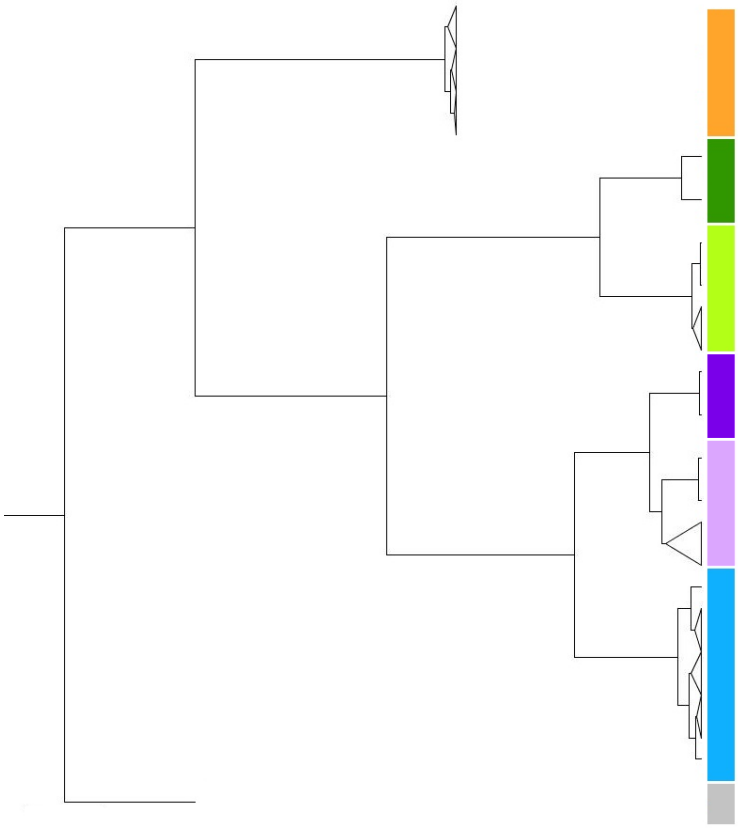

Phylogenetic and ecological characterization of monitor lizards in Northern Africa

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

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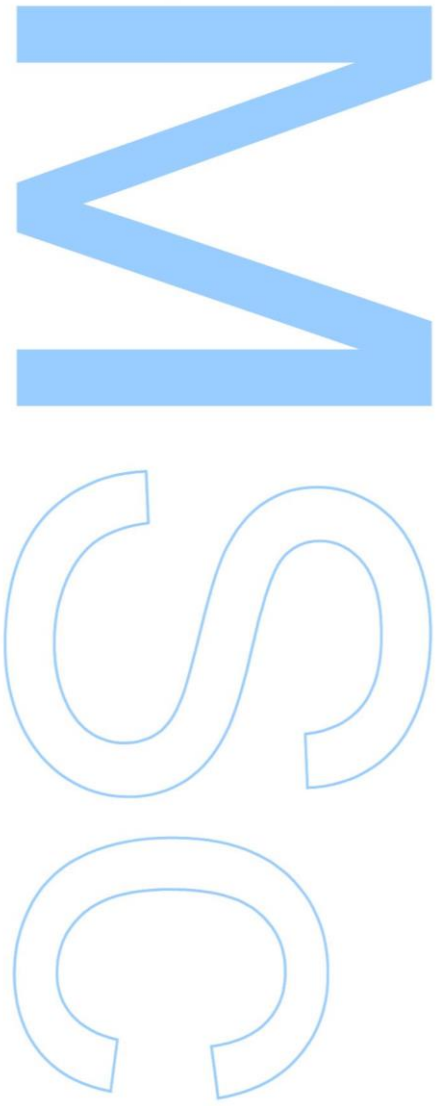

"*Ipsa scientia potestas est*." - Bacon, 1597

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To me, knowledge itself is the most important human possession and there is little purpose in life if not to contribute, even if only by a little, to the sum of all human knowledge.

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Without you, this work would not have been possible.

Resumo

A actual crise na biodiversidade por causas antrópicas directas e indirectas realça a urgência em se estudar a Biodiversidade global. Uma região particularmente pouco estudada é o Norte de África, onde a instabilidade política, os conflitos sociais, o baixo índice de desenvolvimento e de uma forma geral a inacessibilidade, dificultam o trabalho científico. O Norte da África (acima do Equador) abriga múltiplas ecorregiões com uma ampla gama de condições climáticas e topográficas, desde os desertos hiperáridos até às florestas tropicais saturadas de água. Estudos recentes mostram que a África do Norte é biologicamente mais diversa do que anteriormente se pensava devido ao seu rico passado de mudanças climáticas, como a formação do Saara e as oscilações periódicas entre os climas áridos e húmidos do Plio-Pleistoceno, e devido às múltiplas barreiras ao fluxo génico como cadeias de montanhas e rios. Neste estudo seleccionaram-se três espécies de lagartos do género *Varanus* como modelo de estudo para melhor compreender os padrões da estrutura genética e da diversidade de répteis grandes e altamente móveis da África do Norte. Cada espécie de *Varanus* seleccionada está adaptada a diferentes condições ecológicas, *V. griseus* está adaptada a regiões áridas, *V. exanthematicus* a ambientes mésicos, e *V. niloticus* dependente de corpos de água permanentes. A fim de compreender a estrutura genética e os padrões de diversidade das três espécies de *Varanus*, para resolver incongruências em relação à variedade de espécies, e para auxiliar o planeamento futuro da conservação destas espécies exploradas, o trabalho conducente a esta dissertação foi desenvolvido tendo por base os seguintes objectivos: 1) actualizar a distribuição geográfica; 2) determinar a estrutura genética e a diversidade genética intraespecífica; 3) mapear a diversidade genética espacial e as potenciais barreiras ao fluxo génico; e 4) caracterizar o nicho ecológico de todas as espécies-alvo e as potenciais linhagens intraespecíficas.

Pesquisa bibliográfica exaustiva e dados recolhidos durante viagens de campo anteriores permitiram calcular a extensão da ocorrência de cada espécie de acordo com as normas da Lista Vermelha da IUCN. A análise filogenética de um segmento de 627 pb do genoma mitocondrial de amostras recolhidas durante o trabalho de campo e sequências do NCBI GenBank revelaram um total de 18 linhagens agrupadas em seis clados entre as três espécies. Um clado em *V. griseus*, dois em *V. exanthematicus* (Oeste e Leste) e os três clados já descritos na literatura em *V. niloticus* (Oeste, Norte e Sul). Analisaram-se também três marcadores genéticos nucleares, totalizando 1218 pb, para criar redes de haplótipos das amostras disponíveis. Nestas redes *V. griseus* não apresentou diversidade e *V. exanthematicus* e *V. niloticus* apresentaram pouca diversidade sem estrutura clara. Foi elaborada uma “Spatial Principal Component Analysis” com nove variáveis

topoclimáticas de África para aceder ao nicho ecológico das espécies e respectivos clados. As comparações resultantes revelaram algumas diferenças ecológicas entre as espécies e nenhuma entre os clados.

A distribuição espacial distinta, a estrutura genética e as diferenças percentuais entre os clados de mtDNA sugerem a existência de diversidade críptica potencial dentro de *V. exanthematicus* e *V. niloticus* que provavelmente apareceram devido a fortes eventos climáticos que moldaram a África do Norte no passado. No entanto, análises dos marcadores genéticos nucleares e do nicho ecológico revelaram pouco suporte a favor de novas espécies crípticas, sugerindo que é necessário mais trabalho para abordar adequadamente esta questão. Este trabalho foi importante para preencher lacunas de conhecimento sobre a distribuição e estrutura genética das três espécies, e abrir caminho para futuros estudos e conservação destas espécies.

Palavras-chave

Squamata, Varanoidea, Varanidae, Xerófilo, Mesófilo, Aquático, ND4, KIAA1549, PRLR, RAG1, SIG, Distribuição, Filograma, Estrutura, Diversidade, Nicho Ecológico, Análise de Componentes Principais

Abstract

The ongoing biodiversity crisis of direct and indirect anthropogenic causes highlights the urgency to study global biodiversity. A particularly understudied region is Northern Africa where political instability, social conflicts, low development index, and generic inaccessibility hamper scientific work. Northern Africa (above the equator) harbours multiple ecoregions with a wide range of climatic and topographic conditions, from the hyper-arid deserts to the water-saturated rainforests. Recent studies show that Northern Africa is more biologically diverse than previously thought due to its rich history of climatic shifts like the formation of the Sahara and the periodic oscillations between arid and humid climates of the Plio-Pleistocene, and due to multiple barriers to gene flow like mountain ranges and rivers. In this study, three monitor lizard species (genus *Varanus*) were selected as model systems to better understand the patterns of genetic structure and diversity of large and highly mobile reptiles of Northern Africa. Each selected species of *Varanus* is adapted to different ecological conditions with *V. griseus* being adapted to arid regions, *V. exanthematicus* to mesic environments, and *V. niloticus* being dependent of permanent water bodies. In order to understand the genetic structure and patterns of diversity of the three *Varanus* species, to resolve incongruencies regarding the species range, and to aid future conservation planning of these exploited species, the work leading to this dissertation was developed under the frame of the following objectives: 1) update the geographic distribution; 2) determine the genetic structure and intraspecific genetic diversity; 3) map the spatial genetic diversity and potential barriers to gene flow; and 4) characterise the ecological niche of all target species and potential intraspecific lineages.

Exhaustive literature research and data collected during past field trips allowed to calculate the extent of occurrence of each species according to IUCN Red List norms. Phylogenetic analysis of a segment of 627 bp of the mitochondrial genome from samples collected during past field work and sequences from NCBI GenBank revealed a total of 18 lineages grouped into six clades among the three species. One clade of *V. griseus*, two of *V. exanthematicus* (West and East) and the three clades already described in the literature of *V. niloticus* (West, North and South). Three nuclear genetic markers, totalling 1218 bp, were also analysed to create haplotype networks of the available samples. In these networks, *V. griseus* showed no diversity, and *V. exanthematicus* and *V. niloticus* presented very little diversity without clear structure. A Spatial Principal Component Analysis of nine topoclimatic variables of Africa was created to access the ecological niche of the species and respective clades. The resulting comparisons revealed some ecological differences between species and none between clades.

Distinct spatial distribution, genetic structure and percentual differences among the mtDNA clades, suggest the existence of potential cryptic diversity within *V. exanthematicus* and *V. niloticus* that likely appeared due to strong climatic events that shaped Northern Africa in the past. However, analyses of the nuclear genetic markers and ecological niche revealed little support in favour of new cryptic species suggesting that more work is needed to properly address this issue. This work was important to fill in knowledge gaps regarding the distribution and genetic structure of the three species, and to path the way for future studies and the conservation of these species.

Keywords

Squamata, Varanoidea, Varanidae, Xeric, Mesic, Aquatic, ND4, KIAA1549, PRLR, RAG1, GIS, Distribution, Phylogram, Structure, Diversity, Ecological Niche, Principal Component Analysis

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Abbreviations

BIC - Bayesian Information Criterion

BPP - Bayesian Posterior Probability

ESS - Effective Sample Sizes

GPS - Global Positioning System

HKY+G - Hasegawa-Kishino-Yano 1985 with Gamma

MCMC - Markov chain Monte Carlo

MCP - Minimum Convex Polygon

mtDNA - Mitochondrial DNA

ND4 – NADH dehydrogenase subunit 4

nDNA - Nuclear DNA

PC - Principal Components

PCR - Polymerase chain reaction

PRLR - Prolactin Receptor

RAG1 - Recombination-activating gene 1

SPCA - Spatial Principal Components Analysis

1. Introduction

1.1. Biogeography of Northern Africa

Northern Africa, in here considered to be the continental Africa above the equator line (0ºN) is an enormous mass of land with nearly 20 million km² (Figure 1). Throughout its range four major biomes can be identified: 1) Mediterranean Forests, Woodlands and Scrubs; 2) Deserts and Xeric Shrublands; 3) Tropical and Subtropical Grasslands, Savannas and Shrublands; and 4) Tropical and Subtropical Moist Broadleaf Forests. Those biomes can be subdivided into several ecoregions. Some of the most important ecoregions are: the African Mediterranean Woodlands, mainly North of the Atlas mountain range in Morocco, Algeria and Tunisia; several subdivisions of the Sahara (North, West, East, and South), the largest warm desert in the world with more than 9 million km² (almost half of Northern Africa); the Sahel (from the Arabic for *Shore* or *Margin* of the great sea of sand), the Semi-Arid transition belt between the Desert and the Savannas at South; West and East Sudanian Savannas South of the Sahel; and the Guinean and Congolian Forests near the equator and the Gulf of Guinea (Olson et al., 2001; Dinerstein et al., 2017).

The distribution of biomes and ecoregions in Northern Africa has not been static over time; the region used to be more humid and fertile in comparison to what can be observed today (Armitage et al., 2015). Throughout the Quaternary, Northern Africa shifted from wetter climates to drier ones (deMenocal, 2004; Schuster et al., 2006; Tabel et al., 2016), starting with the shrinkage of the Tethys Sea during the Tortonian Stage of the Miocene (11 to 7 million years ago), that weakened the summer monsoon in Africa (Zhang et al., 2014). That event likely started the aridification of the region and the emergence of the Sahara, which is estimated to have started in the Chad region during the late Miocene (around 7 million years ago) and ended around late Pliocene to early Pleistocene (2.5 million years ago) in the western regions (reviewed by Brito et al., 2014; Ward, 2016). After its formation, the distribution of the Sahara during the Pliocene (5.3 to 2.5 million years ago) kept getting moulded by geological and climatic factors, like the strong wet-dry climatic oscillations typical of that period (reviewed by Brito et al., 2014; Armitage et al., 2015). Since then, climatic cycles kept affecting Northern Africa until the mid-Holocene with the last known wet phase peaking about 11 thousand years ago and ending around 6 and 5 thousand years ago (reviewed by Brito et al., 2014; Tierney et al., 2017).

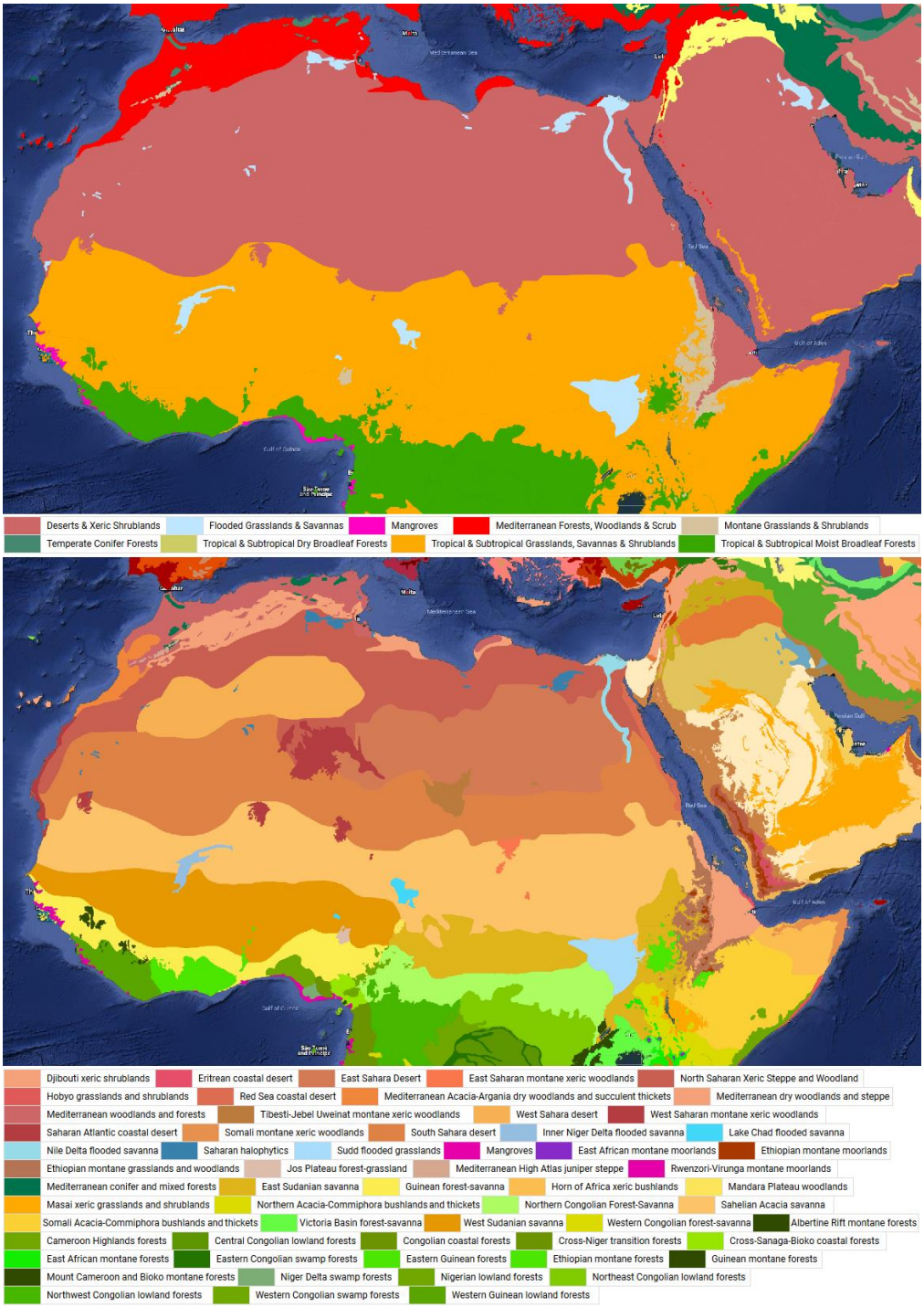

Figure 1.Biomes (above) and ecoregions (below) of Northern Africa, from Biomes©Resolve and Ecoregions2017©Resolve respectively. Adapted from an interactive world map available at ecoregions2017.appspot.com.

Throughout the region, during the wet phase of each wet-dry cycle, major river courses got altered, lakes and wetlands appeared, and with them vegetation followed. The Sahara-Sahel limit shifted northwards, increasing the latitudinal width of the Sahel and the Savannah ecoregions. However, during the dry phase of the cycle, aridity increased, rivers dried, and lake levels decreased, eventually resulting in a regression of mesic vegetation

(reviewed by Brito et al., 2014). Besides vegetation, both the topographic and climatic alterations that shaped Northern Africa throughout its history have been linked to current distribution patterns of biodiversity (reviewed by Brito et al., 2014; 2016).

1.2. Biodiversity in Northern Africa

The study of biodiversity is more urgent than ever; it is estimated that less than a fourth of all eukaryotic species are known (Mora et al., 2011) and that humanity is currently living through, and causing, the sixth mass extinction (Ceballos et al., 2017). This is a more pressing issue in Northern Africa since it is a highly understudied region (Brito et al., 2014; Meyer et al., 2015) due to a multitude of reasons, including amongst others past and present problems of terrorism, trafficking of arms, drugs and human beings (OECD, 2014), general low human development, and long history of political instability (Brito et al., 2014). Biodiversity is threatened due to armed conflicts (Brito et al., 2018), species exploitation and overhunting (Brito et al., 2014; 2016; Joppa et al., 2016; Daskin & Pringle, 2018; Pleguezuelos et al., 2018), direct habitat destruction (Brito et al., 2014; 2016; Joppa et al., 2016; Daskin & Pringle, 2018; Harfoot et al., 2018), and natural resources exploitation (oil, gas, mining), pollution, and climate change (Brito et al., 2014; Harfoot et al., 2018). Climate change, in particular, is expected to highly impact biodiversity due to expected factors like increasing temperatures (Cook & Vizy, 2015; Lelieveld et al., 2016), sea level rise (Dutton et al., 2015), extreme precipitation in dry and wet areas (Lavaysse, 2015; Donat et al., 2016), desertification of many regions like West Africa (Sylla et al, 2016), the Mediterranean Basin (Gao & Giorgi, 2008; Brito et al., 2014), African Forests and the Gulf of Guinea (Sylla et al., 2016). Even species adapted to arid environments would be vulnerable to climate change (Vale & Brito, 2015), lizards in particular could be highly affected by increasing temperatures (Ferri-Yáñez & Araújo, 2015; Buckley et al., 2016).

