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Species Determination of Captive Sugar Gliders (*Petaurus breviceps* s.l.) Bred in Europe

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Species Determination of Captive Sugar Gliders (*Petaurus breviceps* s.l.) Bred in Europe independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague April 25, 2024

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Bc. Miroslav Mulko

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Abstract

Molecular genetics and bioacoustics have been proven to be very efficient monitoring methods, especially for passive monitoring. Recent advancements in these techniques together with morphometric analyses have led to the discovery of new cryptic species. This phenomenon is particularly common in nocturnal mammals, such as the sugar glider (*Petaurus breviceps* s.l.). Based on morphology, it was believed that the sugar glider is a polytypic species with seven subspecies. However, molecular studies suggested the existence of cryptic species within this taxon. From the conservation perspective, this is crucial as the species with widespread distribution in the past is now limited only to certain areas. With the increasing popularity of sugar gliders in the pet trade, the evaluation of the species' status and origin is important to prevent hybridization between differentiated lineages. In the thesis, we have focused on determining the species status and origin of sugar gliders in captivity by molecular genetics using the ND2 and ND4 mitochondrial genes and ω-globin, along with assessing the species-specificity of the barking calls of wild populations and comparing them with recordings from captivity. The phylogenetic analysis revealed a close relationship between captive populations from Europe and the USA. Both populations were closely associated to the samples from Kai Besar Island suggesting that the origin of both captive populations might be from Indonesia or Papua New Guinea. Based on mitochondrial genes, high haplotype and relatively low nucleotide diversity were observed within the captive populations, implying the potential of repeated imports of animals from several source localities. Moreover, the barking call was described as a stereotypical call, which is characterized by the repetition of uniform syllables. However, no clear specificity of this call was found under the utilization of comparative analyses of recordings between the wild and captive populations. Nevertheless, vocalizations differed in a single parameter, suggesting that wild sugar gliders bark more rapidly.

Key words: bioacoustics, DNA markers, Marsupiala, Petaurus breviceps, sugar glider, systematics

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List of the Abbreviations Used in the Thesis

AIID - acoustic identification of individuals

ANOVA – analysis of variance

ARU - autonomous recording unit

AZA – Association of Zoos and Aquariums

bp – base pairs

CLU - hierarchical cluster analysis

DNA – deoxyribonucleic acid

Dur – duration of call

Dur_b – bark duration

EAZA – European Association of Zoos and Aquaria

eDNA – environmental DNA

gDNA – genomic DNA

Hd – nucleotide diversity

Int – interval

IUCN – International Union for Conservation of Nature

KG – Krefft's glider

Max_f0 – maximum of fundamental frequency

mtDNA – mitochondrial DNA

Mya – million years ago

Nb – number of barks

ND2 - NADH dehydrogenase subunit 2

ND4 - NADH dehydrogenase subunit 4

Nh – haplotype diversity

PAM - passive acoustic monitoring

PC - principal component

PCA – principal component analysis

PCR – polymerase chain reaction

Peak_f – peak frequency

Peak_f0 – peak of fundamental frequency

PNG – Papua New Guinea

Sd – standard deviation

SG – sugar glider

SGE – sugar glider - Europe

SNP – single nucleotide polymorphism

UPGMA - unweighted pair group method with arithmetic mean

USA – United States of America

VWF – von Willebrand factor

1. Introduction

Molecular genomics and bioacoustics offer an alternative approach to monitoring that can be cost-efficient (Hale et al. 2022). Moreover, both methods benefit from the potential for non-invasive monitoring (McCowan & Hooper 2002; Braune et al. 2008; Foote et al. 2012; Hasiniaina et al. 2018; Rosti et al. 2020; Whisson et al. 2021; Linhart et al. 2022). In the case of passive acoustic monitoring, large amounts of acoustic data are collected via autonomous recording units with minimal human disturbance (Teixeira et al. 2019; Whisson et al. 2021). This can be utilized in studies of species detection, abundance, or density (McCowan & Hooper 2002; Teixeira et al. 2019; Hasiniaina et al. 2020; Hale et al. 2022). Additionally, non-invasive genetic sampling is a highly efficient method for assessing the overall health of study populations through the estimation of genetic diversity (Rasolonjatovo et al. 2022). Environmental DNA (eDNA) proves particularly effective in such studies, encompassing various materials containing biological remnants, including urine, feces, or, in the case of marine species, blow water or sloughed skin (Foote et al. 2012).

Recent advancements in molecular genetics, bioacoustics, and morphological analyses have facilitated the delineation of new cryptic species, particularly those with an elusive lifestyle (Russo & Jones 2000; Munds et al. 2013; Svensson et al. 2017; Baldwin et al. 2021). Cryptic species refer to two or more species that have overlapping phenotypic traits but are biologically distinct, often misclassified as a single species (Bickford et al. 2007); this phenomenon is common among nocturnal mammals (e.g. Thabah et al. 2006; Munds et al. 2013; Hotaling et al. 2016; Pozzi et al. 2019) and also for the sugar glider (*Peaturus breviceps* s.l.).

