármintája *Citrobacter freundii* DNS-ét tartalmazta. A *Citrobacter*-fajok számos gerinces fajban a normál bélflóra alkotóiként ismertek, azonban opportunistá kórokozókként is számontartják őket. Még fontosabb lelet, hogy három bélsárminta (sztyeppi varánusztól: *Varanus exanthematicus*, szőnyegmintás pitontól: *Morelia spilota*, szalagos tigrispitontól:

*Python bivittatus*) a *Gordonibacter* nem DNS-ét tartalmazta, melyet mai tudásunk szerint ezidáig csupán emberi bélfloórából azonosítottak. A *G. pamelaee* azonban bakteriémiát is képes okozni emberekben. A fent említett, oportunista kórokozókra vonatkozó eredmények a kedvtelésből tartott hüllők bélsarának protozoonokra és baktériumokra irányuló szűrésének jelentőségére hívják fel a figyelmet, főként a közelükben élő más állatok és emberek szempontjából. Továbbá, ezen adatoknak a természetes ökoszisztémában járványtani jelentőségük is lehet, amikor például olyan gyümölcsökből készítenek emberi fogyasztásra szánt nyers gyümölcslevet, amelyek a fák lombkoronájában élő hüllők bélsarával szennyeződhetnek.

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