As of right now, biodiversity hotspots in the Sahara are found in North-South corridors (like the Atlantic and the Red Sea coastal areas and the Nile River), mountain refugia, oasis and mountain lagoons. This distribution pattern is likely related to the wet-dry cycles that pushed species upwards in mountains and towards the extremities of the Sahara. These cycles fragmented habitats, creating vicariant events that eventually led to allopatric differentiation, a pattern even visible through molecular analyses of cryptic species that were once thought to be wide-range single taxonomic units (reviewed by Brito et al., 2014). In the Sahel and the Guinean and Sudanian Savannas, vertebrate biodiversity is often distributed in longitudinal-wise. Such patterns have been attributed to the major river basins of the region, like the Niger and Volta rivers, acting as barriers to gene flow (e.g. Brouat et al., 2009; Bryja et al., 2010; Nicolas et al., 2010; 2011). Genetic diversification appeared

mostly by allopatric differentiation mechanisms that operated in different directions depending on the habitat requirements of each species (reviewed by Brito et al., 2014) (Figure 2) rather than by environmental adaptation. Xeric species likely colonized large sections of the Sahara-Sahel during the dry phase of the climatic cycles and diversified during the humid period when their habitat became fragmented.

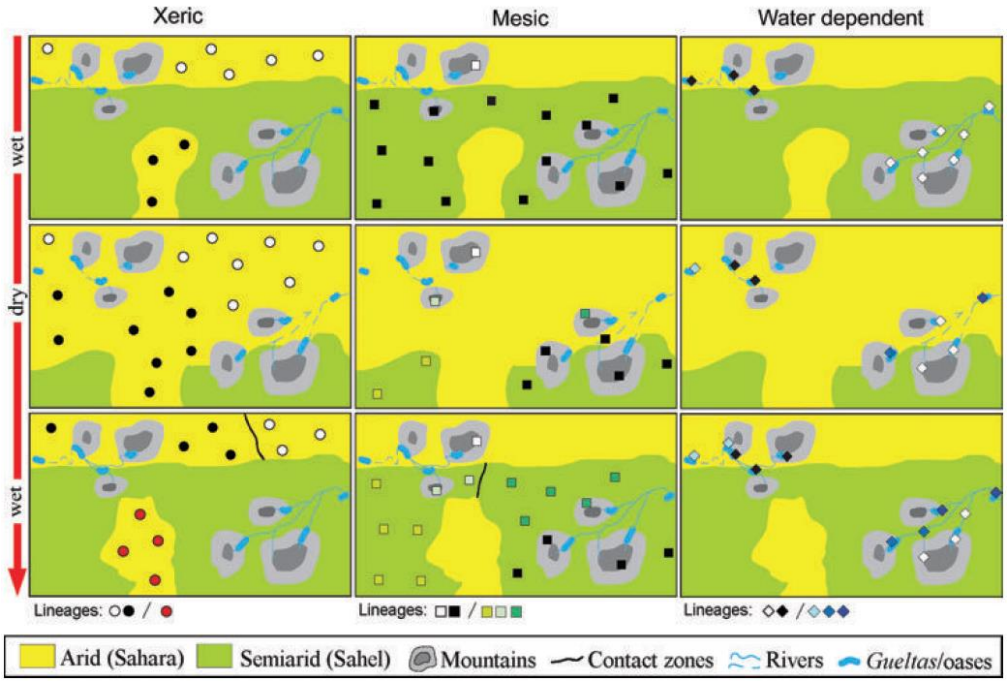

Figure 2. Scheme of the wet-dry cycles and subsequent hypothetical diversification mechanisms through allopatric processes for three functional groups of species: xeric (circles), mesic (squares), and water-dependent (diamonds). Adapted from Brito et al. (2014).

Examples include reptiles (*Uromastyx spp.*, Tamar et al., 2017) and mammals (*Jaculus spp.* Boratyński et al. 2014; *Vulpes rueppellii* Leite et al., 2015). On the contrary, Mesic species likely occupied the region during the humid phase of the cycle and diversified during the peak of the dry phase as they migrated south or upwards into mountain refugia. Examples include reptiles (*Ptyodactylus spp*. Metallinou et al., 2015; *Agama spp*. Gonçalves et al., 2018a; *Psammophis spp*. Gonçalves et al., 2018b; *Acanthodactylus aureus* Velo-Antón et al., 2018) and mammals (*Vulpes vulpes* Leite et al., 2015). Similarly to mesic species, water-dependent species colonized the region during the wet period and these species are expected to have diversified during arid phases as water bodies become disconnected and scarce (e.g. *Crocodylus spp*. Hekkala et al., 2011; Velo-Antón et al., 2014; Cunningham et al., 2016). A species ability to disperse can also greatly influence diversification among species and populations since species with high mobility (that can disperse more) can easily surpass obstacles and unfavourable conditions in order to reach a new area, unlike species with reduced mobility. The ability to reach suitable places and therefore populations mitigates the possible effects of the mechanisms of speciation

previously mentioned by increasing gene flow between populations, resulting in admixture of populations and homogenisation of the species genetic pool (Bohonak, 1999; Payne et al., 2011). An example of large mammals with high mobility that presents shallow genetic structure are *Gazella spp*. (Lerp et al., 2011).

1.3. Old World Monitor Lizards (Genus: *Varanus*)

Monitor lizards, mostly known worldwide by the emblematic Komodo dragon (*Varanus komodoensis*) and for being the closest living relatives to snakes (Lee, 1997; Fry et al., 2006; Streicher & Wiens, 2017), compose the Varanoidea superfamily of the Anguimorpha suborder (Streicher & Wiens, 2017), that together with iguanians and snakes form the clade of Toxicofera, a proposed clade of the Squamata order (Fry et al., 2006). Within Varanoidea, "old world monitor lizards" form the Varanidae family (Ast, 2001), a family with *Varanus* as its only extant genus. It is believed the genus has a Laurasian (Pianka, 1995) or Asian origin (Fuller et al., 1997) and that it is composed by three major clades, an African, an Indo-Asian, and an Indo-Australian (Ast, 2001). While most species fall within the last two mentioned clades, the African clade is composed by five species: *Varanus griseus*, the first divergent lineage; *Varanus niloticus*, the second divergent lineage; *Varanus exanthematicus*, the third divergent lineage; and *Varanus albigularis* and *Varanus yemenensis*, the last two on the line (Pyron et al., 2013). By the year 2000 slightly more than 50 species of the *Varanus* genus had been described (Böhme, 2003), a number that today has already surpassed 70 recognised species due to genetic analysis of previously known taxa (Doughty et al., 2014; Maryan et al., 2014), examples of new species identification can be seen in the works of Aplin et al. (2006), Welton et al. (2013; 2014), Doughty et al. (2014), Dowell et al. (2015a), and Weijola et al. (2016).

Despite occupying highly diverse habitats and displaying a wide range of body sizes (Pianka,1995), *Varanus* have highly conserved morphologies (Ast, 2001). These fast and highly mobile lizards (Rome, 1982; Stanner & Mendelssohn, 1987) have large home ranges (Bennett, 1995; Pianka & King, 2004) and are predators and scavengers that will eat nearly all they can swallow (Smith & Hylander, 1985; Bennett, 1995; Arbuckle, 2009).

Many species of *Varanus* are exploited by humans mostly for either leather or pet trade (Jenkins & Broad, 1994; Bennett, 1995; Dowell et al. 2015b; Dowell & Hekkala, 2016). With *V. niloticus* and *V. exanthematicus* being the two most traded monitor lizard species in the world (Pernetta, 2009).

This work focuses on three species of the African clade present in Northern Africa, each belonging to one of the functional groups mentioned above. *V. griseus*, a xeric species, *V. exanthematicus*, a mesic species, and *V. niloticus*, a water-dependent species.

1.3.1. *Varanus* (*Psammosaurus*) *griseus* (Daudin, 1803)

Varanus griseus (Figure 3), commonly known as Desert Monitor, is a big lizard that can reach more than a meter long and is highly adapted to hyper-arid environments making deserts and surrounding areas its preferable habitat (Bennett, 1995; Ilgaz et al., 2008; Houssaye et al., 2010; Trape et al., 2012).

Figure 3. *Varanus griseus*; Mauritania, 2010 (Photo by Z. Boratyński)

There are no specific studies made about its global distribution and many publications have significant discrepancies in the proposed species range (Pianka & King, 2004, Sindaco & Jeremcenko, 2008; Trape et al. 2012). This lizard is known to spread over a vast area, occupying all arid and semi-arid regions from the Western coast of the Sahara, South of the Atlas Mountains, to the Thar Desert in India including the entire Arabian Peninsula and reaching as far North as South Kazakhstan by the Aral Sea (Bennet, 1995; Bayless 1997; Pianka & King, 2004; Sindaco & Jeremcenko, 2008; Trape et al., 2012) (Figure 4).

Based on morphological traits, *V. griseus* is subdivided in three subspecies: *V. g. griseus*, also known as Grey Monitor, has the biggest range of the three, spanning from the Atlantic Coast of the Sahara to the Zagros Mountains in Iran; *V. g. caspius*, commonly referred to as Caspian Monitor extends from the East side of the Zagros Mountains to the

Western side of the mountain ranges of Pakistan; and *V. g. koniecznyi*, known as the Indian Desert Monitor is present in the East side of Pakistan all the way to the Thar Desert and surrounding semi-arid areas in India (Bennett, 1995; Pianka & King, 2004).

Considering that *V. griseus* is a xeric animal, it is expected to have experienced diversification during humid periods (Brito et al., 2014). However, considering that *Varanus* usually exhibit large home ranges and high mobility (Bennett, 1995), it is plausible that *V. griseus* has shallower, genetic structure than other xeric reptiles of the region. Because of this, a genetic pattern similar to species within the *Uromastyx* genus can be expected, considering that *Uromastyx* are medium-large size reptiles highly adapted to arid environments that have an extremely shallow genetic structure at the intraspecific level (Tamar et al., 2017).

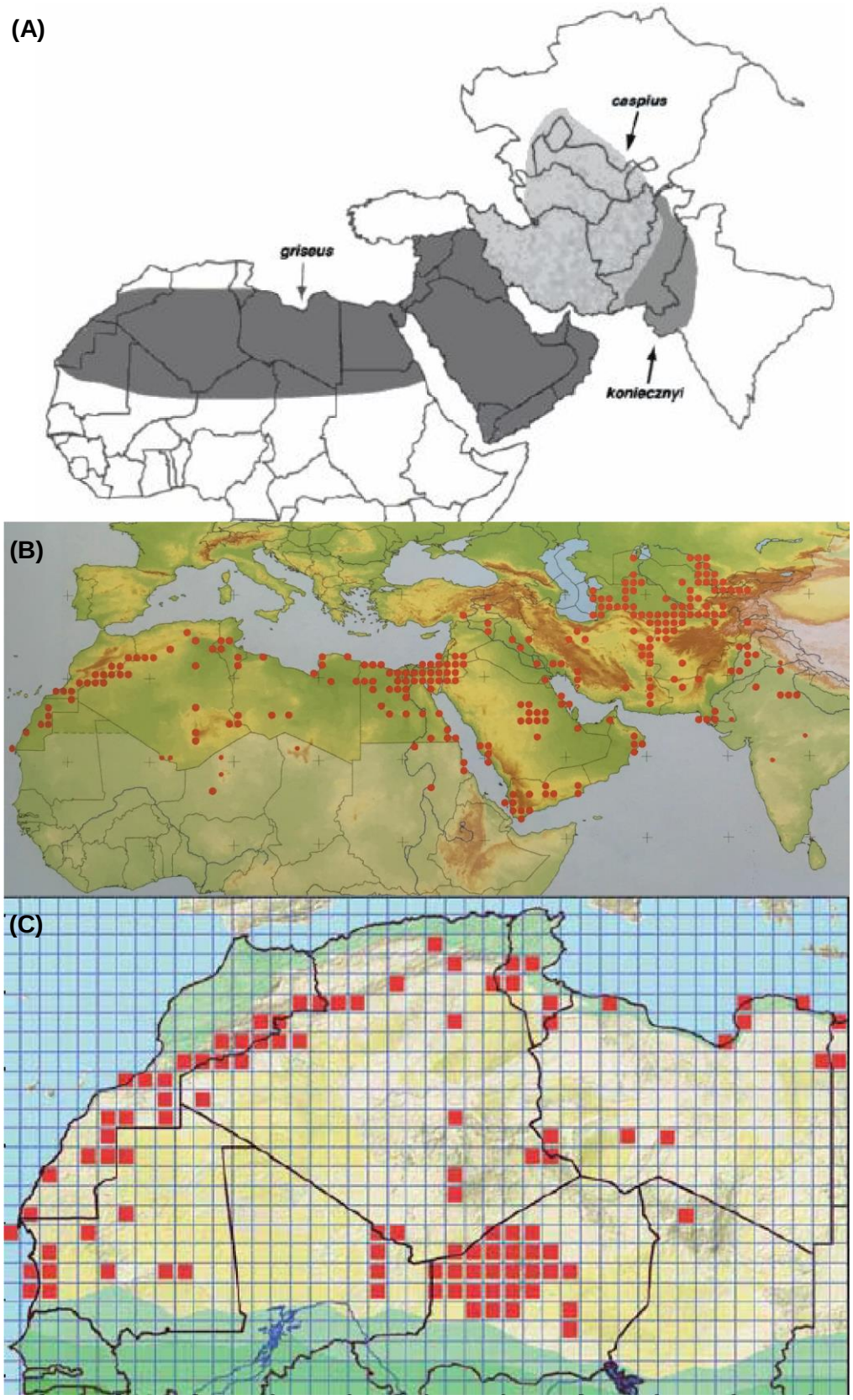

Figure 4. Known geographical distribution of *Varanus griseus* and its three subspecies (A) and some known points of occurrence throughout its range (B) and in North and West Africa (C). (A) adapted from Pianka & King (2004), (B) from Sindaco & Jeremcenko (2008), and (C) from Trape et al. (2012).

1.3.2.*Varanus* (*Polydaedalus*) *exanthematicus* (Bosc, 1792)

Varanus exanthematicus (Figure 5), usually referred to as Savannah Monitor or Bosc's Monitor after its first describer, is the smallest monitor in Africa rarely reaching one meter in length (Bennett 1995; Pianka & King 2004; Houssaye et al., 2010; Trape et al., 2012). While tolerant of some aridity, *V. exanthematicus* depends on trees and other types of vegetation.

Figure 5. *Varanus exanthematicus*; Mauritania, 2010 (Photo by Z. Boratyński)

Its preferred habitats are the sub-Saharan semi-arid regions North of the equator, including the Sahel, the Sudanian and Guinean Savannas, and even reaching the upper limit of tropical forests (Bennett, 1995; Bayless, 1997; Pianka & King 2004; Bennett & Sweet, 2010; Trape et al., 2012). However, while this is the only of the three species that is listed in IUCN (Bennett & Sweet, 2010), there is no consensus in published distributions of the species. Some authors consider its distribution from Senegal to Sudan (e.g. Trape et al., 2012), others from Senegal to Ethiopia (e.g. Pianka, 2004; Bennett & Sweet, 2010), while others claim it extends from Senegal all the way to Eritrea (e.g. Bennett, 1995). Even the range available at IUCN (Bennett & Sweet, 2010) failed to include many known points of occurrence (Pianka & King, 2004; Trape et al., 2012) (Figure 6).

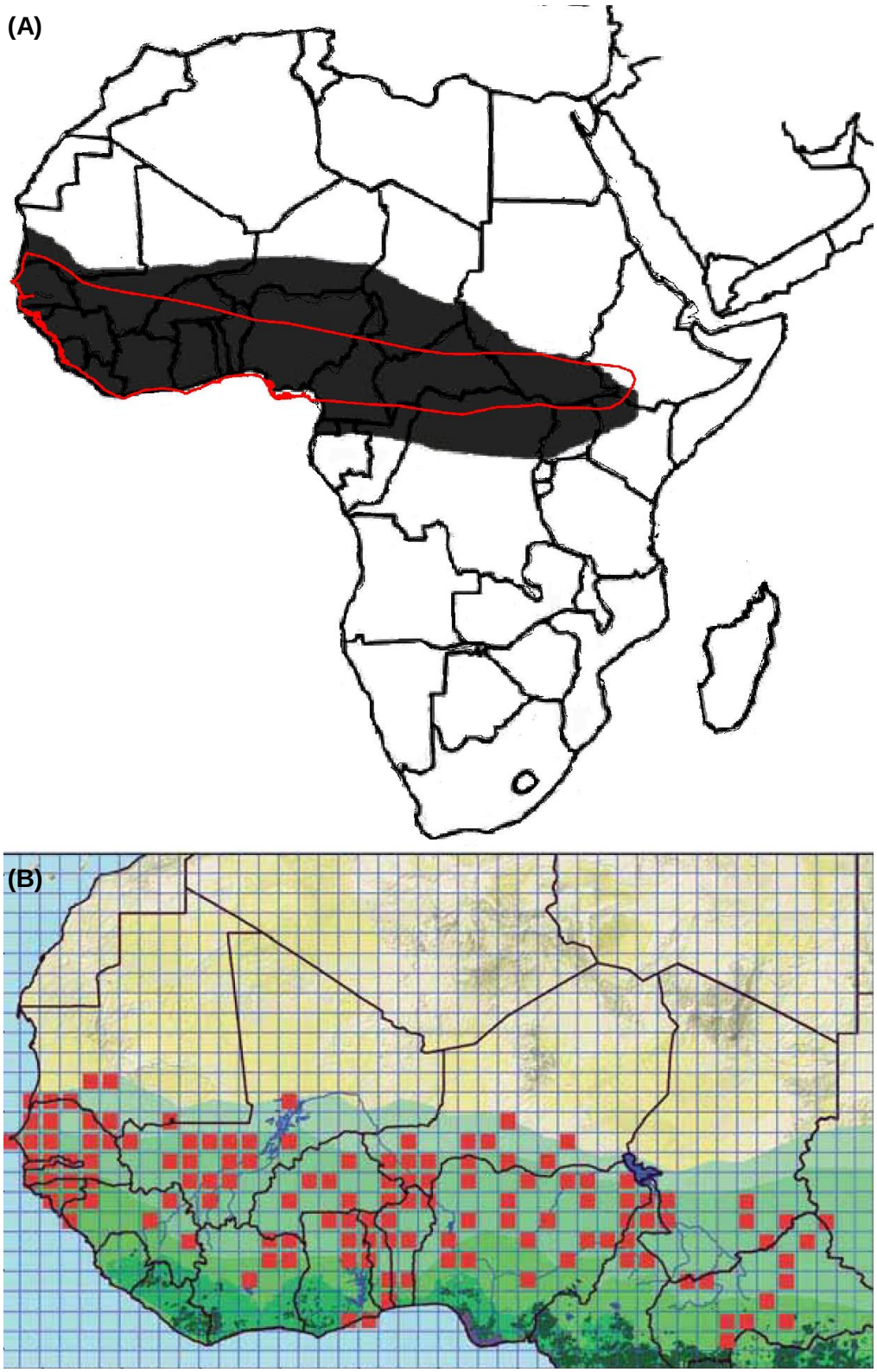

Figure 6. Known geographical distribution of *Varanus exanthematicus* (grey area) with a representation of the IUCN range (red polygon) (A) and known points of occurrence in West Africa (B). (A) adapted from Pianka & King (2004) and Bennett & Sweet (2010), and (B) from Trape et al. (2012).