Until recently, based on slight differences in morphology, it was thought to have seven subspecies (Smith 1973), but Malekian et al. (2010) revealed uncertainties in taxonomy, necessitating further studies to resolve phylogenetic relationships and uncover potential cryptic species. In 2021, Cremona et al. (2021) confirmed this hypothesis, dividing the three Australian subspecies into distinct species.

These findings are crucial for species conservation, as the actual distribution ranges are narrower than previously assumed. Additionally, the conservation status

should be reassessed promptly, given the current classification as Least Concern (Salas et al. 2016). One of the primary threats for the species is habitat destruction and fragmentation of forests (Gracanin et al. 2023), as gliding species are often highly dependent on the presence of trees and their dispersal abilities out of the forest are very limited (Caryl et al. 2013; Knipler et al. 2022). Furthermore, extensive bushfires and land clearings have significantly reduced suitable habitat areas as well (Jolly et al. 2022). It is important to implement conservation measures to determine the distribution ranges of new glider species, the Krefft's glider (*Petaurus notatus*) and the savannah glider (*P. ariel*), as well as for the sugar glider (*Petaurus breviceps*). Estimating the population status, abundance, and density of the species is crucial and can be achieved through the integration of multiple methods, such as molecular genetics and acoustic monitoring, as mentioned earlier.

The sugar glider has become increasingly popular as a pet due to its charismatic nature. A study investigating the origin of captive sugar gliders in the USA revealed the Indonesian origin of all sampled individuals (Campbell et al. 2019). They are also widely featured in zoo collections globally. Therefore, accurately identifying the species' origin (Franke et al. 2013) and classifying them into subspecies is essential to prevent hybridization or outbreeding depression (Schmidt et al. 2015; Palmer et al. 2021). With the relatively undefined phylogenetic relationships, the potential for hybridization between different species in captivity is quite likely (Franke et al. 2013; Hvilsom et al. 2013; Schmidt et al. 2015; Shirley et al. 2015).

2. Literature Review

2.1. Combination of Genetic and Bioacoustic Approaches

Genetic and bioacoustic methodologies have emerged as promising alternatives to traditional monitoring techniques, particularly for non-invasive monitoring purposes. The monitoring of population sizes of solitary and elusive species presents notable challenges due to their secretive behavior and scattered distribution (Whisson et al. 2021; Hale et al. 2022). In some cases, these secretive species tend to communicate through ultrasonic frequency calls as an antipredator mechanism (Hasiniaina et al. 2018; Miard et al. 2019).

Combining multiple methodologies, such as systematic acoustic sampling and genetic analyses, offers a comprehensive understanding of population status and trends, as well as revealing species richness, abundance, density, and overall genetic diversity (McCowan & Hooper 2002; Teixeira et al. 2019; Hasiniaina et al. 2020; Hale et al. 2022). Recent advancements in molecular, acoustic, and morphological analyses have led to the discovery of several cryptic species, such as hyraxes (Oates et al. 2022), bats (Russo & Jones 2000; Thabah et al. 2006; Baldwin et al. 2021), tree shrews (Esser et al. 2008), and primates such as galagos (Masters & Couette 2015; Svensson et al. 2017; Pozzi et al. 2019), lemurs (Craul et al. 2007; Olivieri et al. 2007; Mittermeier et al. 2010; Hotaling et al. 2016), or lorises (Munds et al. 2013). Cryptic species refer to two or more species that have overlapping phenotypic traits but are biologically distinct, often misclassified as a single species (Bickford et al. 2007).

For instance, cryptic primates rely on vocalizations not only for locating potential mating opportunities but also for distinguishing between sympatric cryptic species (Braune et al. 2008). Species-specific signals play a crucial role in mate recognition and can prevent admixture with other species (Pozzi et al. 2019).

Environmental DNA (eDNA) has emerged as a highly efficient tool for large-scale genetic monitoring, especially when combined with DNA barcoding or metabarcoding techniques (Filippi-Codaccioni et al. 2018; Beng & Corlett 2020). DNA barcoding utilizes species-specific primers to identify DNA fragments of a single species within an environmental sample, while metabarcoding employs universal primers to

simultaneously detect DNA fragments from a wide range of species (Beng & Corlett 2020; Antil et al. 2023).

The evolutionary processes leading to the changes in the composition of acoustic signals and the species specificity can be influenced by several aspects. One cause can be ecological selection, where the speciation on a specific diet together with morphological changes might cause a later differentiation in vocalization (Wilkins et al. 2013). From a morphological point of view, body size plays a crucial role in shaping the vocal structure, as larger animals tend to vocalize at lower frequencies (Wilkins et al. 2013). Other factors are sexual selection, habitat structure, or genetic drift (Schneiderová & Policht 2012; Wilkins et al. 2013).

2.1.1. Application in Different Species

2.1.1.1. Species Presence

In several studies, both methodologies were utilized to ascertain the presence of targeted species within specific geographical locations. This practice is particularly prevalent in the realm of marine mammal research. An illustrative example is evident in the study conducted by Foote et al. (2012), where they explored the potential of eDNA as a tool for genetic monitoring in marine mammal populations. To assess the viability of employing eDNA for genetic monitoring, the researchers employed specific primers designed to amplify short mitochondrial DNA sequences. Their objective was to detect the presence of the harbor porpoise (*Phocoena phocoena*) across controlled and natural marine environments. The researchers employed static acoustic monitoring devices to detect echolocation clicks emitted by the targeted species, thereby verifying the presence of the harbor porpoise. Under the controlled conditions the genetic detection was more successful than in the natural conditions, where the acoustic monitoring was more efficient. Interestingly, despite the absence of long-finned pilot whale (*Globicephala melas*) clicks in the acoustic data, genetic sequencing successfully identified the species' presence.

Another method that could complement genetic sampling is bioacoustic stimulation, which can be employed to determine the presence of targeted species. This was demonstrated in a study focusing on the golden jackal (*Canis aureus*), as jackals are known to reliably respond to howling stimulation throughout the year (Hatlauf et al.

2021). By utilizing bioacoustic stimulation techniques, researchers can elicit vocal responses from the targeted species, providing additional evidence of their presence in a given area.

2.1.1.2. Species Determination

Crypticity in echolocating bats is very common (Filippi-Codaccioni et al. 2018), thus they are great case species for the integration of different methodologies for species determination. This is exemplified in the study of the *Hipposideros caffer* complex, a group of West African Old-World leaf-nosed bats, where four distinct lineages were delineated through mitochondrial DNA analysis. Notably, significant differences were observed not only in nuclear microsatellites but also in echolocation calls, with peak frequencies ranging from 1.5 kHz to 28.8 kHz, confirming the existence of four cryptic species (Baldwin et al. 2021).

However, the process of species delineation is full of challenges. For instance, Sun et al. (2016) investigated the big-eared horseshoe bats (*Rhinolophus macrotis* complex) in China, revealing discrepancies between mitochondrial DNA and microsatellite analyses, leading to differing lineage assessments. Bioacoustic and morphological analyses corroborated the presence of two distinct lineages, suggesting potential factors such as paleoclimatic oscillations or interspecific hybridization influencing the observed patterns. Similarly, in Japan, acoustic differences were observed among the colonies of Okinawa least horseshoe bat (*Rhinolophus cornutus pumilus*), despite the evidence of sufficient gene flow between the colonies (Yoshino et al. 2008). This suggests the possibility of the presence of cultural drift and maternal transmission of constant frequency.

In French Guiana, coexisting colonies of *Pteronotus alitonus* and *P. rubiginosus* in four caves provided a unique opportunity for comparative analysis. Distinct peak frequencies of echolocation calls (*P. rubiginosus* – 53 kHz; *P. alitonus* – 59 kHz) coupled with genetic evidence of separate haplogroups underscored the presence of two distinct species. While limited hybridization and asymmetric introgression were observed, the absence of spatial genetic structure between caves suggested other mechanisms, possibly acoustic and morphological, influencing species isolation (Filippi-Codaccioni et al. 2018).

Traditionally, species identification in nocturnal primates relied heavily on vocal signals. Pozzi et al. (2019) investigated two cryptic Eastern dwarf galago species, *Paragalago cocos*, and *P. zanzibaricus*, revealing distinct genetic lineages corresponding to species differentiation. Moreover, acoustic analyses played a pivotal role in confirming species identity, with a high percentage of vocal recordings accurately assigned. Similarly, in Taita Hills, Kenya, Rosti et al. (2020) utilized vocal repertoire analysis to confirm the species identity of a galago population as *P. cocos*. Thus, these findings underscore the importance of acoustic signals in species identification.

In conclusion, integrating genetic, acoustic, and morphological analyses provides a robust framework for uncovering cryptic diversity and comprehending the mechanisms behind species differentiation and coexistence across various ecological contexts.

2.1.1.3. Individual-based Recognition

Traditionally, genetic analyses were used for individual determination by the application of microsatellites and SNPs (single nucleotide polymorphisms) (e.g. Mondol et al. 2009; Costa et al. 2012; Monteiro et al. 2014; Abdul-Muneer 2014; Bach et al. 2022; Pérez-González et al. 2023), which are bi-parentally inherited (Abdul-Muneer 2014). The microsatellites are highly polymorphic, and their main advantage is in the cost-effectiveness and easy-to-use technique. Nowadays, SNPs are increasing in popularity with technological advancements, such as next-generation sequencing techniques (Pérez-González et al. 2023). However, the determination of the individuals based on vocalization can be suitable in some cases.

Vocalizations play a pivotal role in communication among mammalian species, with emerging evidence highlighting their individual distinctiveness. This individual recognition serves as a prerequisite for various complex social interactions, including territorial behavior, parent-offspring interactions, mate choice, and the allocation of potentially altruistic behavior (Schneiderová & Policht 2011; Linhart et al. 2022). Spectral characteristics of vocalizations emerge as the most important variables in distinguishing among individuals, although features such as call length, intensity, or the number of notes per call can also aid in individual classification (Schneiderová & Policht 2011).

Consequently, acoustic identification of individuals (AIID) has garnered attention as a non-invasive and labor-efficient alternative to traditional mark-recapture identification methods. Successful AIID hinges upon the presence of strong and stable acoustic signatures, a feature particularly prevalent in cetaceans and primates (Linhart et al. 2022).