While today *V. exanthematicus* is considered to be a single species with no subspecies, other taxonomic units have been grouped with *V. exanthematicus* in the past (Bennett, 1995; Pianka & King, 2004). *Varanus albigularis* used to be considered a subspecies of *V. exanthematicus* until Böhme (1988) re-elevated it to species level (Pianka & King, 2004), and *V. ocellatus*, firstly described as its own species and later classified as a subspecies of *V. exanthematicus* endemic to Sudan and South Sudan, is no longer accept by most authors as they claim there are no morphological differences to the typical *V. exanthematicus* (Bennett, 1995; Pianka & King, 2004). Considering that many historical studies grouped *V. albigularis* and *V. exanthematicus* under the same name, and that both species are sympatric in Ethiopia, Kenya and other surrounding areas, it is possible that *V. albigularis* may be behind the disagreement between authors regarding the Eastern end of the range of *V. exanthematicus*, as some authors may have confused records of both species recognised nowadays.

As a mesic species, *V. exanthematicus* likely spread during humid periods and became isolated during hyper-arid periods which could have caused diversification (Brito et al., 2014). Such genetic structure can be observed in other mesic reptiles, like *Agama spp.* (Gonçalves et al., 2018a) and *Ptyodactylus spp*. (Metallinou et al., 2015), and in mammals, such as *Vulpes vulpes* (Leite et al., 2015). The habitat of *V. exanthematicus* is mostly continuous at a large scale and considering its high dispersal ability, it could be expected little to no genetic diversification. However, at a finer scale, non-occurrence areas and natural barriers (like major river valleys) may constrict gene flow, resulting in a deeper genetic structure. This is a pattern already observed in small mammals of western Africa, such as *Praomys spp.* (Bryja et al., 2010; Nicolas et al., 2010; 2011), and *Mastomus erythroleucus* (Brouat et al., 2009).

1.3.3. *Varanus* (*Polydaedalus*) *niloticus* (Linnaeus, 1766)

Varanus niloticus (Figure 7), best known as Water Monitor or Nile Monitor, is the biggest African lizard. This enormous reptile frequently reaches over two meters in length (Bennett, 1995; Pianka & King, 2004; Trape et al., 2012) and is highly dependent of permanent water bodies due to its semi-aquatic nature (Bennett, 1995; Pianka & King, 2004; Sindaco & Jeremcenko, 2008; Trape et al., 2012; Dowell & Hekkala, 2016).

Most studies agree that *V. niloticus* is present in most of sub-Saharan Africa where water is permanently present plus the Nile River (Bennett, 1995; Pianka & King, 2004; Sindaco & Jeremcenko, 2008; Trape et al., 2012). However, some authors, like Bennett (1995) and Pianka & King (2004), failed to exclude the Namib and/or the Kalahari deserts of Southern Africa from their presented distributions (Figure 8).

Figure 7. *Varanus niloticus*; Mauritania, 2010 (Photo by J.C. Brito)

Varanus ornatus, previously thought to be a subspecies of *V. niloticus* occurring in rainforests and mangroves of the Gulf of Guinea, used to be considered a sister species of *V. niloticus* until a recent study by Dowell et al. (2015a) showed that there was no phylogenetic data to support its status of species or subspecies. Because of this, *V. ornatus* and *V. n. ornatus* should be considered synonyms of *V. niloticus* in future studies. In the same publication, Dowell et al. (2015a) identifies three major lineages of *V. niloticus* that she names of North, with specimens mostly from the eastern Sahel and the Nile, South, with specimens mostly from East, Central, and Southern Africa, and West, with specimens mostly present in West Africa (Figure 9). Based on molecular data, Dowell et al., (2015a) argues that the West Lineage of *V. niloticus* is a cryptic species that she nominates of *V. stellatus*. Since no morphological comparison was made and that no formal description of the species was presented, all previously mentioned lineages should be considered as being part of *V. niloticus* for time being.

Dowell et al. (2015a) estimates that the separation of the Western clade happened approximately 7.7 million years ago and that the Southern and Northern clades separated around 4.7 million years ago. Considering that water dependent species, like *V. niloticus*, are likely to have experienced diversification during arid periods (Brito et al., 2014), it is plausible that the first lineage diverged during the late Miocene, when aridity was rapidly increasing in Northern Africa, which eventually resulted in the formation of the Sahara. The separation of the Northern and Southern lineages could be attributed to the wet-dry cycles of the Pliocene that shifted the latitudinal ranges of the Savannas and Forests of Northern Africa.

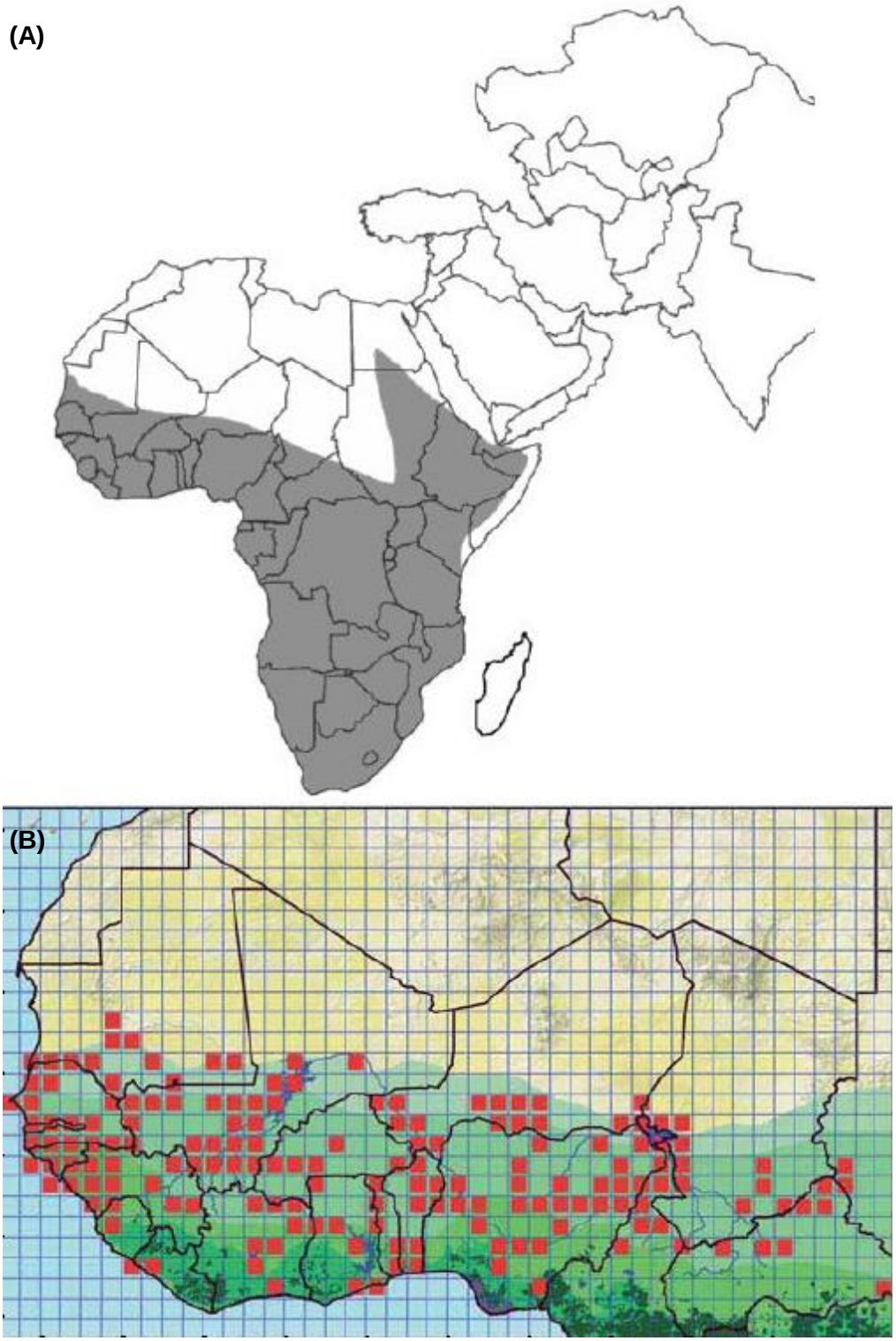

Figure 8. Known geographical distribution of *Varanus niloticus* (A) and known points of occurrence in West Africa (B). (A) adapted from Pianka & King (2004), and (B) from Trape et al. (2012).

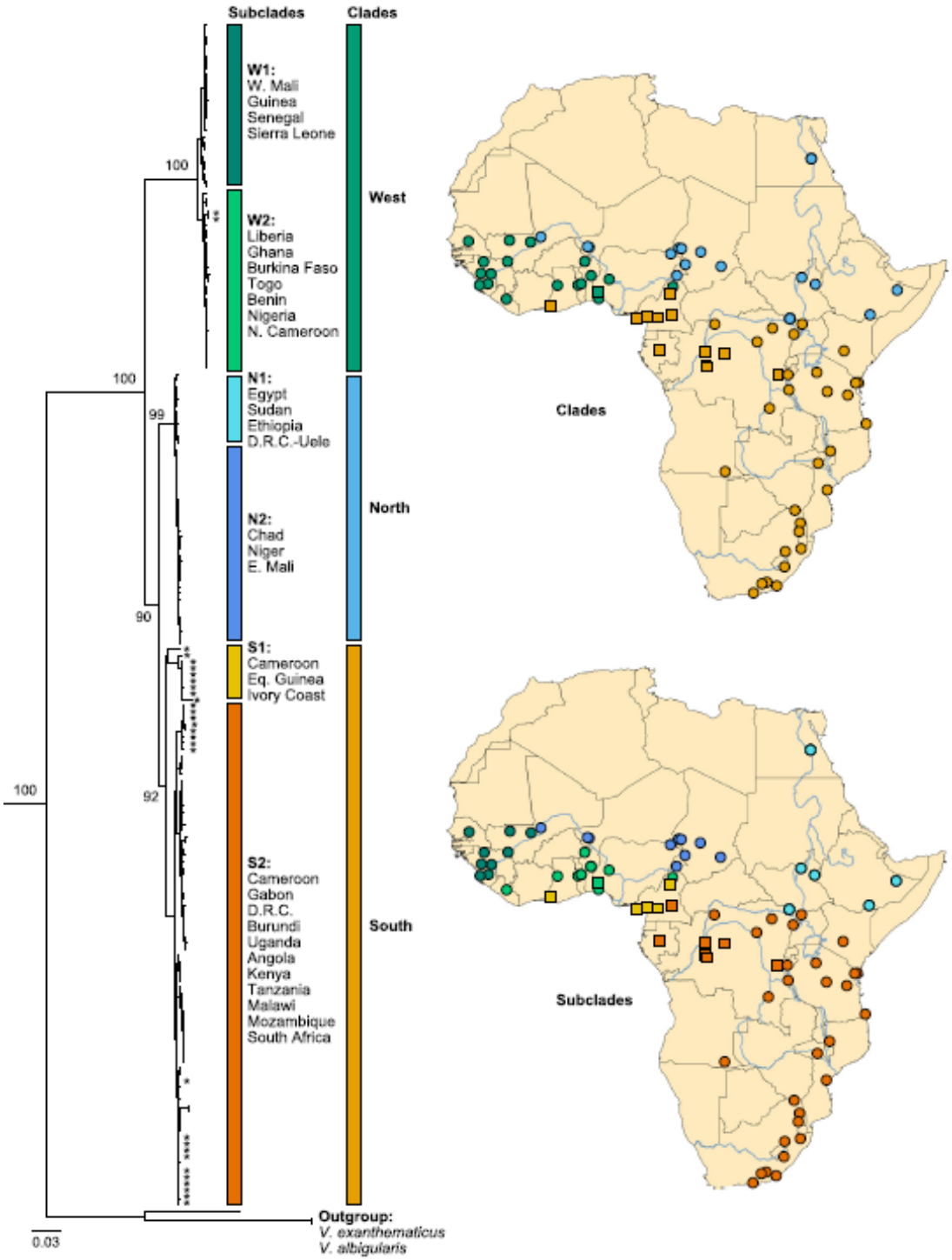

Figure 9. Maximum likelihood tree of *Varanus niloticus*. Coloured bars denote the major clades and subclades. Maps show the locality of individuals belonging to each of the major clades and subclades. Localities were represented as squares for specimens identified as *V. ornatus* and circles for *V. niloticus*. Adapted from Dowell et al. (2015a).

1.4. Research Hypothesis and Objectives

As previously explained, there is generalised uncertainty about the distribution of *V. griseus*, *V. exanthematicus*, and *V. niloticus* in Africa, which translates into lack of consensus in available distribution maps and coarse distribution limits. While for *V. niloticus* there is published information about the three major lineages found throughout the African continent that can be linked to past climatic changes, no data are available about the genetic structure and distribution of genetic diversity of *V. griseus* and *V. exanthematicus*. On the one hand, their large body size, wide home range, and generalised unfragmented habitat along their distribution area, lead to the expectation of shallow intraspecific structure. On the other hand, past climatic changes in Northern Africa have been linked to genetic divergence amongst taxa of multiple functional groups, as previously shown for *V. niloticus*. While ecological differences between *V. griseus*, *V. exanthematicus* and *V. niloticus* should be expected, since they all share different habitats, the same cannot be said for intraspecific lineages considering that the range of each species is mostly contiguous and climatically homogenous, thus no major ecological differences are expected.

To shed some light on the hypotheses here formulated, this work was conducted with the following objectives in mind:

1) Update the geographic distribution of the three target African *Varanus*;
2) Determine its intraspecific genetic structure and diversity;
3) Map the geographic distribution of the genetic diversity and identify potential barriers to gene flow;
4) Characterise the ecological niche of species and potential intraspecific lineages.

2. Methodology

2.1. Study Area

In this study we focused on the African continent North of the equator line (0ºN), an area that includes 26 countries in its totality and parts of 8 other internationally recognised souverain states comprising of almost 20 million km² (Figure 10). This region has a wide spectrum of topographic and climatic conditions, ranging from the negative 155 meters in Lake Assal in Djibouti to 5109 meters in Mount Stanley in the Democratic Republic of Congo, and from one of the hottest and most arid places on Earth (Sahara) to the extremely humid rainforests of the Congo region. This study area was selected for being an easy to define area that included considerable portions of the known species distribution, including the entire range of *V. exanthematicus*, and a wide variety of climates. The entire range of the three species was not considered to be the area used in this study due to the lack of genetic samples available outside of Northern Africa.

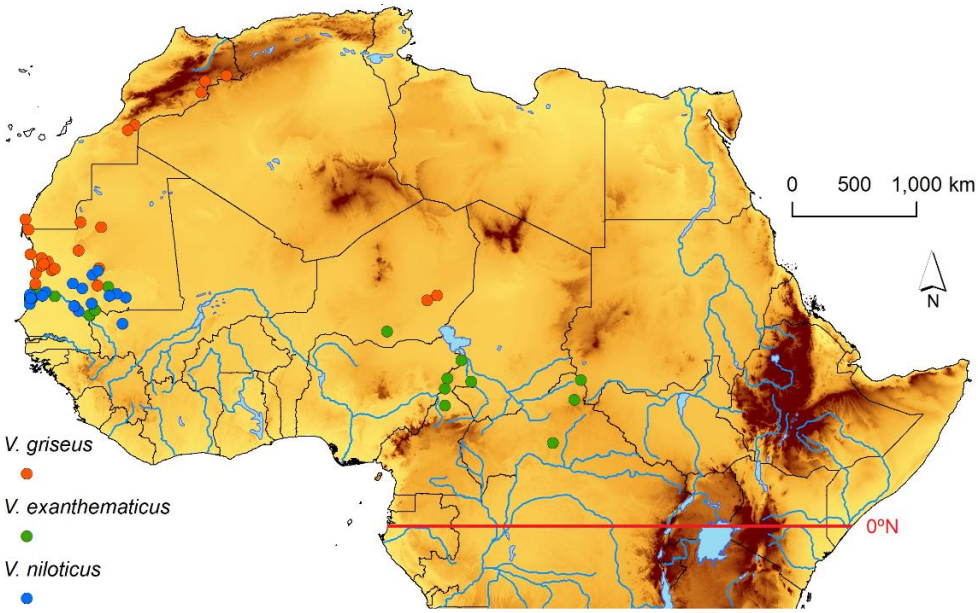

Figure 10. Altitudinal map of Northern Africa with national frontiers (Black) and major water bodies (Light Blue). Red line represents the equator (0ºN) and circles represent available genetic samples of *Varanus griseus* (Orange), *Varanus exanthematicus* (Green), and *Varanus niloticus* (Blue) for the development of this study.

2.2. Geographic Distribution

Over 50 peer reviewed articles and books were thoroughly analysed to create a dataset of the geographical distribution of *V. griseus*, *V. exanthematicus*, and *V. niloticus* throughout their entire range. When presented in the articles, Global Positioning System (GPS)

coordinates were directly recorded. When only maps with presence points were available, those maps were georeferenced in ArcGIS (ESRI, 2011) under the WGS 84 datum and coordinates for all points were retrieved. However, for most of the articles consulted, only names and descriptions of the locality were available. In such cases, every single locality was searched for in online databases such as the National Geospatial-Intelligence Agency GEOnet Names Server (NGA, 2016) (available at geonames.nga.mil/gns/html), Google Maps (available at www.google.com/maps/), and Geographical Names (available at geographic.org/geographic_names/), and a GPS coordinate was associated to it with an assumed error of 10 km². Localities with descriptions that were ambiguous or too broad to be identified were removed resulting in a robust database of multiple points of occurrence throughout the natural range of the three species from 47 publications (references in Annex 1). GPS coordinates recorded in field from observational and sampling data collected by researchers and collaborators of the CIBIO-InBIO's (Research Center in Biodiversity and Genetic Resources - InBIO Associate Laboratory, in Portuguese *Centro de Investigação em Biodiversidade e Recursos Genéticos*) research group BIODESERTS during multiple field expeditions to North Africa (biodeserts.cibio.up.pt/expeditions), and from samples ceded by the French National Museum of Natural History in Paris (*Muséum national d'Histoire naturelle*) were added to the dataset.

Extent of occurrence of each species was created according to the IUCN Red List standards for range maps. All georeferenced distribution points were plotted onto the WGS 84 datum using ArcGIS (ESRI, 2011) and using the Minimum Bounding Geometry tool within ArcGIS (ESRI, 2011) a Minimum Convex Polygon (MCP) or Convex hull was created. Resulting polygons were then manually edited in ArcGIS (ESRI, 2011) to remove non-terrestrial areas, oceanic islands, and terrestrial regions of known absence.

2.3. Genetic Analyses

2.3.1. Sampling

In this study a total of 88 biological samples were used. Of those, 78 were collected by researchers and collaborators of the CIBIO-InBIO's research group BIODESERTS during multiple field expeditions to North Africa from 2004 to 2016. All samples were assigned to previously established taxonomical units based on morphological characteristics and all geographic coordinates were recorded in the field with a GPS under the WGS 84 coordinate reference system. Of all biological samples, 34 were obtained from live animals captured in the field by toe or tail clipping, while the remaining 44 were collected from individuals found dead, either killed by locals, ran-over, or from unknown causes. The remaining 10 samples

and their respective location of capture were gently provided by the French National Museum of Natural History in Paris. Another European museum was contacted but they chose not to help in this research. All samples were stored in 2 ml tubes filled with 100% ethanol to preserve the DNA. All biological samples originated from seven countries (Figure 10), as discriminated in Table 1.