In social-living ground squirrels, alarm calls serve as prominent vocalizations, conveying species-specific warnings of potential threats and predators. Schneiderová & Policht (2011) demonstrated the potential for individual recognition within ground squirrels based on these calls. Their analysis revealed a remarkable accuracy, with over 90% of calls correctly assigned through discriminant and cross-validation analyses. This highlights the efficacy of acoustic cues in facilitating individual identification and underlines its significance in understanding social dynamics and communication patterns within mammalian populations.

2.1.1.4. Passive Monitoring

Passive acoustic monitoring (PAM) has emerged as a valuable remote sensing method increasingly utilized for surveying species that prominently signal their presence, identity, and behavioral traits through vocalizations. In PAM, autonomous recording units (ARUs) are strategically deployed at survey sites for extended periods, programmed to capture recordings during times when the target species is likely to vocalize (Whisson et al. 2021). Subsequently, these recordings are scrutinized either manually or through automated detection and classification algorithms to identify species-specific vocalizations. PAM offers several advantages, including its efficacy in challenging terrains and vegetation, applicability across large spatial and temporal scales, non-invasiveness, and enhanced detection probability of small, nocturnal, elusive, or rare species (Lambert & Mcdonald 2014; Heinicke et al. 2015; Campos-Cerqueira & Aide 2016).

To maximize the effectiveness of PAM, it is essential to construct a comprehensive vocal repertoire for the target species, understanding not only the quantity but also the functional significance and contextual cues associated with each vocalization (Dorph & McDonald 2017). Whisson et al. (2021) endeavored to establish PAM guidelines for detecting the Yellow-bellied glider (*Petaurus australis*). Employing Songmeter: SM4 as the ARU, recordings were scheduled for 11 hours post-sunset over

14 consecutive days. Their findings revealed peak vocalization activity occurring within four hours post-sunset, affirming the effectiveness of PAM in detecting the presence of Yellow-bellied gliders. Despite challenges posed by the species' variable vocalizations, the study demonstrated the feasibility of automating call detection, potentially enhancing the efficiency of PAM surveys.

On the other hand, passive genetic monitoring is very efficient as it is feasible in the case of most species. For this purpose, eDNA could be used (Thomsen et al. 2012; Foote et al. 2012; Allen et al. 2023), as mentioned above. In the case of terrestrial mammals, the collection of faeces or urine is usually performed (Lampa et al. 2015; Velli et al. 2015; Arandjelovic & Vigilant 2018; Ferreira et al. 2018; López-Bao et al. 2018; Aylward et al. 2022; Hulva et al. 2024).

2.1.2. Benefits & Constraints

One of the primary advantages, as noted earlier, is the ability to get a more comprehensive understanding of biodiversity, particularly concerning species identification (e.g. Russo & Jones 2000; Svensson et al. 2017; Pozzi et al. 2019; Baldwin et al. 2021), population dynamics (McCowan & Hooper 2002), and evolutionary relationships (Filippi-Codaccioni et al. 2018). This is especially crucial for species that cannot be reliably distinguished based on morphology alone. For instance, in the case of tree-roosting bat species, acoustic surveys have been deemed the most suitable method for monitoring due to their relatively low cost and the capability to survey multiple species simultaneously (Hale et al. 2022). Acoustic surveys can be conducted in challenging terrains and vegetation, and they can be applied across large spatial and temporal scales (Rosti et al. 2020; Whisson et al. 2021).

Moreover, the combination of these methodologies can enhance conservation efforts as the inclusion of genetic analyses can provide insights into the genetic diversity of populations and cryptic speciation (McCowan & Hooper 2002; Pozzi et al. 2019; Hale et al. 2022; Rasolonjatovo et al. 2022), identifying priority conservation areas (Law et al. 2018), and evaluating the efficacy of conservation interventions.

Both genetic and bioacoustic methods often enable non-invasive sampling, which is advantageous for studying elusive or endangered species without causing disturbance or harm (McCowan & Hooper 2002; Braune et al. 2008; Foote et al. 2012; Hasiniaina et

al. 2018; Rosti et al. 2020; Whisson et al. 2021; Linhart et al. 2022). For some species, bioacoustics may be the only feasible approach to acquire behavioral data, such as for cetaceans' and galago's cryptic behaviors (Bearder et al. 2003; Teixeira et al. 2019).

However, a significant constraint lies in sampling limitations. Genetic analysis often requires tissue samples, such as blood or tissue biopsies (Foote et al. 2012), which may not always be feasible to obtain. Additionally, in the case of faecal samples, there is a risk of collecting scats from different species (Hatlauf et al. 2021). Nevertheless, biological excretory processes like skin sloughing, urination, and defecation can be sources of eDNA and provide a record of species' presence over time (Foote et al. 2012). eDNA can also be detected from soil or bark samples (Allen et al. 2023). In freshwater aquatic environments, the use of eDNA for genetic monitoring has shown promise, as it is homogeneously distributed and can effectively detect and quantify species presence (Thomsen et al. 2012). However, seawater samples present challenges due to larger source water bodies, strong tide and current action, and high salinity, which may inhibit eDNA amplification by polymerase chain reactions (PCRs) (Foote et al. 2012).