Table 1. Country of origin of available samples of Varanus lizards.

	Cameroon	CAR*	Mali	Mauritania	Morocco	Niger	Senegal
V. griseus	–	–	–	17	10	3	–
V. exanthematicus	7	3	1	18	–	1	2
V. niloticus	–	–	1	23	–	–	2

*Central African Republic

2.3.2. Genomic DNA Extraction

DNA extractions were performed in the CTM's (Centre for Molecular Analysis, in Portuguese *Centro de Testagem Molecular*) facilities. Total DNA was extracted under sterile conditions following two different protocols. All samples were first processed using the EasySpin kit from Citomed as follows.

In a clean and sterile glass surface, 30 mg of every sample were cut one at a time with sterile scissors and scalpel and put in a 1.5 mL tube. Then, 300 µL of ACL Solution and 20 µL of Proteinase K were added to each tube. All tubes were then incubated at 55 ºC with agitation overnight. After digestion the tubes were vortexed and centrifuged. Then, 300 µL of supernatant from each tube was transferred to the columns and 300 µL of AB solution was added. After incubation at room temperature, columns were centrifuged and all flow through was discarded. Columns membranes were cleaned twice by centrifuging with 500 µL of wash solution, all flow through was discarded. Each sample was eluted twice with 50 µL of elution buffer each time. Both elutions were stored in different tubes.

Samples that showed no DNA in the agarose gel electrophoresis after PCR amplification (2.3.3. Genetic Marker Amplification) were re-extracted using Rohland & Hofreiter (2007) protocol that was created specifically for samples with old and highly degraded DNA, and for tissues with small amounts of DNA such as bone samples. The entire process of the extraction was performed in a clean and sterile room that is entirely dedicated to difficult and non-invasive DNA extractions. All material and reagents were also sterilized. Samples were washed in a PBS solution overnight to clean any leftover ethanol, and hypothetical contaminants like foreign DNA. Samples were cut into small fragments and 50 to 150 mg of each sample was added to a 2.0 mL tube containing extraction buffer (ultrapure water, 0.5 M EDTA, Tween 20 and Proteinase k (New England Biolabs)).

Digestion occurred overnight at 37 ºC. Along with the samples, a negative control was also added to test for any contaminations in the reagents. After digestion samples were centrifuged and the supernatant was transferred to a 50 mL tube containing binding buffer (ultrapure water, isopropanol, tween 20 and guanidine hydrochloride) and sodium acetate. Volume extensions were added to the columns, so the entire mixture could be transferred to the columns. Samples were centrifuged twice inverting the tubes between both centrifugations until all liquid had pass through the column, all flow through was discarded. PE Buffer (Qiagen) was added and centrifuged twice to clean the membrane of the column. Next, DNA was eluted twice with 25µL of TET buffer to a 1.5mL low retention tube. Both elutions were transferred to the same tube. A new elution with 50µL of TET buffer was performed to a different tube.

The quality and quantity of extracted DNA were assessed by agarose gel electrophoresis. Each sample of 2 µL of extracted DNA was mixed with 3 µL loading dye and analysed in a 0.8 % (w/v) agarose gel in TBE - (89 mM Tris, pH 8, 89 mM boric acid, 2 mM EDTA) prestained with GelRed (Biotarget) for DNA visualization. The electrophoresis was performed at 300 V in 0.5x TBE. Gels were visualized in a BioRad Universal Hood II Quantity One 4.4.0.

2.3.3.PCR Amplification

Mitochondrial DNA (mtDNA) has many advantages for phylogenetic studies at shallow levels. It shows maternal inheritance, lack of recombination, abundant presence in cells, low effective population size compared to nuclear DNA (nDNA) and high mutation rate (De Mandal et al., 2014). Considering that altogether mtDNA is more able to resolve relationships among recently diverged species and lineages a fragment of 932 bp (expected size) of the mitochondrial genome, including a partial NADH dehydrogenase subunit 4 (ND4) gene and its adjacent tRNA-His and tRNA-Ser genes and partial sequence of tRNA-Leu gene (hereafter referred as ND4) was amplified using the primers VarND4F and VarND4RLeu (Table 2). ND4 was chosen because it is considered a genetic marker of good quality for phylogenetic analysis of closely related species (De Mandal et al., 2014) and has been used with success, being more informative than other mtDNA markers, to study many species, including reptiles like snakes (e.g. Figueroa et al., 2016; Martínez-Freiría et al., 2017; Maddock et al., 2017; Kindler et al., 2017; Portillo et al., 2018), and lizards (e.g. Freitas et al., 2016; Díaz-Cárdenas et al., 2017; van den Burg et al., 2018), including *Varanus spp.* (e.g. Dowell et al., 2015a; Lin & Wiens, 2016).

Considering mtDNA phylogenies limitation to recent divergences and that a phylogeny of a single locus tells the evolution of a gene and not the species, analysis of multiple unlinked loci with different mutation rates is important to properly understand historic populations dynamics. This was exemplified by Godinho et al., (2008) using *Lacerta schreiberi* as species model to compare observed patterns of mitochondrial DNA analysis with nuclear DNA. Therefore, to complement the analyses, three protein coding genetic markers of the nuclear DNA (nDNA) were chosen based on published results with *Varanus spp.* and other reptiles at the inter- and intraspecific level: KIAA1549, already used with *Varanus spp.* by Dowell et al. (2015a); Prolactin Receptor (PRLR), used with snakes at the inter- (Maddock et al., 2017) and intraspecific (Freitas et al., 2018) level; and Recombination-activating gene 1 (RAG1), used in many studies of reptiles, including *Varanus spp.* (Portik & Papenfuss, 2012; Dowell et al., 2015a; Lin & Wiens, 2016). A segment of the KIAA1549 gene, with an expected size of 781 bp was amplified using the pair of primers VarKIAA1549F and VarKIAA1549R. A segment of 541 bp (expected size) of PRLR was amplified using the primer forward PRLR.F1 and the primer reverse PRLR.R3. And lastly, a fragment with an expected size of 980 bp of RAG1 was amplified using the primers Amp-RAG1 F and Amp-RAG1 R1. The primers used, and their respective sequences and references are depicted in Table 2.

Table 2. List of primer pairs used in amplification and sequencing of genetic markers.

Genetic Marker	Primer Name	Primer Sequence (5'-3')	Reference
ND4	VarND4F	TGACTACCAAAAGCCCATGTAGAAGC	Arèvalo et al., 1994
	VarND4RLeu	TGCTTTTACTTGGAGTTGCACCA	Arèvalo et al., 1994
KIAA1549	VarKIAA1549F	RTAACAAGYAATGAGGCAKTCTTAA	Portik et al., 2012
	VarKIAA1549R	RTATGRTCTMGTGAAAGGCACTG	Portik et al., 2012
PRLR	PRLR.F1	GACARYGARGACCAGCAACTRATGCC	Townsend et al., 2008
	PRLR.R3	GACYTTGTGRACTTCYACRTAATCCAT	Townsend et al., 2008
RAG1	Amp-RAG1 F	AGCTGCAGYCARTACCAYAARATGTA	San Mauro et al., 2004
	Amp-RAG1 R1	AACTCAGCTGCATTKCCAATRTCACA	San Mauro et al., 2004

For each selected sample, the amplification process of each genetic marker was prepared with 5.0 µL of an enzyme mix (2 X MyTaq Mix from Bioline for the ND4 and RAG1 genetic markers and MasterMix from Qiagen for KIAA1549 and PRLR), 0.4 µL of each primer (0.4 µM final concentration of each primer), 1.0 µL of extracted DNA, and ultra-pure water for a final volume of 10 µL. PCR amplification programs were optimized for each marker in a thermocycler (T100™ Thermal Cycler by BIO-RAD) with PRLR genetic marker surprisingly requiring a different program for each species (Table 3).

The results of each PCR were tested for contaminations using negative controls and analysed by electrophoresis of 2% agarose gels in the same conditions as described above (2.3.2. Genomic DNA Extraction). Amplified DNA was also quantified in agarose gel electrophoresis and diluted accordingly for sequencing. NZYDNA Ladder V from NZYTech was used as molecular marker.

Table 3. Touchdown PCR programmes used for the amplification of ND4, KIAA1549, PRLR and RAG1. PRLR required a different programme per species. Number of cycles, duration in minutes (m) or seconds (s), and temperature (ºC) is shown for the Denaturation, Primer Annealing and Extension steps of each PCR.

Genetic Marker	Cycle	Denaturation	Primer Annealing	Extension
ND4	Start	95 ºC for 10 min	–	–
	1 - 9	95 ºC for 30 s	62ºC for 30 s (-0.5ºC/cycle)	72 ºC for 1 min
	10 - 40	95 ºC for 30 s	58ºC for 30 s	72 ºC for 1 min
	End	–	–	72 ºC for 10 min
KIAA1549	Start	95 ºC for 15 min	–	–
	1 - 9	95 ºC for 30 s	62ºC for 20 s (-0.5ºC/cycle)	72 ºC for 1 min
	10 - 40	95 ºC for 30 s	58ºC for 20 s	72 ºC for 1 min
	End	–	–	60 ºC for 10 min
PRLR (for *V. griseus*)	Start	95 ºC for 15 min	–	–
	1 - 9	95 ºC for 30 s	62ºC for 30 s (-1ºC/cycle)	72 ºC for 25 s
	10 - 40	95 ºC for 30 s	54ºC for 30 s	72 ºC for 25 s
	End	–	–	60 ºC for 10 min
PRLR (for *V. exanthematicus*)	Start	95 ºC for 15 min	–	–
	1 - 8	95 º C for 30 s	62ºC for 30 s (-1ºC/cycle)	72 ºC for 25 s
	9 - 40	95 ºC for 30 s	55ºC for 30 s	72 ºC for 25 s
	End	–	–	60 ºC for 10 min
PRLR (for *V. niloticus*)	Start	95 ºC for 15 min	–	–
	1 - 7	95 ºC for 30 s	62ºC for 30 s (-1ºC/cycle)	72 ºC for 25 s
	8 - 40	95 ºC for 30 s	56ºC for 30 s	72 ºC for 25 s
	End	–	–	60 ºC for 10 min
RAG1	Start	95 ºC for 10 min	–	–
	1 - 9	95 ºC for 30 s	62ºC for 25 s (-1ºC/cycle)	72 ºC for 1 min
	10 - 40	95 ºC for 30 s	54ºC for 25 s	72 ºC for 1 min
	End	–	–	72 ºC for 10 min

2.3.4. Sequencing

Of the 88 samples, all successful amplifications of ND4 and KIAA1549 and a selection (based on the mtDNA results) of 14 successfully amplified samples of PRLR and RAG1 were sequenced. Sequencing was performed using only the primer forward of each genetic marker (Table 2) and it occurred in two batches, one in CTM facilities and the other one was outsourced to GENEWIZ laboratory in London, United Kingdom. At CTM, sequencing

started by purifying PCR products enzymatically by removing primers and nucleotides that were not incorporated in the new DNA strands with ExoSAP (USB® ExoSAPIT® PCR Product Cleanup, Affymetrix) following manufacturer's instructions, with 15 min at 37°C followed by 15 min at 85°C. Sequencing reactions were prepared following the protocol from Big-Dye cycle sequencing kit (BigDye® Terminator v3.1 Cycle Sequencing Kits, AB Applied Biosystem), using 0.4 µL of BigDye, 0.5 µL of the respective primer forward, 1 µL of sequencing buffer (TRR) and 1 µL of purified PCR product for a final volume of 10 µL. The products were then cleaned of dNTPs (deoxynucleotide triphosphates) and other reagents using Sephadex, following manufacturer's protocol. Sequencing by the Sanger's method was performed in an ABI 3130xl Genetic Analyser (AB Applied Biosystems).

2.3.5. Data Processing and Phylogenetic Analyses

All chromatograms were manually checked, edited, and aligned in SeqScape Software v3.0 (Applied Biosystems). Heterozygous positions in nuclear sequences were coded using the IUPAC nucleotide ambiguity code, a Basic Local Alignment Search Tool (BLAST) was performed on NCBI to sequences that were visibly different to check for alien DNA. The alignments were then transferred to MEGA v7 (Kumar et al. 2016) and translated to amino acids to check for stop codons.

Available sequences in NCBI GenBank for the target markers and species were added to the alignments. Sixteen sequences added to the ND4 alignment: fourteen *V. niloticus* (access codes: KT720864.1, KT720866.1, KT720890.1, KT720892.1, KT720894.1, KT720904.1, KT720905.1, KT720909.1, KT720912.1, KT720946.1, KT720949.1, KT720950.1, KT720955.1 and KT720957.1) and one *V. exanthematicus* (access code: KT720982.1) from Dowell et al. (2015a), and one *V. salvator* (access code: AY033776.1) from Huebinger et al. (unpublished). Four sequences to the KIAA1549 alignment: three *V. niloticus* (access codes: KT721198.1, KT721200.1 and KT721217.1) and one *V. exanthematicus* (access code: KT721289.1) published by Dowell et al. (2015a).

Alignments were trimmed to maximize the number of sequences for the following analyses while trying not to cut them too short in order to keep relevant polymorphisms. Sequences too short were removed from the alignment, this resulted in 74 sequences of ND4 trimmed to 627 bp, 36 sequences of KIAA1549 trimmed to 249 bp, 11 sequences of PRLR trimmed to 157 bp, and 12 sequences of RAG1 trimmed to 812 bp.

Bayesian inference was employed to construct a mtDNA phylogenetic tree using BEAST v1.8.3 software (Drummond et al., 2012). The substitution model Hasegawa-Kishino-Yano 1985 with the site heterogeneity model of Gamma (HKY+G) was selected as the best nucleotide model based on Bayesian Information Criterion (BIC) previously

calculated on MEGA v7 (Kumar et al. 2016). Two independent runs were performed, one assuming a lognormal mutation rate (Uncorrelated Relaxed Clock with Lognormal Relaxed Distribution) and other assuming a constant mutation rate (Strict Clock of mutation). Both runs had Speciation by the Yule Process as the prior shared by all tree models and were performed with a total of 10 million generations using the Markov chain Monte Carlo (MCMC) algorithm, sampling trees and parameters every one thousand generations, and discarding 10% of the trees as burn-in (first 1000 trees). Quality of each run was accessed by observation of the posterior trace plots and Effective Sample Sizes (ESS) of all parameters using Tracer v1.6.0 (http://tree.bio.ed.ac.uk/software/tracer). A final consensus tree with the maximum clade credibility of all remaining trees and Bayesian posterior probabilities (BPP) for each node was obtained using TreeAnnotator v1.8.3 (Drummond et al., 2012). This was only done for the run with Strict Clock of mutation based on Tracer v1.6.0 (http://tree.bio.ed.ac.uk/software/tracer) results. The resulting phylogenetic tree was visualized and edited (rooted with the sequence from *V. salvator* access code: AY033776.1)) with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree). Only nodes with a posterior probability of 95% or above were considered to be strongly supported (BPP ≥ 0.95). Therefore, all nodes with a posterior probability below that value were collapsed and resulting lineages were here considered as subclades. Based on the literature, a threshold of 4.5% uncorrected p-distance was used to define clades of the phylogram. To achieve this, uncorrected p-distances between samples were calculated using MEGA v7 (Kumar et al. 2016) and monophyletic lineages were considered to belong to the same clade if divergence between each other showed less than 4.5% uncorrected p-distance.

Each nDNA alignment (KIAA1549, PRLR, RAG1) was loaded to DnaSP v5 (Librado & Rozas, 2009) where sequences were unphased to account for the variability in the heterozygote positions. Resulting phased alignments were individually loaded to TCS v1.2.1 software (Clement et al. 2000) to generate a haplotype network of each nuclear genetic marker using statistical parsimony and considering a connection limit of 95%. Resulting networks were mapped against the previously obtained mtDNA clades on TcsBU (Múrias dos Santos et al., 2015).

2.4. Ecological Analyses

Seven climatic variables were downloaded from the Worldclim database available at www.worldclim.org (Hijmans et al., 2005; Trabucco & Zomer, 2009) with a spatial resolution of 2.5-arc seconds and one topographic variable, a Digital Elevation Model downloaded from the Shuttle Radar Topography Mission database available at lta.cr.usgs.gov/SRTM (USGS, 2000) that was used to derive a Terrain Ruggedness Index using the ArcGIS (ESRI,

2011) tool Topographic Ruggedness Index (Riley et al., 1999). After data standardization of all nine topoclimatic variables, a Spatial Principal Components Analysis (SPCA) of the African continent was performed using the Principal Components tool of ArcGIS (ESRI, 2011) to summarize the data into independent Principal Components (PC). The variables used in the SPCA were Annual Mean Temperature (ºC), Annual Precipitation (mm), Precipitation of the Wettest Month (mm), Maximum Temperature of Warmest Month (ºC), Minimum Temperature of the Coldest Month (ºC), Temperature Annual Range (ºC), Potential Evapo-Transpiration (mm), Altitude (m), and Terrain Ruggedness index. The three PCs that accounted for most of the variability (highest eigenvalues) were used to create a composite map. Distribution points (collected in 2.2. Geographic Distribution) were then plotted onto the composite map and using the Extract Multi Values to Points tool from ArcGIS (ESRI, 2011) values of each PC were retrieved for each point that was within the study area. Each species and retrieved species clades were compared against each other along the three axes of the SPCA (PC1, PC2, and PC3). Points that had no molecular data were assumed to belong to the same clade as the nearest point that had been previously associated to a clade after the phylogenetic analyses. However, when no clear association could be made, like points that were nearby multiple clades, said points were not considered for the comparison.

3. Results

3.1. Geographic Distribution

The revision of published data resulted in a total of 1056 curated points that together with observational points collected by CIBIO-InBio's group BIODESERTS, totalled 1358 points of occurrence for the three species. The resulting 489 points of *Varanus griseus*, 245 points of *V. exanthematicus*, and 624 points of *V. niloticus* were used to create MCPs of the extent of occurrence of each species. All resulting polygons are in conformity with the IUCN Red List norms and had an area of 21,137,090 km² for *V. griseus* (Figure 11), 6,799,659 km² for *V. exanthematicus* (Figure 12), and 16,534,229 km² for *V. niloticus* (Figure 13).

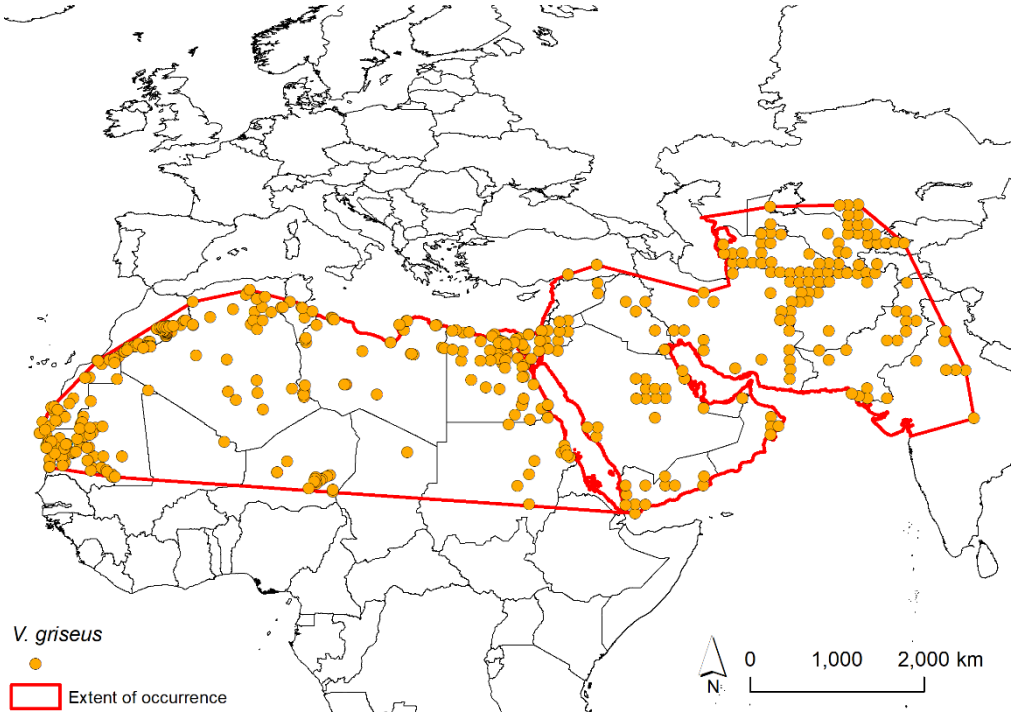

Figure 11. Verified points of occurrence of *Varanus griseus* (Orange circles) and its extent of occurrence (Red polygon).

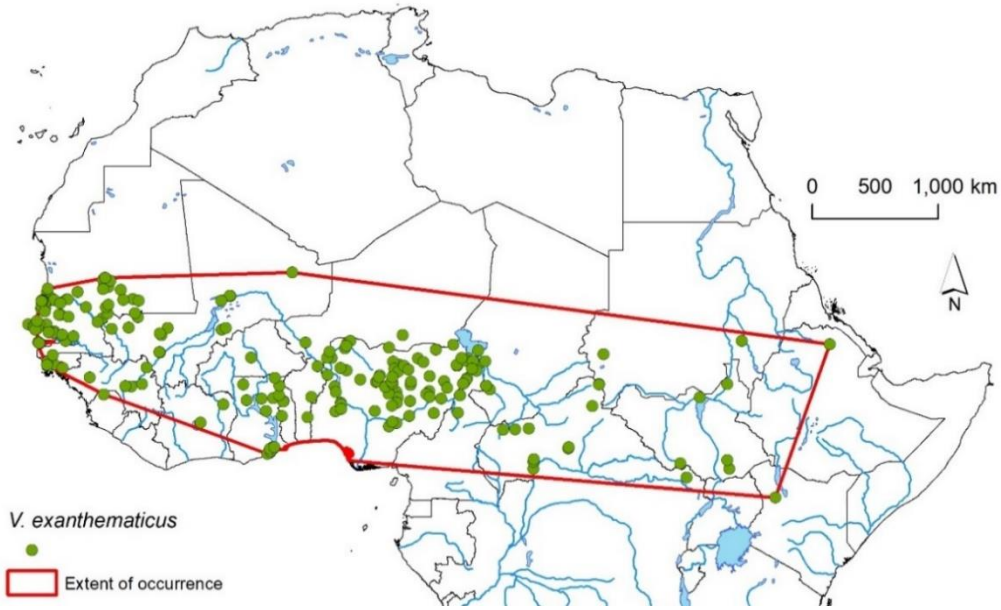

Figure 12. Verified points of occurrence of *Varanus exanthematicus* (Green circles) and its extent of occurrence (Red polygon).

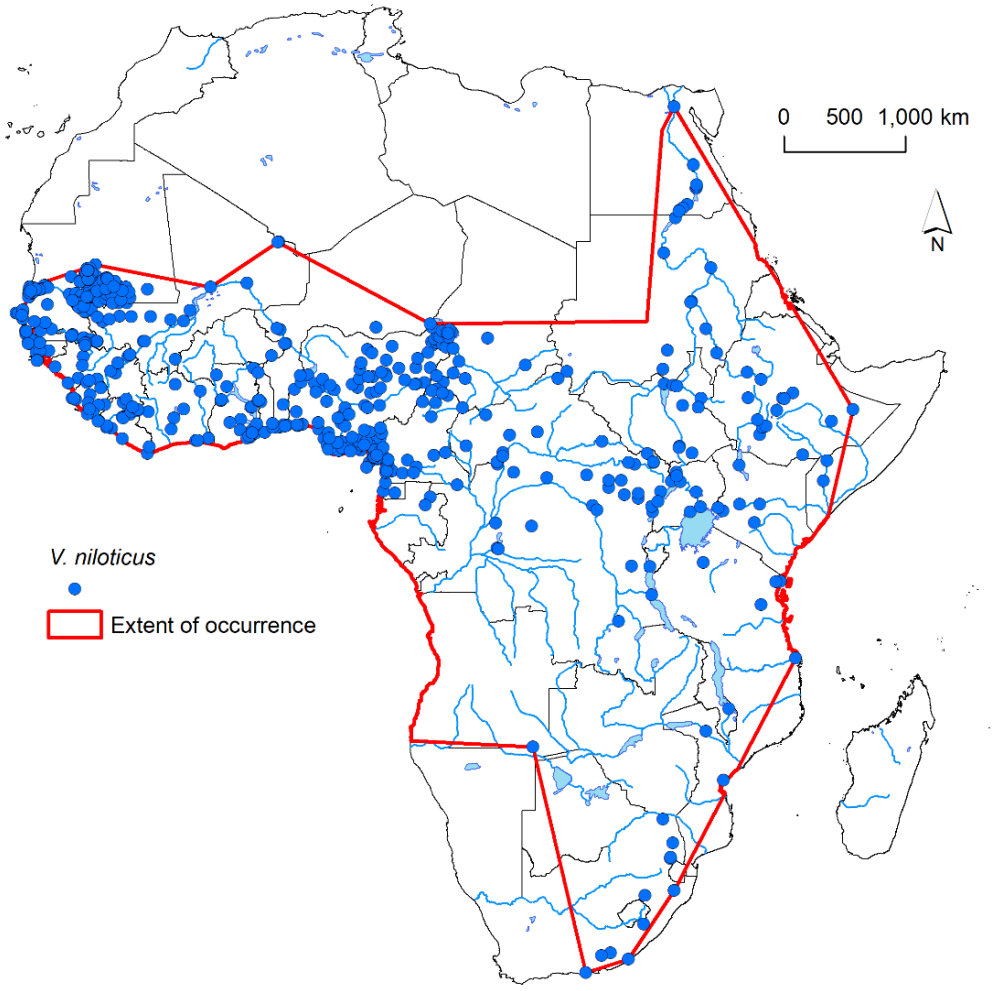

Figure 13. Verified points of occurrence of *Varanus niloticus* (Blue circles) and its extent of occurrence (Red polygon).

3.2. Genetic Analyses

Genomic DNA extraction was performed for all the 88 biological samples. All extractions were used to amplify the ND4 marker, which resulted in 76 amplifications with no signs of contaminations, as observed in the agarose gel. All successful amplifications were then sequenced which resulted in sequences of varying length, with a maximum length of 820 base pairs (bp). BLAST results of the four successfully amplified museum samples revealed PCR contaminations (ND4 gene of *Homo sapiens*). All sequences that were contaminated or too short were removed resulting in 58 sequences. Sequences from NCBI GenBank were added to the alignment which was trimmed to 627 bp (62.7% of the expected size). After adding the sequences available in NCBI GenBank, a final alignment of 74 sequences was used for downstream analysis.

The test for nucleotide substitution and site heterogeneity models of the ND4 alignment returned the model HKY+G, which was used as priors for the phylogenetic analysis. Parameter convergence was achieved with the Strict Clock model (ESS of all parameters well above 200), but not with the Lognormal Clock model (ESS of all parameters below 100), and thus this run was discarded. Obtained consensus phylogram resulted in 18 lineages (excluding outgroup) with a posterior probability equal or higher than 95% on each node (Figure 14) (unedited phylogram can be seen in Annex 2 Figure A1). All lineages with a posterior probability below the 95% threshold were collapsed into one lineage. Lineages were classified as subclades and named after each species. The first divergent subclades belonged to *V. griseus* and were named G1, G2, and G3, and the last two species diverged into five subclades of *V. exanthematicus*, named from E1 to E5, and ten subclades of *V. niloticus*, named from N1 to N10.

Sequences were also grouped together if they had an uncorrected pairwise p-distance score of less than 4.5% between one another. Retrieved groups were classified as clades and included one clade of *V. griseus*, two clades of *V. exanthematicus*, and three clades of *V. niloticus*. Among species, mtDNA differences ranged from 19.5% to 26.3%, and between clades of the same species, *V. exanthematicus* clades differed in 10.5% and *V. niloticus* first diverging clade differed in 10.7% of the other two clades that had 4.8% differences between each other (Table 4). Clades were named after each species and their general geographical distribution. Since *V. griseus* exhibited only one clade, it was named solely after the species. However, the clades in *V. exanthematicus* were longitudinally distributed and were named *V. exanthematicus* East and *V. exanthematicus* West. Samples from the West clade were from Mauritania, Mali, and Senegal, while the East clade contained samples from Ghana and Niger (Figure 15). Retrieved clades of *V. niloticus* were exactly the same as previously described by Dowell et al. (2015a) and were named accordingly.

The first diverging clade was *V. niloticus* West and is present in West Africa, from Mauritania and Senegal to the Cameroon. While the last two diverging clades (*V. niloticus* North and *V. niloticus* South) show a latitudinal distribution with the division roughly around northern Cameroon, northern Democratic Republic of Congo and Kenya. However, the North and South clades extend westwards to Mali and Ivory Coast, respectively (Figure 16).

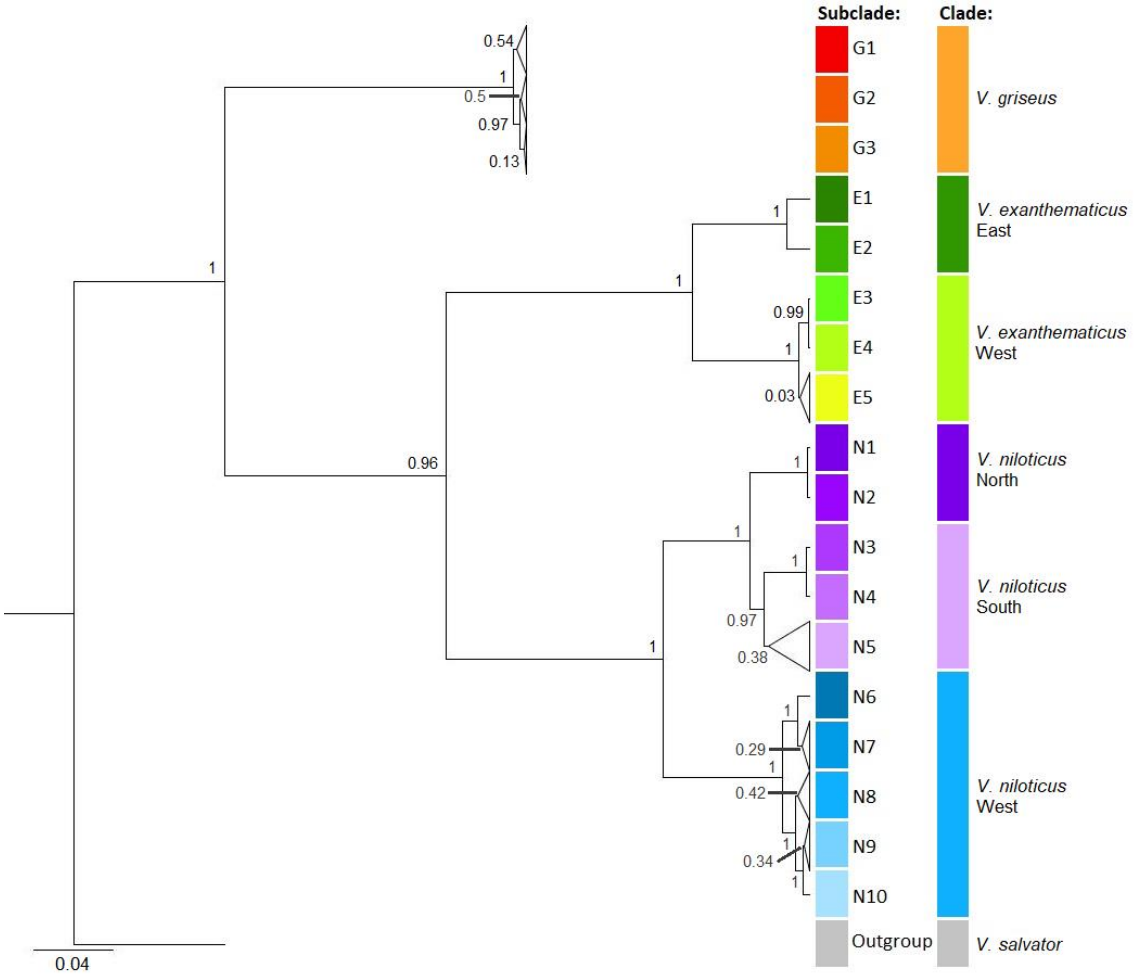

Figure 14. Bayesian phylogram of *Varanus griseus*, *V. exanthematicus*, and *V. niloticus* obtained from the ND4 mitochondrial marker rooted in *V. salvator*. Bayesian posterior probability (BPP) of each node is depicted next to the respective node, and nodes with a BPP < 0.95 were collapsed. Scale bar denotes 0.04 substitutions per site and coloured bars denote subclades and clades.

Table 4. Uncorrected proportion of nucleotide differences (p-distance) between all retrieved mtDNA clades of *Varanus griseus*, *V. exanthematicus* and *V. niloticus* within Northern Africa, plus *V. salvator* outgroup

	Uncorrected p-Distance	1	2	3	4	5	6	7
1	*V. griseus*							
2	*V. exanthematicus* East	0.243						
3	*V. exanthematicus* West	0.225	0.105					
4	*V. niloticus* North	0.206	0.203	0.203				
5	*V. niloticus* South	0.208	0.211	0.211	0.048			
6	*V. niloticus* West	0.213	0.212	0.196	0.099	0.109		
7	*V. salvator* - Outgroup	0.195	0.257	0.263	0.226	0.232	0.239	

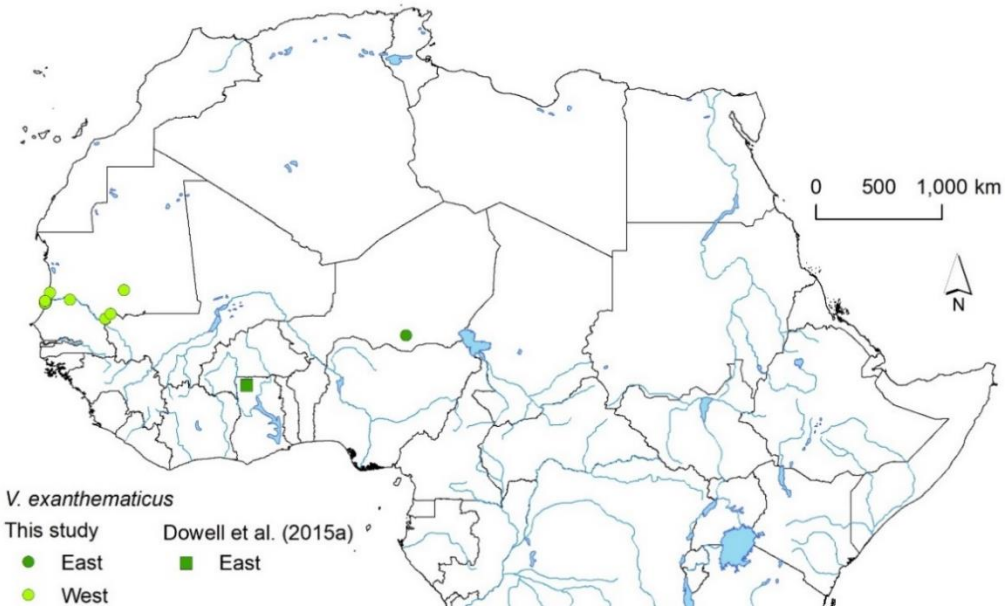

Figure 15. Distribution of individuals belonging to each clade of *Varanus exanthematicus*. Circles represent new sequences from this study and squares represent sequences obtained by Dowell et al. (2015a).

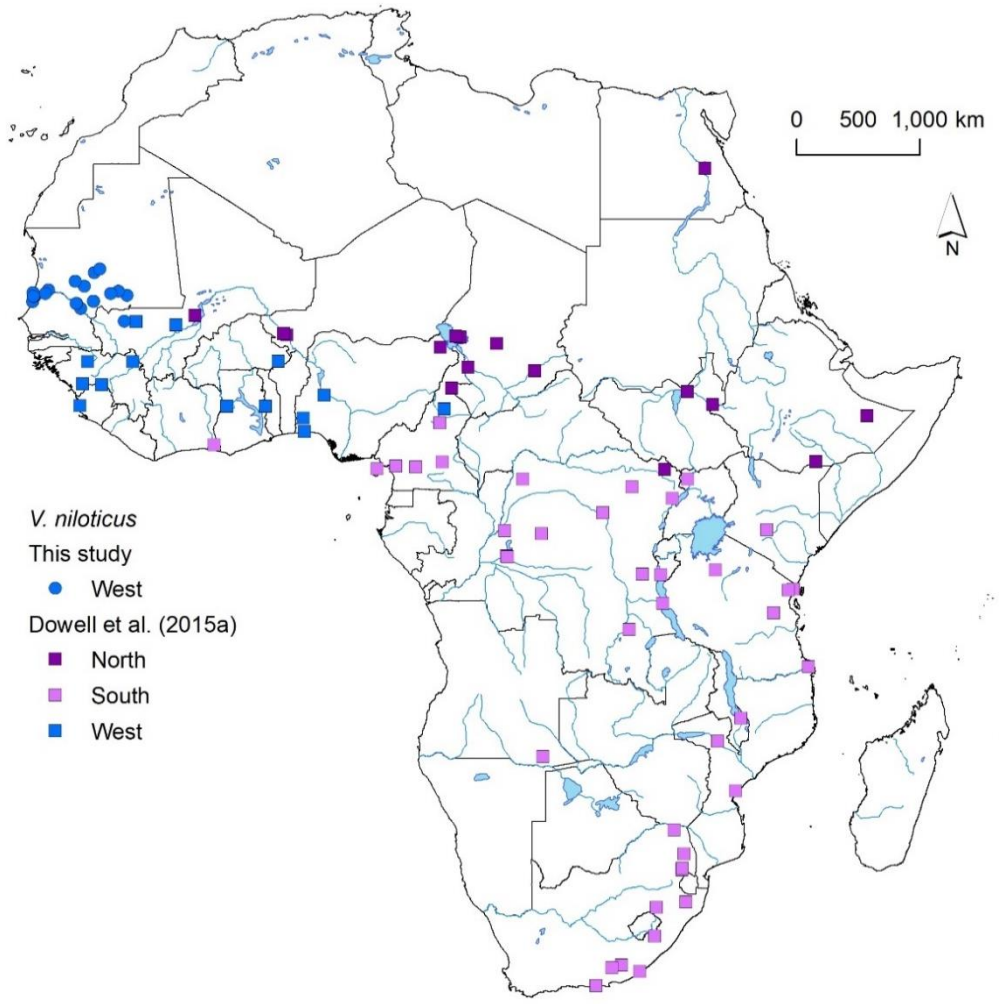

Figure 16. Distribution of individuals belonging to each clade of *Varanus niloticus*. Circles represent new sequences from this study and squares represent sequences obtained by Dowell et al. (2015a).