Similarly, bioacoustic data collection may be restricted by factors such as environmental conditions, species behavior, and equipment capabilities. For instance, in the case of the Yellow-bellied glider, weather conditions greatly influence the detection probability of vocalizations, as background noise, such as wind or rain, may mask vocalizations. Rainfall, in particular, negatively affects detection probability, especially during the autumn/winter sampling period (Whisson et al. 2021). Even though, this method could serve as a cost-effective alternative (Hale et al. 2022), the data processing is still very time-consuming. Therefore, it is important to know the vocal repertoire of the studied species (Dorph & McDonald 2017) to easily detect the species' vocalization in the recordings.

2.2. The Genus *Petaurus*

The genus *Petaurus* consists of a group of marsupials characterized by a gliding membrane – the patagium. The patagium extends from the forelimbs to the hindlimbs and is tightly bound together by connective tissue. The muscles play a crucial role in controlling the attitude of the airfoil (Jackson 2000a). The gliding membrane has likely

evolved as an adaptation to open forests and the transition to a lower quality diet such as plant exudates (Jackson 2000a; Malekian et al. 2010; Byrnes & Spence 2011). Moreover, this type of fast and energetically cheap movement is very efficient in the avoidance of terrestrial predators, but also in foraging activities (Byrnes & Spence 2011). Nevertheless, the dispersal activities are quite limited. For example, the mean glide distance of *P. breviceps* is 20.4 m, *P. norfolcensis* 21.5 m, and *P. gracilis* 29.7 m, but it is dependent on the habitat (Jackson 2000a; Goldingay & Taylor 2009).

The first fossils of specimens similar to *P. norfolcensis* are dated to 4.46 million years ago (mya) (Malekian et al. 2010). Malekian et al. (2010) have performed a molecular dating analysis and suggested that the most recent common ancestor of existing *Petaurus* species emerged around 18-24 mya, therefore during the early Miocene. The following major split within the genus occurred approximately 9-12 mya, distinguishing *P. abidi* from the Australian species *P. norfolcensis* and *P. gracilis*, as well as the New Guinean and Australian populations of *P. breviceps*.

The species are distributed in northwestern and eastern Australia, Papua New Guinea, and parts of Indonesia (Cremona et al. 2021). The distribution is highly correlated with its natural habitat (e.g. wet and dry sclerophyll forests). However, since European colonization, Australia has lost approximately 40% of its forests, with the remaining native vegetation highly fragmented. Eucalypt forests have experienced the highest rate of deforestation, with 80% of the remaining woodlands altered by human activities (Bradshaw 2012; Lancaster et al. 2016). From a global perspective, 27% of mammal species are under the threat of extinction due to habitat loss and degradation, largely driven by habitat fragmentation, wherein smaller, disconnected continuous habitat is reduced into patches (Gracanin et al. 2023). This fragmentation poses a significant risk to certain taxa, particularly in Australia's tropical savannas, where habitat fragmentation combined with extensive grazing can threaten small mammals (Bradshaw 2012).

2.2.1. Taxonomy

Until recently, seven species were recognized within the genus *Petaurus*. Namely, the yellow-bellied glider (*P. australis*), the squirrel glider (*P. norfolcensis*), the mahogany glider (*P. gracilis*), the northern glider (*P. abidi*), the Biak glider (*P. biacensis*), and the

sugar glider (*P. breviceps* s.l.) (Malekian et al. 2010; Burnett et al. 2016; Leary et al. 2016a, 2016b; Salas et al. 2016; Winter et al. 2016; Woinarski et al. 2016).

However, the taxonomy within this genus is not fully resolved and for example, the situation within the sugar glider (*P. breviceps* s.l.) is very uncertain. *Petaurus breviceps* s. l. has been recognized as a polytypic species with originally seven subspecies (Smith 1973). The taxonomy was primarily based on morphological characteristics, as some subspecies exhibit significant morphological variation, with many populations displaying two distinct color morphs and notable differences in body and skull sizes (Colgan & Flannery 1992).

Malekian et al. (2010) conducted a study to determine phylogenetic relationships among the *Petaurus* species. Their findings revealed notable diversity in mitochondrial DNA (mtDNA) within *P. breviceps* s. l., identifying two distinct clades in Australia and five in New Guinea. One of the subspecies of *P. breviceps* from northern Australia exhibited a closer phylogenetic relationship to the *P. norfolcensis* and *P. gracilis*. Moreover, the distribution of these lineages did not align with morphologically described taxa and previously proposed distributions, as they often overlapped in the ranges of different subspecies. Notably, the reciprocal monophyly was not supported when combining mtDNA and ω-globin analyses, suggesting potential genetic complexities within the genus and hinting at the existence of cryptic species (see Figure 1).

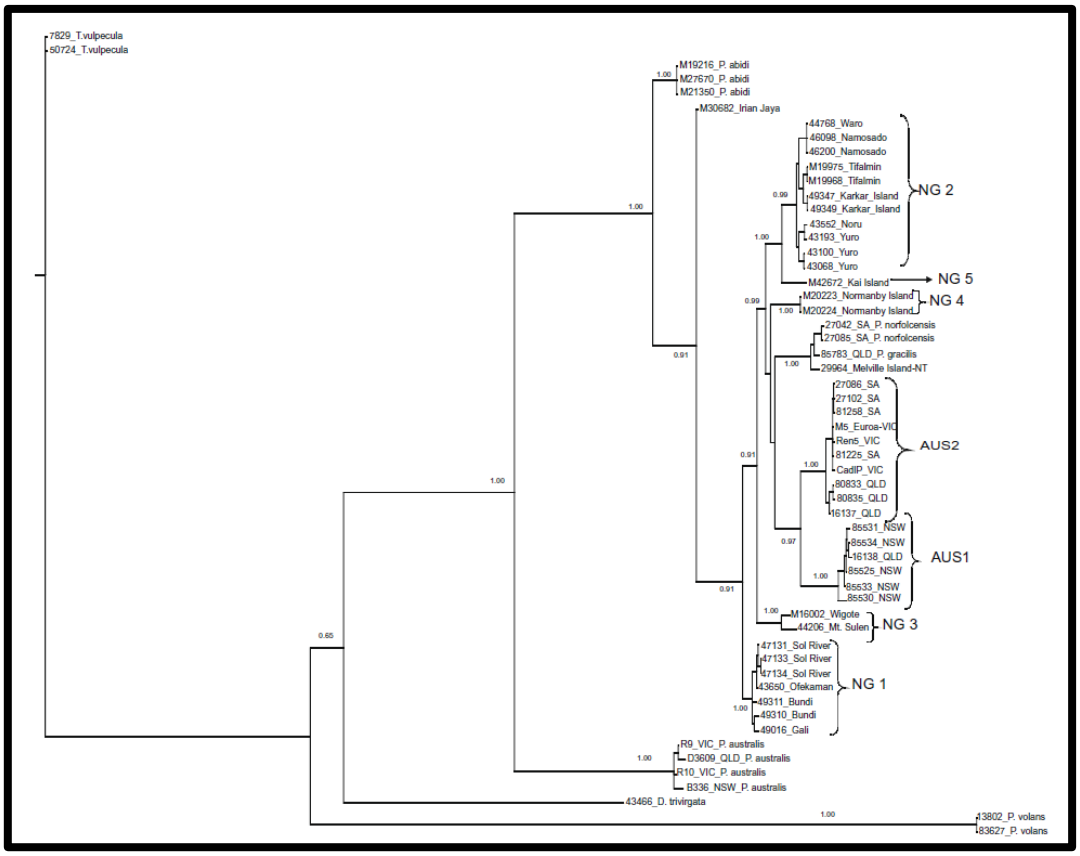

Figure 1: Bayesian consensus tree demonstrating the phylogenetic relationships of *Petaurus breviceps* s.l. and other *Petaurus* species, which are indicated on the label (Malekian et al. 2010).

Cremona et al. (2021) using NADH dehydrogenase subunit 2 (ND2) mitochondrial marker confirmed the presence of three cryptic species within *P. breviceps* s.l.: the sugar glider (*P. breviceps*), the Krefft's glider (*P. notatus*), and the savannah glider (*P. ariel*). This taxonomic reassessment has significant implications for conservation and management, as a species previously perceived as widespread consists of multiple distinct species with considerably narrower distributions. The three Australian species are differentiated by small changes in morphology and coloration, but also by their distribution area (Smith 1973; Cremona et al. 2021).

Petaurus ariel can be identified by its notably cylindrical and thinly furred tail, exhibiting minimal variation in fur length from the base to the tip. It possesses a distinct dorsal stripe, typically terminating between the hind legs. In terms of cranial features, P. ariel displays a significantly smaller maximum skull length, rostral height, intra-orbital width, and rostral width compared to the other new Australian species. Additionally, its pelage tends to display warmer tones in comparison to *P. breviceps*. The savannah glider

is completely separated from the other two species, restricted to the northern part of Australia (see red distribution in Figure 2) (Cremona et al. 2021).

Petaurus notatus potentially has the widest distribution among the three species, as it is distributed in the eastern part of Australia and in Tasmania (see blue area in Figure 2). It is distinguished by its elongated tail, featuring longer fur at the base that gradually shortens towards the tip. A well-defined middorsal stripe is evident, diminishing gradually as it extends towards the hind legs. *P. notatus* typically exhibits a larger intra-orbital width compared to the other two Australian species. While nasal width is smaller than in *P. breviceps*, other cranial parameters remain similar. Its pelage is characterized by a greyish pelage, with a distinctive white tip at the tail (Cremona et al. 2021).

Petaurus breviceps, on the other hand, exhibits less variability, largely attributed to its confined distribution. The distribution range extends across eastern coastal areas of Australia (see green distribution area in Figure 2) and there is a possibility of overlap with *P. notatus* at the borders of their distributions. Its dorsal stripe is less pronounced and usually concludes before reaching the hind legs. Skull size is smaller, with a relatively larger nasal width compared to *P. notatus*. Zygomatic arches tend to be smaller in this species, and its pelage typically ranges from grey to brown (Cremona et al. 2021).