All genomic DNA extractions were also used to amplify all three nuclear markers (KIAA1549, PRLR, and RAG1), resulting in a tota of 49, 42, and 43 successfully amplified samples for KIAA1549, PRLR, and RAG1 respectively, with no detectable contaminations in the agarose gel. Amplification of the KIAA1549 genetic marker resulted in two bands of similar size (around 800 bp) visible in the agarose gel for the *V. exanthematicus* West clade (Figure 17). Such patter was not observed in any other clade for this or other genetic. Sequencing of KIAA1549 resulted in sequences of varying size, with the maximum being of approximately 750 bp. However, all sequences from the *V. exanthematicus* West clade had a discernible length of approximately 250 bp due to background noise by the end of the sequence. Final alignment resulted in 36 sequences of 249 bp (approximately 32% of the expected size) including GenBank sequences. PRLR sequences had a maximum size of approximately 490 bp. However, the only available sequence of the *V. exanthematicus* East clade had a length of 157 bp due to background noise making it difficult to properly visualize it. Because of this final alignment resulted in 11 sequences of 157 bp (approximately 29% of the expected size). RAG1 sequencing resulted in sequences with maximum size reaching approximately 900 bp. Alignment was trimmed to 812 bp (approximately 83% of the expected size) to maximize number of sequences in the alignment (12 in total) and minimize missing data.

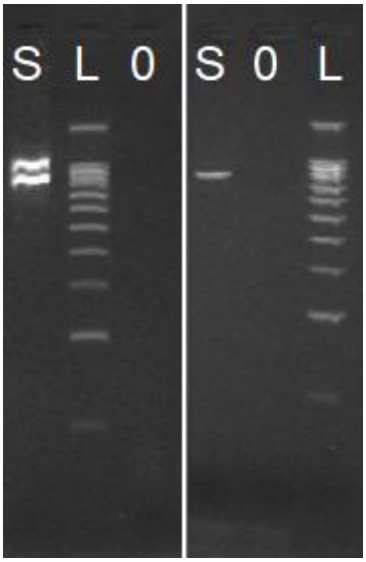

Figure 17. Side by side comparison of the results of the amplification of the KIAA1549 genetic marker in the *Varanus exanthematicus* West (Left) and *V. exanthematicus* East (Right) clades in agarose gel (2%) after electrophoresis. Molecular marker used was NZYDNA Ladder V (NZYTech). Amplified samples are marked with an “S”, molecular marker with an “L”, and negative control with a “0”.

TCS haplotype network for KIAA1549 resulted in a network of three clusters, each of them belonging to a species. Clusters were separated by tree hypothetical haplotypes. While *V. griseus* was composed by only one haplotype, *V. niloticus* and *V. exanthematicus*

were composed by three haplotypes each. Both clades within *V. exanthematicus* had sequences belonging to the major haplotype while the two minor haplotypes had sequences only from *V. exanthematicus* West. For *V. niloticus*, the major haplotype was composed by sequences from both the *V. niloticus* West clade and from the *V. niloticus* South clade while the *V. niloticus* North clade comprised of a single haplotype separated by one polymorphism from the major haplotype. For PRLR, TCS returned a haplotype network with only three haplotypes with each one belonging to a different species with no distinction between clades. RAG1 haplotype network resulted in three haplogroups completely separated at the 95% threshold. Each haplogroup belongs to a species with *V. griseus* having one single haplotype, while *V. exanthematicus* had five haplotypes, three belonging entirely to the clade *V. exanthematicus* West and the other two to the *V. exanthematicus* East. The *V. niloticus* haplogroup was composed of three haplotypes, all belonging to the only available clade (*V. niloticus* West) as there were no available sequences of this region of the RAG1 gene for the two other clades. Haplotype networks are depicted in Figure 18.

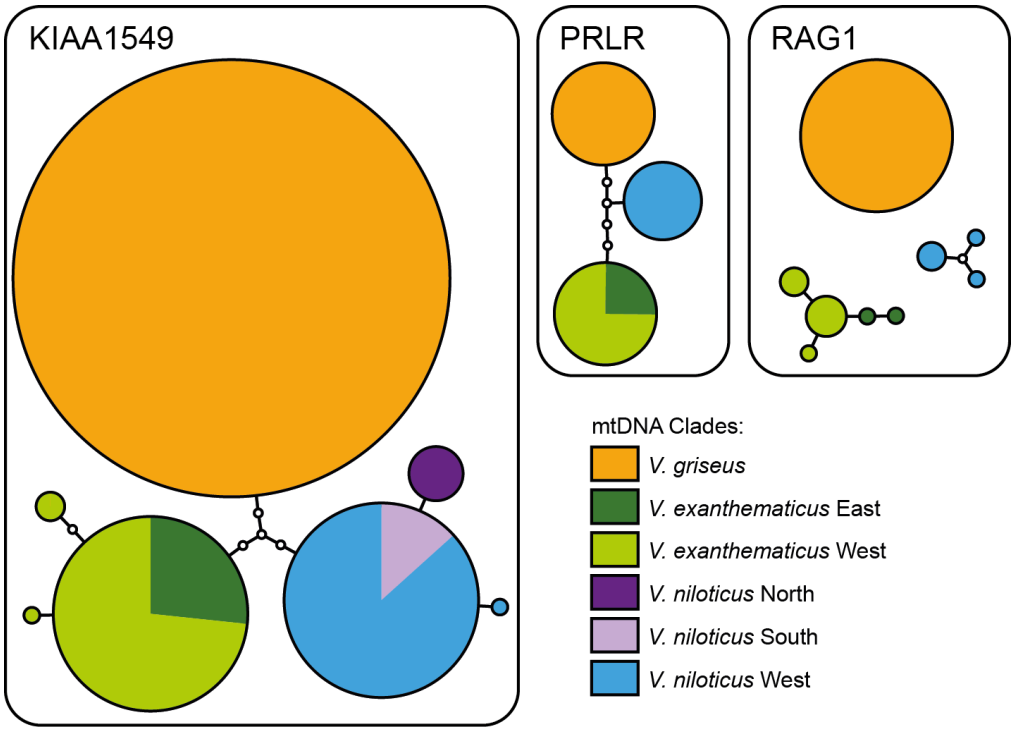

Figure 18. TCS haplotype networks for KIAA1549, PRLR, and RAG1 genetic markers, mapped against the available retrieved mtDNA clades of *Varanus griseus*, *V. exanthematicus* and *V. niloticus*. White circles represent hypothetical haplotypes. Circle size is proportional to the number of sequences that share the same haplotype.

3.3. Ecological Analyses

Spatial Principal Components Analysis (SPCA) of the nine topoclimatic variables for the African continent resulted in nine independent Principle Components (PCs) (Table 5).

The principal component that accounted most of the variation (PC1) was mostly explained by annual precipitation, precipitation of the wettest month and temperature annual range and explained 44% of the total variation. The second most important principal component (PC2) accounted for 33.4% of the total variation and was explained mostly by altitude, annual mean temperature and minimum temperature of coldest month. The third principal component (PC3) represented 9.4% of the total variation and was mainly explained by the terrain ruggedness index. These three principal components accounted for 86.8% of the total variation and were used to generate a composite map of the African continent (Figure 19).

Each presence point was located within the three axes of the SPCA (PC1, PC2, and PC3) and species and clades within species were compared against each. Comparison between the three species along all axes (PC1 vs PC2, PC1 vs PC3, and PC2 vs PC3) resulted in points all clustered together with a slight tendency for *V. griseus* to be more separated from the other two (Figures 20, 21, and 22). No differences were visible in the comparison of inferred clades of each species along the three axes (PC1 vs PC2, PC1 vs PC3, and PC2 vs PC3) as both *V. exanthematicus* clades (Figures 23, 24, and 25) and *V. niloticus* clades (Figures 26, 27, and 28) were clustered together.

Table 5. Loading scores and variance (percentage and cumulative) explained by each component extracted according to the Spatial Principal Components Analysis of nine topoclimatic variables from Africa.

Variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**
Altitude	-0.139	0.481	0.023	-0.432	0.732	0.156	-0.042	0.028	0.000
Annual Mean Temperature	0.049	-0.571	-0.227	-0.177	0.310	0.040	0.100	-0.694	-0.003
Annual Precipitation	-0.489	-0.062	0.097	-0.319	-0.306	0.129	-0.713	-0.165	-0.001
Precipitation of Wettest Month	-0.466	-0.102	0.059	-0.516	-0.270	-0.120	0.632	0.127	0.001
Max Temperature of Warmest Month	0.395	-0.332	-0.229	-0.421	-0.021	0.222	-0.144	0.465	0.466
Min Temperature of Coldest Month	-0.311	-0.459	-0.147	0.191	0.309	0.130	-0.074	0.492	-0.523
Temperature Annual Range (P5-P6)	0.487	0.122	-0.042	-0.415	-0.242	0.050	-0.039	-0.054	-0.713
Potential evapo-transpiration	0.053	-0.084	-0.052	-0.154	0.157	-0.935	-0.229	0.112	0.000
Terrain Ruggedness Index	-0.170	0.293	-0.925	0.068	-0.149	-0.025	0.002	-0.036	0.000
% variation	44.0	33.4	9.4	6.3	4.7	1.0	0.9	0.3	0.0
cumulative	44.0	77.4	86.8	93.1	97.8	98.8	99.7	100.0	100.0

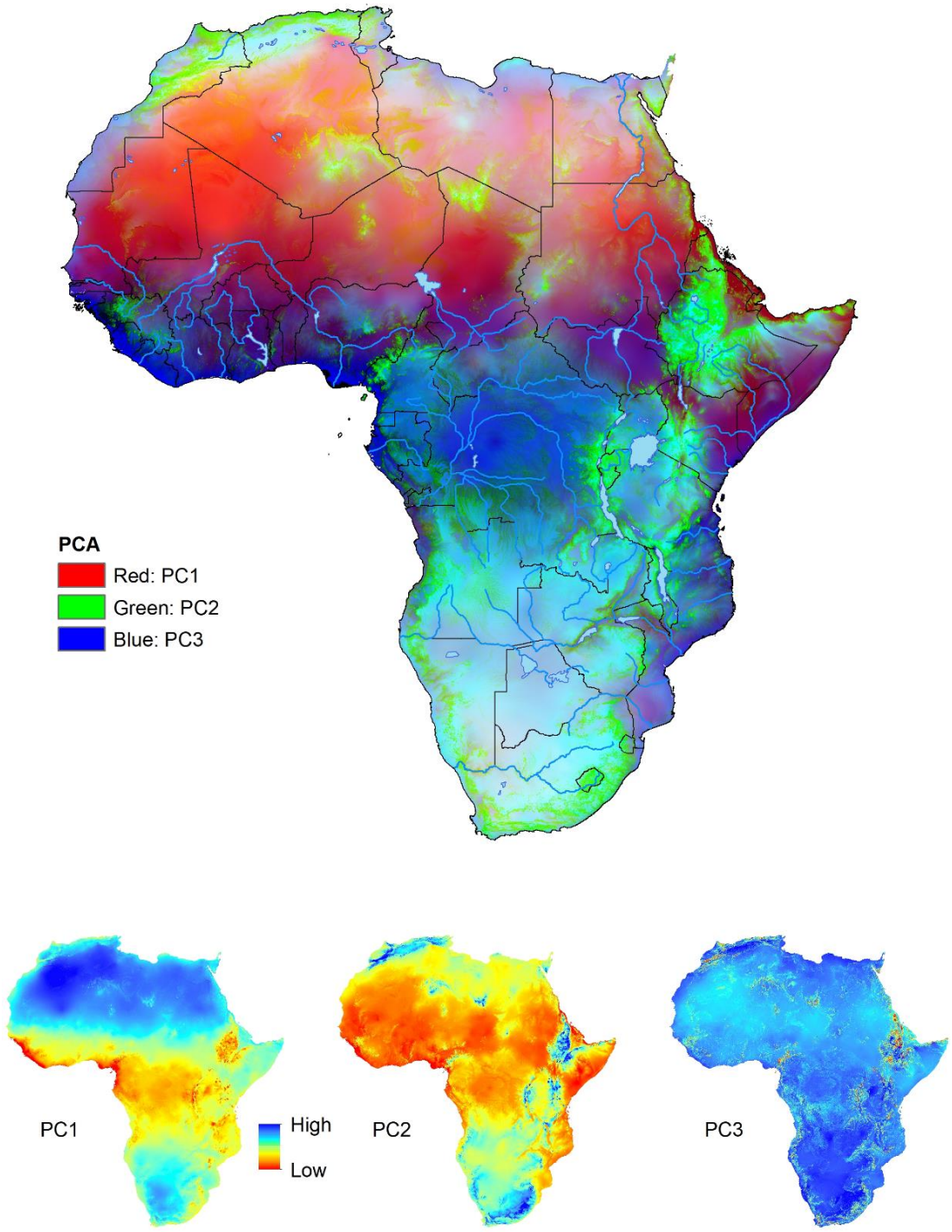

Figure 19. Composite map of the environmental variability in the African continent (Top) derived by Spatial Principal Components Analysis where each colour represents one of the first three Principal Components (PC1 in Red, PC2 in Green, and PC3 in Blue). And three individual maps for each of the first three Principal Components (Bottom). PC1 (44.0% total variation) mainly accounts for annual precipitation, precipitation of the wettest month and temperature annual range. PC2 (33.4% total variation) mainly accounts for altitude, annual mean temperature and minimum temperature of coldest month. PC3 (9.4% total variation) mainly accounts for terrain ruggedness index.

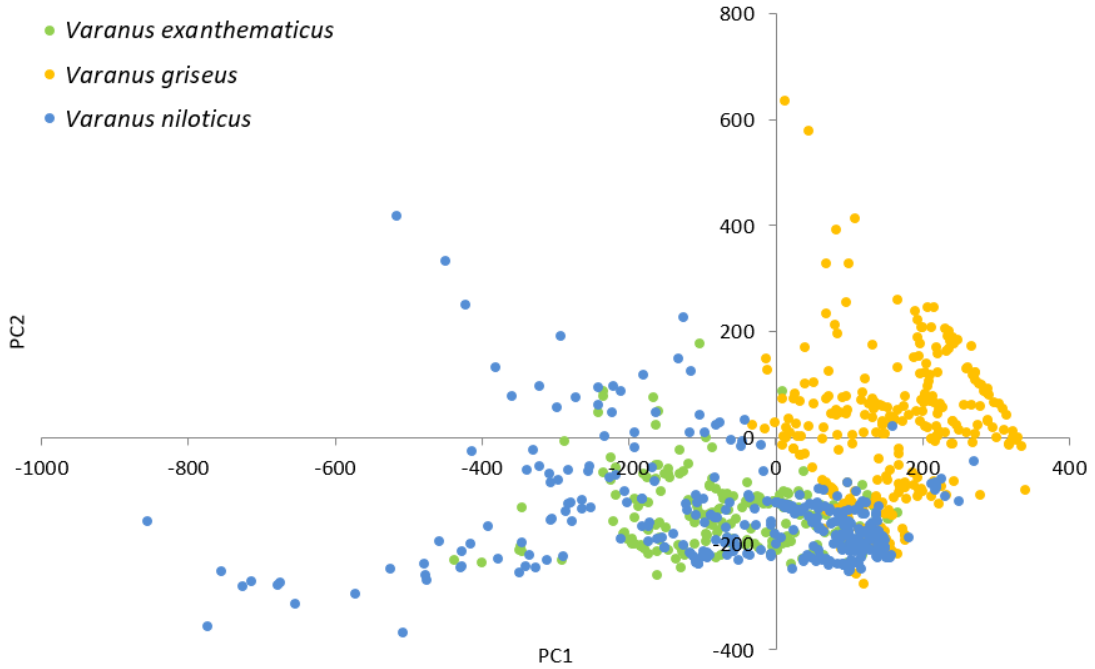

Figure 20. Presence points of *Varanus exanthematicus*, *V. griseus*, and *V. niloticus* along the PC1 and PC2 axes of the Spatial Principal Components Analysis.

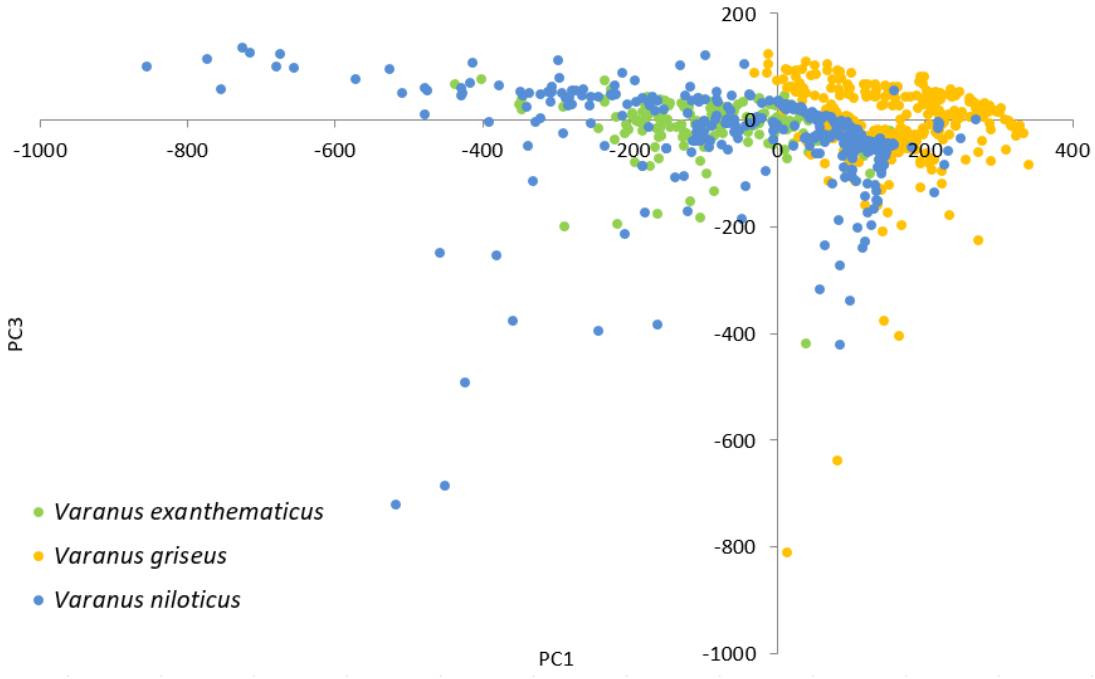

Figure 21. Presence points of *Varanus exanthematicus*, *V. griseus*, and *V. niloticus* along the PC1 and PC3 axes of the Spatial Principal Components Analysis.