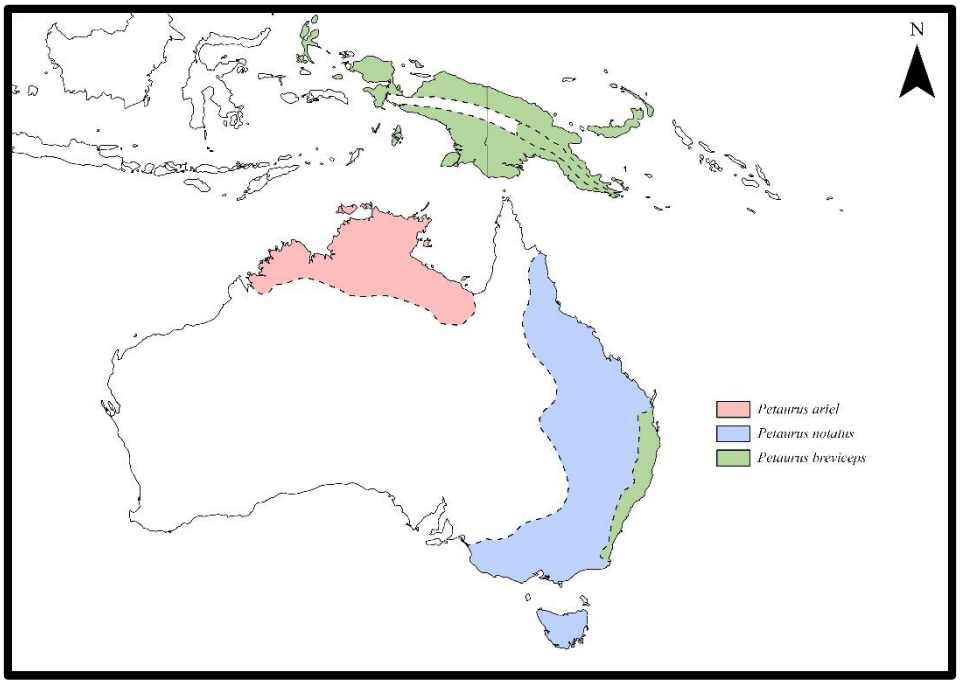

Figure 2: Proposed distribution of *Petaurus breviceps* together with new Australian species (Smith 1973; Cremona et al. 2021). The map was redrawn in the ArcGIS Pro 3.0.2 software (Esri 2022, 2023).

On the contrary, no recent taxonomic revision was conducted in the case of the subspecies located on the islands of New Guinea. Therefore, the classification is still based on subtle differences in external morphology, including variations in coloration and body size (Smith 1973; Malekian et al. 2010). The proposed subspecies were *P. b. flavidus*, *P. b. papuanus*, *P. b. tafa*, and *P. b. biacensis* (Smith 1973). However, the *P. b. biacensis* was later elevated to the species level and currently, it is just provisionally retained as a species by the IUCN Red List and taxonomic revision is advised to fully understand its relationship to *P. breviceps* (Leary et al. 2016a).

In this study, the main focus was on the sugar glider (*Petaurus breviceps* s.l.) due to the fact, that it was thought to be the species kept in captivity all around the world. The origin of captive animals was also uncertain, but it was proven to be Indonesian in the case of the population in the USA (Campbell et al. 2019).

2.3. Sugar Glider (*Petaurus breviceps*)

The uncertainty about the ecology of the species arises, as all information were described at the time before the recent split into three distinct species (Cremona et al. 2021). Therefore, the information below summarises knowledge about *Petaurus breviceps* s.l.

It occupies an insectivore-exudivore feeding niche throughout many habitats including various types of forest, such as wet and dry sclerophyll forests, primary montane forests, or open woodlands with the preference for vegetation mainly composed of Acacia species (Smith 1973; Suckling 1984; Quin 1995; Jackson & Schouten 2012). In the study of Jackson (2000b), the presence of sugar gliders was highly associated with a lot of stems, especially with *Corymbia intermedia* and *Acacia mangium* together with other potential food sources, rainforest vegetation, and a dense mid and upper canopy cover.

In the wild, their energy needs are fulfilled by exudates like *Acacia* gum, eucalypt sap, or nectar, while invertebrates and pollen contribute to their protein requirements (Smith 1982; Quin 1995). Their diet adaptation includes the ability to enter torpor to cope with food source shortages (Körtner & Geiser 2000).

Open woodland habitats are crucial for the species, as they rely on tree hollows for nesting, breeding, and refuge (Quin 1995; Campbell et al. 2018). The species exhibits a polygynous mating system with social groups typically comprising several males, females, and offspring, often led by one or two dominant males (Suckling 1984; Jackson & Schouten 2012). These dominant males take on primary roles in various social activities, including mating, territory maintenance, patrolling, and displaying aggression toward individuals from outside the group (Malekian et al. 2015). Body weight fluctuates throughout the year, with no distinct breeding season; on average, sugar gliders produce litters of one or two offspring and can raise two litters of young per year (Jackson 2000c).