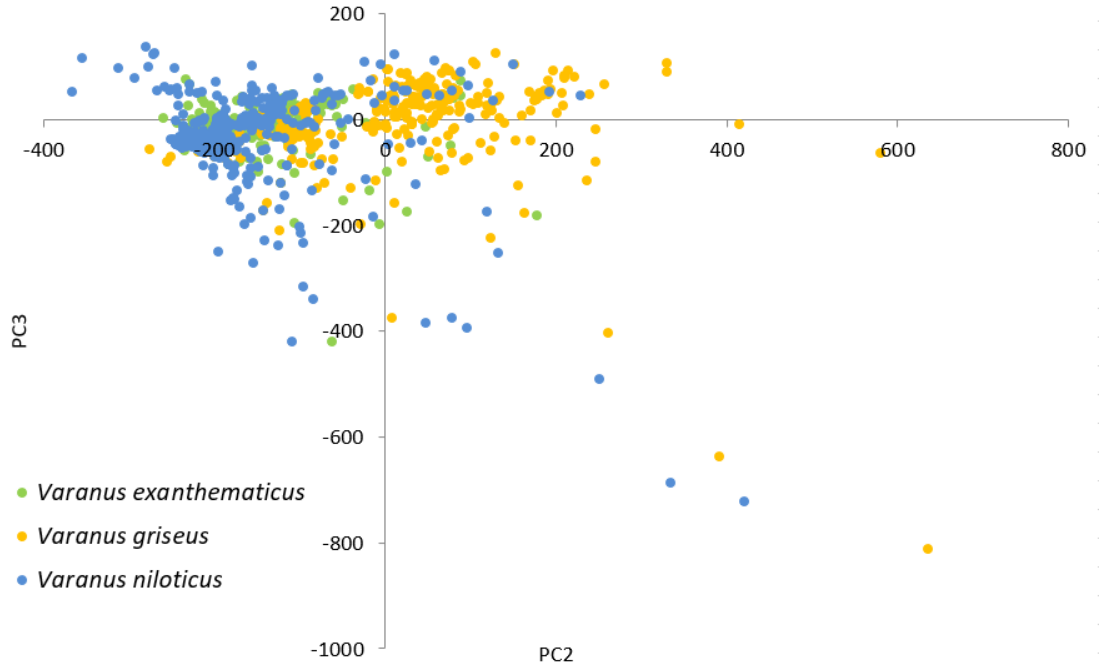

Figure 22. Presence points of *Varanus exanthematicus*, *V. griseus*, and *V. niloticus* along the PC2 and PC3 axes of the Spatial Principal Components Analysis.

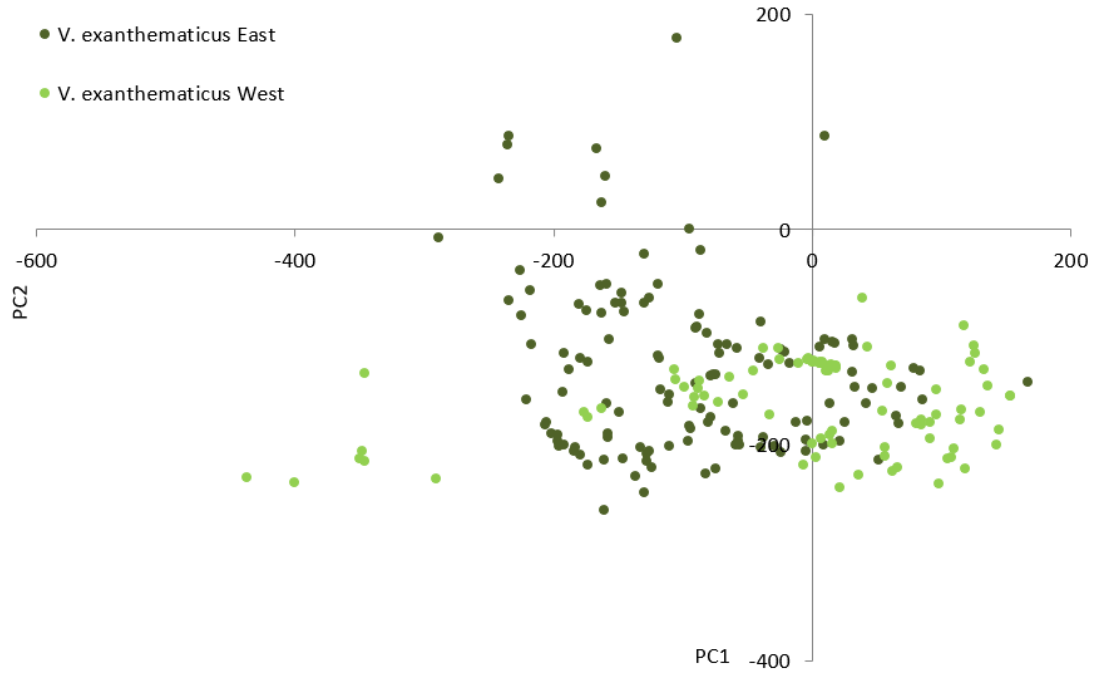

Figure 23. Presence points inferred to belong to *Varanus exanthematicus* clades along the PC1 and PC2 axes of the Spatial Principal Components Analysis.

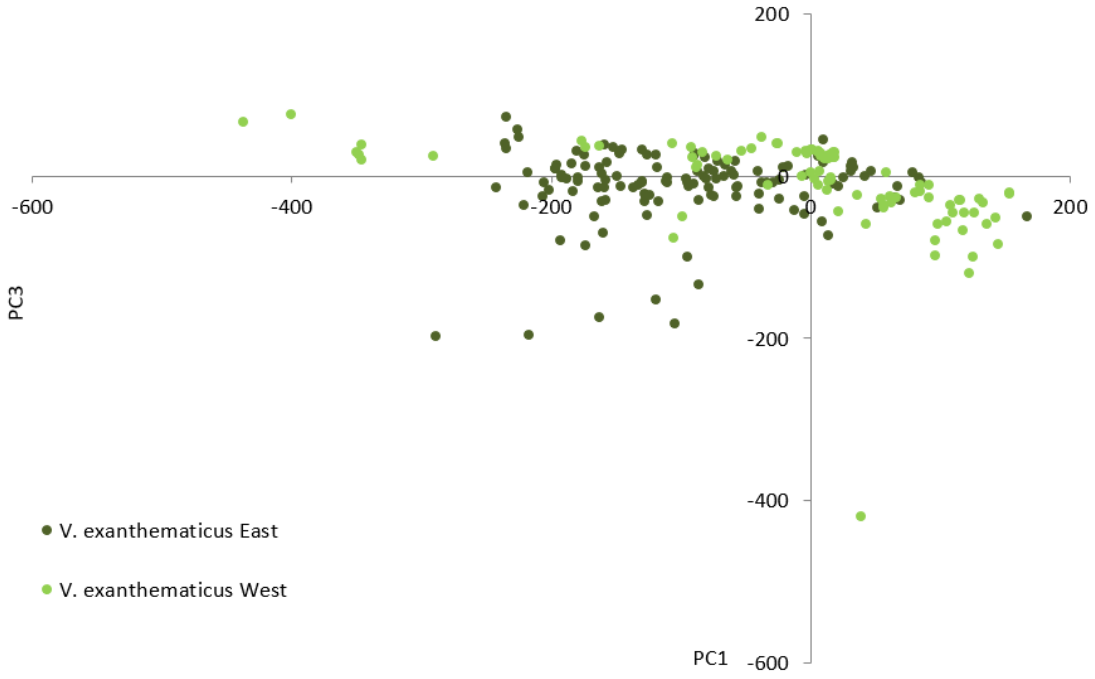

Figure 24. Presence points inferred to belong to *Varanus exanthematicus* clades along the PC1 and PC3 axes of the Spatial Principal Components Analysis.

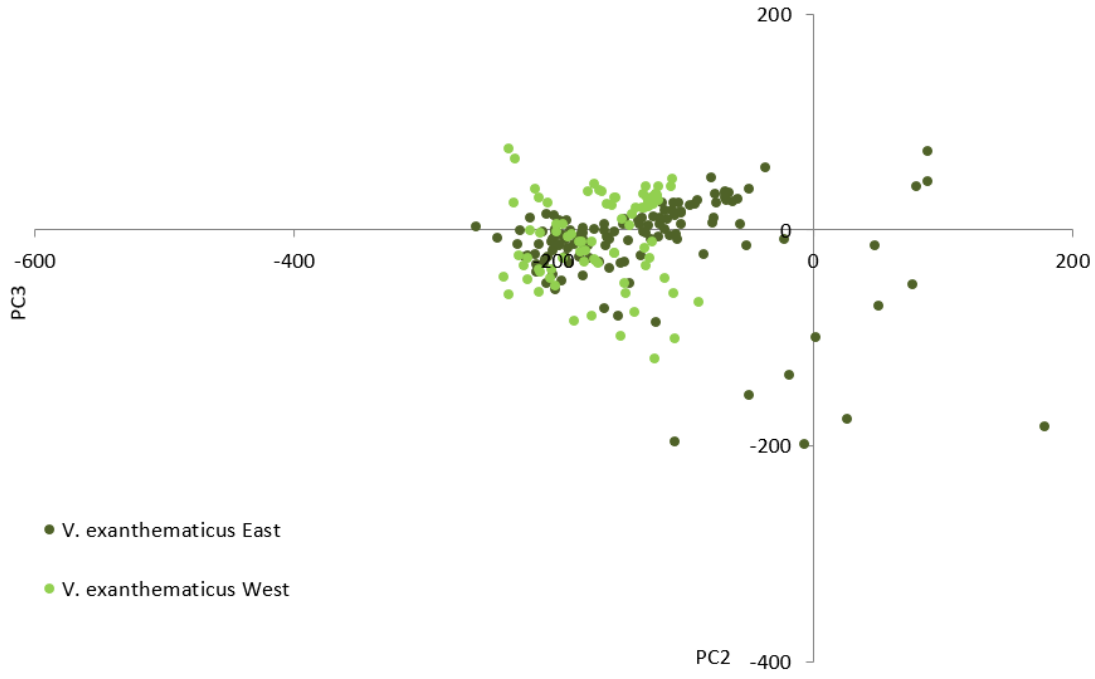

Figure 25. Presence points inferred to belong to *Varanus exanthematicus* clades along the PC2 and PC3 axes of the Spatial Principal Components Analysis.

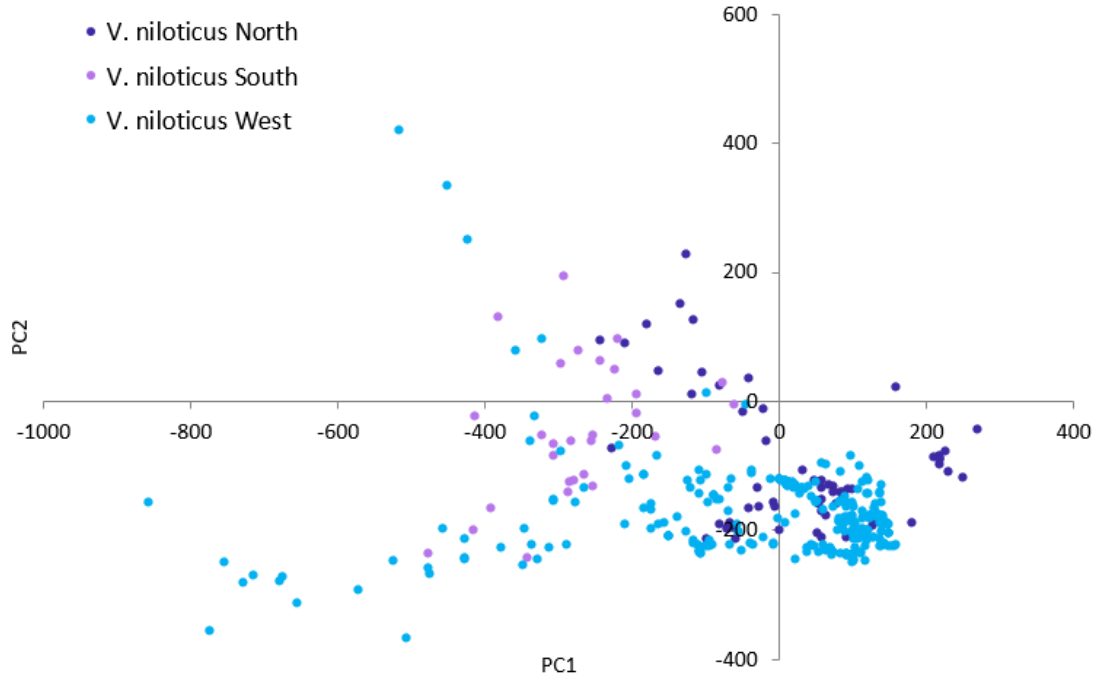

Figure 26. Presence points inferred to belong to *Varanus niloticus* clades along the PC1 and PC2 axes of the Spatial Principal Components Analysis.

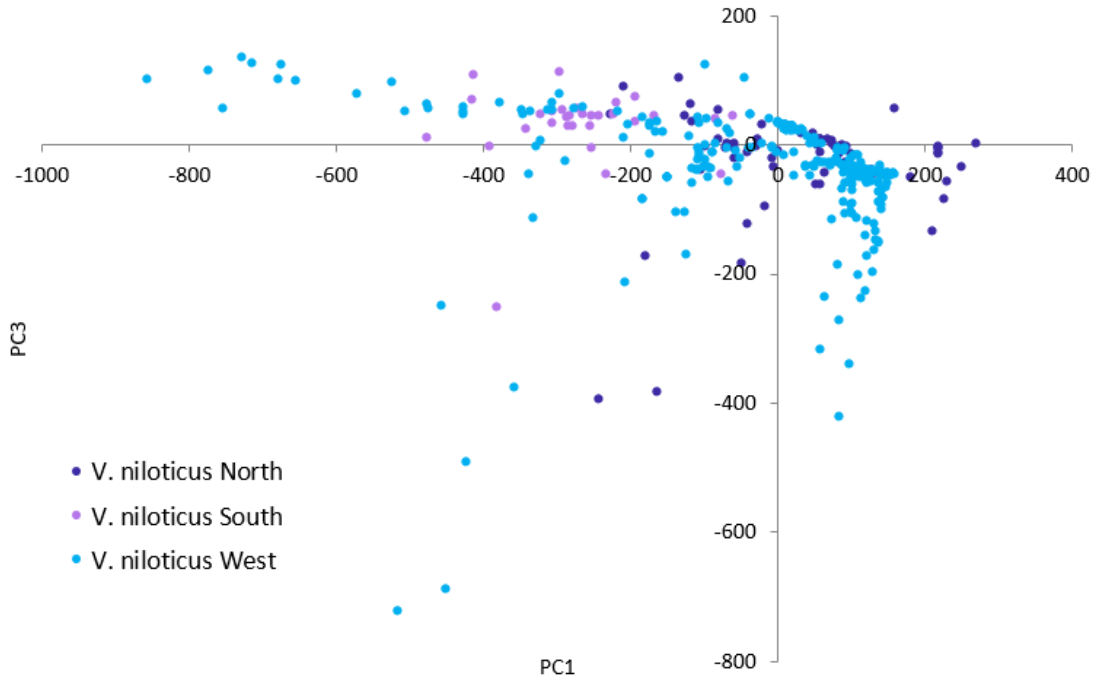

Figure 27. Presence points inferred to belong to *Varanus niloticus* clades along the PC1 and PC3 axes of the Spatial Principal Components Analysis.

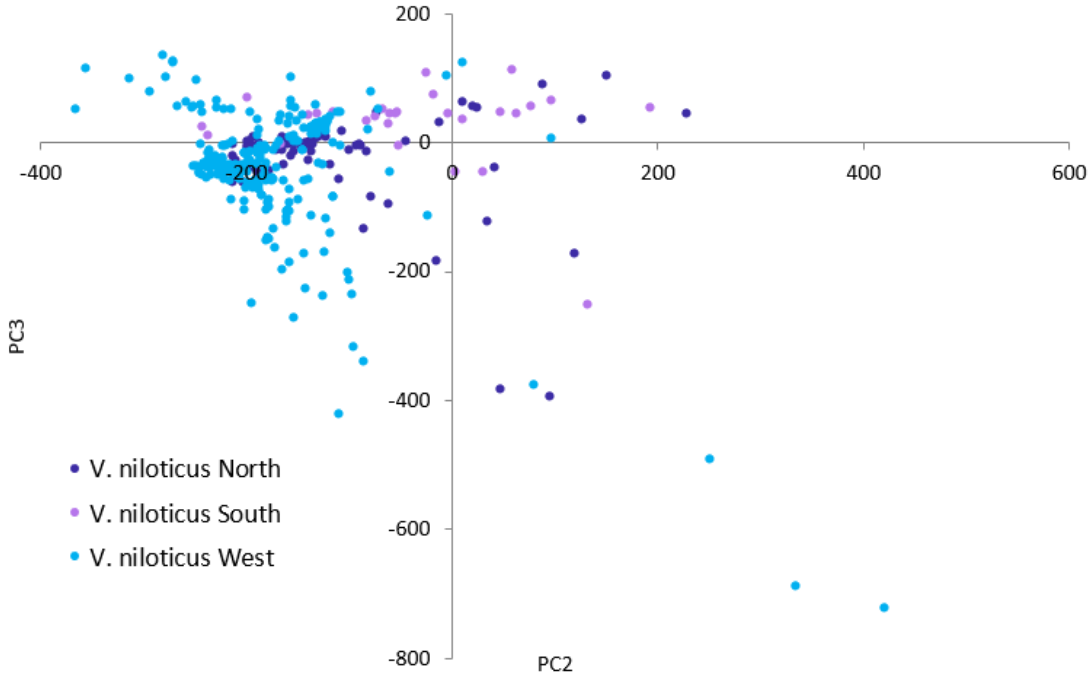

Figure 28. Presence points inferred to belong to *Varanus niloticus* clades along the PC2 and PC3 axes of the Spatial Principal Components Analysis

4. Discussion

This work resulted in an update of the distribution of *Varanus griseus*, *V. exanthematicus* and *V. niloticus*, resolving incongruences in the literature regarding their global distribution. It also allowed for a better understanding of the phylogenetic structure and diversity of *Varanus* in the sampled area and possible causes of divergence. This study also revealed that the distribution of genetic diversity is not structured according to the topoclimatic variability in Northern Africa.

While these analyses reveal that a lot more work can and should be done, especially regarding the overall diversity and realized niches of the three species, it was also important to bridge previous knowledge gaps, laying the foundations for future studies and better conservation planning of this exploited genus.

4.1. Geographic Distribution

The revised distribution of the three species allowed having a better understanding of the total extent of the species range and resolved some discrepancies in the available literature.

As expected, *V. griseus* showed to have the largest range of the three species (21,137,090 km²) spanning all the way from the west coast of the Sahara to India. However, considerable differences in relation to the published global distribution were encountered. Data collected shows that the species range extends into south-eastern Turkey and considerably further inland in India, beyond the Thar Desert, unlike what was described by Pianka & King (2004). The southern limit of the species extent of occurrence was also moved southwards, especially in Mauritania, when comparing to the current results with the works of Pianka & King (2004) and Sindaco & Jeremcenko (2008). Also, no distributional points were found to support the species presence north of the Aral Sea in Kazakhstan, like it was suggested by Pianka & King (2004). The range of *V. exanthematicus* was unsurprisingly the smallest of the three analysed species (6,799,659 km²) and it was mostly within the extent of the Sahel and Savannas ecoregions. However, many important discrepancies can be noted between the newly obtained range and the previously published works. The northern limit extends a lot further northwards than what is shown in the current IUCN range (Bennett & Sweet, 2010), as collected data supports its presence in eastern Mali, southern Niger, Lake Chad, southern Sudan, and northern Ethiopia. Eastwards, the recorded occurrence points were located considerably further on than what is described by Pianka & King (2004), Bennett & Sweet (2010), and Trape et al. (2012), but no evidence

was collected corroborating its extent to Eritrea (Bennett, 1995). No points were recorded as far south as north Gabon and Equatorial Guinea, zones considered to belong to the species range by Pianka & King (2004). The obtained range of *V. niloticus* (16,534,229 km²) also had its discrepancies when compared to the literature. In particular, points recorded extended the limit northwards in south-eastern Mauritania and eastern Mali, as well as in the Nile River, when compared to Pianka & King (2004). No support was found for the distribution to reach as further east as in Ethiopia, as shown by Pianka & King (2004). The Deserts of the Kalahari and the Namib also appear in the literature as where the species may be present (Bennet, 1995; Pianka & King, 2004), but no distribution points were found for these areas and a recent study by Dowell & Hekkala (2016) modelling the ecological niche of *V. niloticus* was also considered and thus these areas were discarded from the final range.

All resulting polygons were made in conformity with the IUCN Red List norms in order to present a more updated extent of occurrence of all three species. However, these polygons should keep being updated as more data are collected. In the future, additional distribution points should be gathered, in particular from the extremities of the range and preferably from direct field work to obtain recent and precise data that helps delimiting the extent of the range for each species. Fine-scale niche modelling should also be done in order to exclude from the range zones that are not viable to the species.

4.2. Genetic Structure and Diversity

Mitochondrial DNA phylogram of the three species revealed a total of 18 well supported subclades (BPP ≥ 0.95). Of those, three very short branches belonged to *V. griseus*, the first diverging species; five subclades belonged to the two clades of *V. exanthematicus*; and the ten remaining to the tree clades of *V. niloticus*. The haplotype networks of the nuclear markers showed that *V. griseus* presented only one haplotype for each of the genetic markers, *V. exanthematicus* presented three haplotypes for KIAA1549, one for PRLR and five for RAG1, and *V. niloticus* presented three haplotypes for KIAA1549, one for PRLR and three for RAG1. Unfortunately, the size of the sequences used for the KIAA1549 and PRLR haplotype networks was around 30% of the expected size, which may compromise any drawn conclusions. Besides short sequences due to background noise and lack of land cover, this work was also deeply hampered by the inability to successfully sequence museum samples. While amplification success rates are expected to be low when using museum samples due to old and likely degraded DNA, it was unexpected to detect contaminations considering that there were no traces of contaminations during extraction

and amplification. This suggests that the contamination likely occurred within the museum when curating or subsampling.

The relationships between species was expected considering the work published by Pyron et al. (2013). However, *V. griseus* was surprisingly close to *V. salvator* percentage-wise (p-distance) considering the two had only 19.5% differences between the two, around 1 to 5% less than the differences between *V. griseus* and the other two African *Varanus* present in this study. This, allied to the fact that *V. griseus* is also present in India, could raise questions regarding the placement of *V. griseus* in the African clade instead of in one of the other two clades (Indo-Asian or Indo-Australian). However, a much more likely hypothesis could be the phenomenon of long-branch attraction that typically happens when two evolutionarily distant species acquire similar mutations by mere chance, appearing to be closer than they actually are (Bergsten, 2005). While it is safe to assume that *V. griseus* belongs to the African clade based on works like the one from Pyron et al., (2013), it would be more conclusive to perform a global analysis of all *Varanus* clades using multiple molecular markers with slow rates of mutation.

As previously hypothesised, *V. griseus* presents an extremely shallow structure and low diversity. This was expected considering the species distribution in the Sahara, where few major barriers to gene flow are present (when compared to other ecoregions of Northern Africa like the Sahel and Savannah) especially for species adapted to arid and hyper arid habitats with high dispersal abilities like *V. griseus*. Because of this it was no surprise to see a genetic structure similar to what was observed in other medium sized xeric species with big home range sizes, like *Uromastyx dispar* and *U. aegyptia* (Tamar et al., 2017) and *Vulpes rueppellii* (Leite et al., 2015). What was surprising in the current work was the complete lack of genetic diversity in the three nuclear markers analysed, in particular RAG1, despite the considerably large sampled area. Generalized lack of diversity can be a pattern of rapid and recent colonization of the Sahara or west Africa, but more sampling is needed to properly determine the phylogeography of the species. The structure of *V. exanthematicus* was slightly unexpected considering the seemingly continuous range and high mobility. However, a pattern similar to other mesic species of Northern Africa was also expected. A particularly similar species structure-wise is *Psammophis schokari* (Gonçalves et al., 2018b) that, like *V. exanthematicus*, exhibits a deep structure throughout the extension of its large range. Considering the reduced number of available samples due to uneven sampling and museum contaminations, the observed genetic diversity was within the expected when comparing to other fairly diverse mesic species, like the previously mentioned *P. schokari* (Gonçalves et al., 2018b), especially for the RAG1 genetic marker. However, nuclear differentiation showed little support for the retrieved mtDNA clades. While KIAA1549 network showed no support for the two clades of *V. exanthematicus*, the main

reason for the small sequence size of the KIAA1549 genetic marker was most likely the presence of two bands in the agarose gel electrophoresis for the *V. exanthematicus* West clade. This hints that two copies of the same gene, of similar size, are present only in the West clade, which suggests a stronger isolation than what is perceivable by the retrieved levels of diversity. The observed genetic structure of *V. niloticus* fits the one described by Dowell et al. (2015a), which was not surprising considering most sequences used to achieve the phylogram were used and made available by Dowell et al. (2015a) in the first place. The very deep structure of *V. niloticus* in northern Africa is expected considering the species high dependency to permanent water bodies, which can be scarce and fragmented within the study area. While no sequences of the North and South clades were available for the PRLR and RAG1 genetic markers, KIAA1549 network revealed surprisingly low levels of diversity and almost no support for the three retrieved clades since only the *V. niloticus* North clade was separated by one polymorphism. This represents a lot less diversity and clade support than what is published by Dowell et al. (2015a). However, not many conclusions may be drawn from this work regarding that aspect, since KIAA1549 sequences used had only under a third of the total expected size, which restrains the analysis and subsequent conclusions.

Many authors have used wildly different mtDNA uncorrected sequence divergence thresholds to describe new species of *Varanus*: Welton et al. (2014) considered between 1.0% and 3.5%, Portik & Papenfuss (2012) between 9% and 23.9%, and Fitch et al. (2006) between 3.4% and 27.7%. This lead Dowell et al. (2015a) to nominate *V. niloticus* West clade as *V. stellatus*, a cryptic species of *V. niloticus*, based on less than 10% mtDNA differences. Because of this and considering that the differences between all retrieved clades of *V. exanthematicus* and *V. niloticus* range from around 11% to 5%, it could be argued that each clade represents a cryptic species. However, considering the generalized lack of diversity and structure present in haplotype network analysis, there is no support to back up such claim.

Overall, the observed structure and diversity of each species fell within the expected based on the available literature and size of the alignments used. To improve future analyses, more samples covering more of the species ranges, in particular in the East, should be collected and integrated into the phylogenetic analyses. Samples used should always be the most recent possible especially when using museum samples where it may be impossible to determine the quality of their preservation. In order to create haplotype networks that are more informative, genetic markers should also be sequenced from both ends, guaranteeing a large sequence size. Despite this study was not designed to analyse the intraspecific structure of each species at a fine-scale, many subclades were retrieved which should be carefully analysed in the future with the help of other genetic markers of

fast mutation rate, like fast-evolving genes and microsatellites. Future phylogenetic studies could also resort to methods of comparative genomics like Clade Genomics and Single Nucleotide Polymorphisms data gathering with novel tools like Next Generation Sequencing (NGS) and Restriction site Associated DNA Sequencing (RADseq).

4.3. Diversity Distribution

The three *Varanus* species have a parapatric distribution among each other. *Varanus griseus* is mainly allopatric to the other two species with the exception in southern Mauritania, eastern Mali, and southern Sudan, where it is locally sympatric with both *V. exanthematicus* and *V. niloticus*, and in the Nile River where it is sympatric with *V. niloticus*. *Varanus exanthematicus* and *V. niltoticus* are broadly sympatric and the range of *V. exanthematicus* falls inside the *V. niloticus* range, except in some northern regions of the Sahel. Throughout the sampled extent of occurrence of each species, only one clade of *V. griseus* was found; two clades of *V. exanthematicus*, one in the western Sahel and the other to the east of West Africa, in Ghana and Niger; and three clades of *V. niloticus*, one mainly in western Africa, other mostly in sub-Saharan limit and the Nile River, and the last one in most of the African continent south of the other clades.

While the obtained phylogram was not time-calibrated, node age can be inferred to a certain extent by comparison of node ages previously calculated by Dowell et al. (2015a). In that study, Dowell et al. (2015a) estimated that the divergence between the *V. niloticus* West clade from the other two occurred around 7.7 million years ago; that the divergence from *V. niloticus* North from *V. niloticus* South occurred around 4.7 million years ago; and that the subclade divergences occurred between 3.2 (divergence of the N3 and N4 subclades from the N5 subclade) and 0.5 million years ago (divergence of the N1 subclade from the N2 subclade). Comparing these estimations with the obtained phylogram allowed to roughly infer the age of divergence of most clades. While *V. griseus* has a single clade throughout the sampled range that diverged long time ago, it shows, however, a very recent ramification (subclades G1, G2, and G3). Such pattern of ramification could suggest a selective sweep by positive selection or a rather recent and rapid colonization of the sampled area within the Sahara (Avise, 2000). Assuming a constant rate of mutation, the divergence age of the two *V. exanthematicus* clades seems to be very similar to the divergence age between the *V. niloticus* West clade and the *V. niloticus* North and South clades previously estimated by Dowell et al. (2015a). Because of this it could be assumed that the divergence between the *V. exanthematicus* West and East clades happened during the late Miocene (around 7.7 million years ago). This time period is associated to the increased aridity in the region (Zhang et al., 2014) and the subsequent formation of the

Sahara (Schuster et al., 2006), which seems likely to have caused the observed diversification in *V. exanthematicus* considering its mesic nature. The current distribution of the two clades of *V. exanthematicus* seems to be allopatric, with the Niger River putatively acting as a barrier to gene flow between the two clades. Such pattern of longitudinal diversification has been observed multiple times in the Sahel and Savannas and its cause is often associated to major rivers like the Niger (e.g. Brouat et al., 2009; Bryja et al., 2010; Nicolas et al., 2010; 2011). Considering the age estimation of Dowell et al. (2015a), observed subclades of *V. exanthematicus* (E1 to E5) seem to have appeared during the Plio-Pleistocene, a time marked by the expansion and contraction of the Sahara (Brito et al., 2014). As previously stated, Dowell et al. (2015a) estimated that the divergence between the *V. niloticus* West clade and the remainder two to have occurred around 7.7 million years ago, a period that Zhang et al. (2014) associated to the reduction of the monsoons in Africa, leading to what may have cause the formation of the Sahara (Schuster et al., 2006). The divergence between the *V. niloticus* North and South clades was estimated to have occurred around 4.7 million years ago (Dowell et al., 2015a) which coincides with the changes in the distribution of the Sahara, often associated to the wet-dry cycles of the Pliocene (Le Houérou, 1997). Said wet-dry cycles continued until the mid-Holocene (Gasse, 2000; Kröpelin et al., 2008) which may explain the most recent divergences within clades (subclades N1 to N10) around 3.2 to 0.5 million years ago. Considering the water dependency of *V. niloticus*, it makes sense that these divergences occurred around time periods marked by increased aridity, said climate alterations likely changed the course and fragmented rivers, and lowered the water level of permanent water bodies. Nowadays, the distribution of the *V. niloticus* clades seem to be parapatric, with the *V. niloticus* West clade being present in the Senegal, Niger and Volta river basins; the *V. niloticus* North clade being present in the Niger, Lake Chad, north of the Congo, Nile and Jubba basins; and the *V. niloticus* South being present in the Volta, Congo, south of the Nile and most major water bodies south of the Congo. Despite the inexistence of records of the South clade in the Niger river basin, considering that it may be the connection between the Volta and the Congo basins, it is likely that the clade is also present there. This shows a large zone of sympatry between the three clades in the Niger River. Besides the sympatry in the south of the Nile river basin, sympatry between the North and South clades of *V. niloticus* is harder to detect due to insufficient data from the Central African Republic, where it would be expected to be the meeting point between both clades.

Varanus species and their intraspecific clades seem to be mostly parapatric in their distribution in Northern Africa and the observed diversity seems to be heavily linked to past climatic shifts of the region. However, understanding the patterns of genetic diversity of *Varanus* of Northern Africa and the mechanism underlying their intra-specific diversification,

still need further work that involve sampling efforts from important under sampled areas like central and eastern Africa and contact zones like the Niger river basin, as well as well as more robust dated multilocus phylogeny and demographic analysis.

4.4. Ecological Niche

Little to no ecological differences could be observed between the three species and their respective clades from the Spatial Principal Components Analysis (SPCA), with only *V. griseus* being slightly separated from the remaining species when comparing the different Principal Components.

The overlap between species along the SPCA axes is understandable considering that only topographic and climatic variables were used, instead of habitat variables, and that the three species share part of their distribution. This means that the variables that accounted for most of the environmental variation (annual precipitation, precipitation of the wettest month, temperature annual range, altitude, annual mean temperature, minimum temperature of the coldest month and terrain ruggedness index) do not fully explain the ecological niche of the *Varanus* of Northern Africa because all three species coexist in places where water is present but scarce, and therefore are susceptible to the same topoclimatic variations in those areas. Instead, local scale variables like vegetation density, woodlands availability, shrublands availability, water availability, distance to water bodies and sand substrate availability, and biotic factors like food availability and competition could have also been used considering that all these variables are more closely related to the species niche. Having this in consideration it is also understandable why *V. griseus* is the most secluded, even if only slightly, along the SPCA axes as it is the species that shares less of its range among the three species in Northern Africa while *V. exanthematicus* is almost sympatric to *V. niloticus*. While clades within *V. exanthematicus* and *V. niloticus* seem to be allopatric and parapatric respectively, their habitat throughout the species range within the study area is mostly climatically homogenous. Because of this, the total overlap observed along the axes of the SPCA between clades of each species was not entirely surprising.

It is known that the three analysed species have different habitat requirements (Bayless, 1997). However, topoclimatic variables appear to not be enough to modulate the environmental niche of each species. Therefore, future analyses should use more precise distributional data and environmental variables that are more directly related to the habitat requirements of each species. Other types of analyses could also be performed to access the fundamental niche of the species and clades like Ecological Niche Modelling with statistical comparisons between each other. Considering the warnings of imminent climate

changes, models of the species distributions for the future should also be done in order to aid conservation planning.

4.5. Integrative Overview

This study was important to update the global distribution of *V. griseus*, *V. exanthematicus* and *V. niloticus*, and to better understand how they are genetically structured throughout Northern Africa. It also allowed for the identification of zones of sympatry and allopatry among the species and the intraspecific clades which helped to the identification of possible causes of divergence between clades. The outcome of this work is expected to facilitate future conservation planning of these exploited species throughout the understudied region of Northern Africa and pave the road for future studies.

The xeric *V. griseus*, distributed in the arid regions from the west coast of Mauritania to central India, was found to have a shallow genetic structure and reduced diversity throughout its sampled range in Sahara, where few natural barriers can constrict this species gene flow due to its high dispersal capacity and adaptation to arid environments. The genetic analyses of *V. exanthematicus* revealed that the genetic diversity of this mesic species is structured in two clades along the Sahel and the Savannah in a longitudinal distribution. The divergence of the two clades is likely related to the appearance of the Sahara in Africa and current distribution of diversity seems to be dependent of the Niger river, that likely acts as a barrier to the species dispersal. *Varanus niloticus* appears to be the most structured of the three species with three identified clades. Being a water-dependent species with its distribution tied to permanent water bodies, like the main rivers of Africa, it is likely that the observed structure is related to periods of increased aridity, like the appearance of the Sahara, that is estimated to have caused the divergence of the first clade, and the climatic oscillations of the Pliocene, that seem to be behind the divergence between the two remaining clades.

Future studies should focus on both a broad-scale analysis of the African clade of the Varanidae family, including all species of the clade to better understand its history, and on a fine-scale analysis of each individual species to the access their structure and diversity with more detail and test for possible cryptic diversity. To accomplish this, future works should gather more distributional data; should obtain more biological samples to have a good cover of the entire distribution of each species; should use more genetic markers with different mutation rates; should use fossil records to time-calibrate the phylogenetic tree; and should also create models of the ecological niche of each species and respective lineages using precise distribution points and variables related to the required habitat of the species.

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6. Annexes

Annex 1 – Dataset reference list

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Annex 2 – Figure

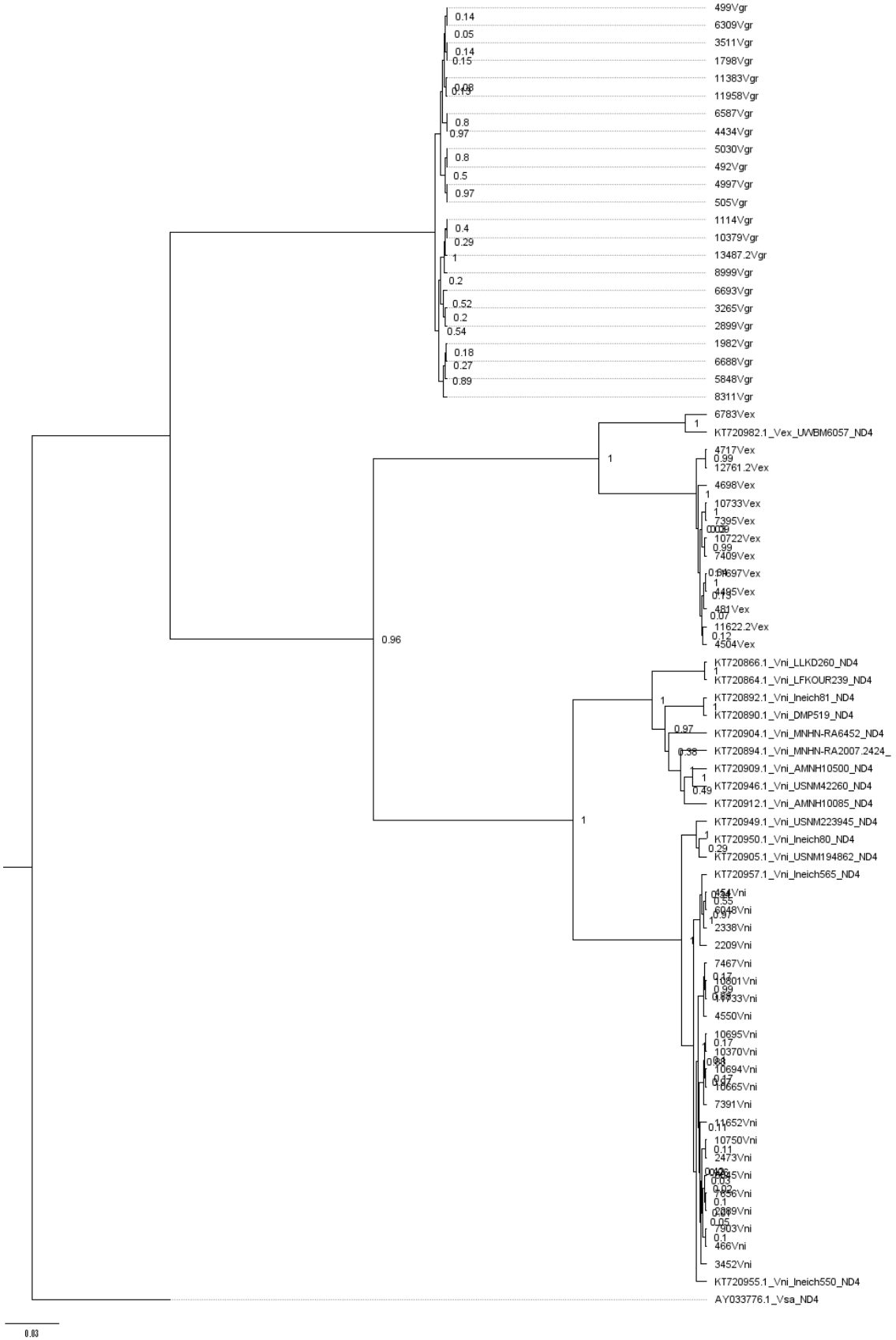

Figure A1. Complete Bayesian phylogram of *Varanus griseus*, *V. exanthematicus*, and *V. niloticus* obtained from the ND4 mitochondrial marker rooted in *V. salvator*. Bayesian posterior probability (BPP) of each node is depicted next to the respective node. Scale bar denotes 0.03 substitutions per site.