2.3.1. Communication

Similar to many other nocturnal animals, the sugar glider relies on a sophisticated chemical communication system for social interactions. Vocalizations, visual signals, and odors are employed to convey individual-specific and community-specific information, with odor being the primary medium for community-level communication (Smith 1973). Secretions from the scent glands, as well as urine, may serve a crucial role in marking and defining the boundaries of their home range (Goldingay 1994). Males generate at least three distinct odors (frontal, sternal, and urogenital), while females produce two (pouch and urogenital) (Smith 1973).

2.3.1.1. Vocalization

Vocal communication in sugar gliders exhibits a diverse range of sounds (Raftery 2015). There are several types of vocalizations, known as crabbing, purring/chirping, hissing, and barking (Smith 1973; Raftery 2015). These signals are associated with various types of behavior. The crabbing sound is used when they are feeling threatened, purring signals satisfaction of the animal, hissing is associated with grooming behavior, and the barking call was proposed to serve as an alarm call among other purposes (Smith 1973; Goldingay 1994; Raftery 2015).

The barking call also signifies curiosity, and it is also expressed during playful behavior (Smith 1973; Goldingay 1994; Raftery 2015). The alarm call usually starts at full volume, gradually reducing its volume to faint grunts, and can be heard over a distance of 200 meters during the night. The calls are more prevalent in the early part of

the night, with individuals often engaging in repeated calls lasting for 20-30 minutes without interruption (Goldingay 1994).

2.3.2. Conservation & Threats

Global extinctions represent a significant threat to biodiversity, with the modern extinction rate for mammals estimated at about 100 genera extinctions per million genera years (Pimm et al. 2014). In Australia, which experiences one of the highest proportional extinction rates globally, more than 30 out of 100 endemic species already extinct (or extinct in the wild) are mammals (Woinarski et al. 2019).

Despite being classified as Least Concern on the IUCN Red List with a stable population trend, the Sugar glider faces various threats to its survival (Salas et al. 2016). Due to the similar life histories and nature, these threats might be crucial also for the survival of the new species, the savannah glider and the Krefft's glider. This could cause a potential problem in future conservation activities, as their conservation status was not assessed yet and there is overall big lack of knowledge about these species.

Factors such as land clearing, the devastating 2019–2020 mega-fires, climate change, and introduced predators collectively raise concerns about its ongoing viability (Pimm et al. 2014; Woinarski et al. 2019; Legge et al. 2022). The wildfires in southeastern Australia alone burned over 10 million hectares (Jolly et al. 2022); impacting 832 native vertebrates, with 70 taxa losing more than 30% of their habitat (Ward et al. 2020). Gliders, in particular, are adversely affected by fires due to direct mortality and the loss of critical resources, such as hollow-bearing trees (Jolly et al. 2022).

2.3.2.1. Habitat Fragmentation

Habitat fragmentation and barriers impact arboreal gliders, like the sugar glider, by extending their glide distance threshold and hindering movement between trees (Caryl et al. 2013; Knipler et al. 2022). Deforestation-induced fragmentation can lead to population isolation, reduced gene flow, genetic drift, decreased genetic diversity, and an increased risk of extinction due to the inability of populations with low genetic diversity to adapt to environmental changes (Malekian et al. 2015; Knipler et al. 2021a, 2022; Gracanin et al. 2023). Tree cover gaps act as barriers to the movement of gliding

marsupials, forcing them to traverse their habitat on the ground where they are more vulnerable to predators and vehicles (Caryl et al. 2013; Knipler et al. 2021a).

Studies, such as that of Knipler et al. (2021a), have identified infrastructure like the Pacific Motorway and pine plantations as dispersal barriers, limiting gene flow among sugar glider populations (Malekian et al. 2015). Isolated populations often experience higher rates of inbreeding, leading to a decrease in body size and genetic diversity (Malekian et al. 2015; Gracanin et al. 2023). Malekian et al. (2015) examined the effects of landscape on the genetic structure of sugar gliders. They estimated the average allelic diversity between 3.46 to 9.87. Within each patch, the heterozygosity was moderate to high with an average of 0.68 among all loci. Also, the degree of relatedness within nests was measured. In nest boxes, the degree of relatedness between adults of the opposite sex was found to be lower compared to pairs of adult males or females. The occurrence of unrelated males and females as potential mates within nest boxes may stem from the species' inherent inclination to select unrelated partners as a mechanism to prevent inbreeding. Sugar gliders employ various mechanisms to mitigate inbreeding, such as male-biased dispersal, a higher number of male migrants than females, and mate selection to avoid kin.

Furthermore, high levels of fragmentation increase the potential for hybridization with conspecific species, as observed between *Petaurus breviceps* and *P. norfolcensis*, resulting in fertile offspring (Colgan & Flannery 1992; Knipler et al. 2021b), posing additional challenges for species conservation.

2.4. Taxonomic Challenges in Ex-situ

The management of breeding programs aims to maintain genetically, physically, and behaviourally healthy populations that closely resemble those from the wild (Hvilsom et al. 2013). This is important as captive populations may serve as reservoirs for potential reintroduction programs.

Conservation breeding typically adopts two approaches. One approach segregates individuals based on naturally isolated populations, while the other combines individuals to avoid inbreeding and preserve genetic diversity (Hvilsom et al. 2013). However, this poses the significant challenge of potential outbreeding depression and hybrid sterility

(Schmidt et al. 2015; Palmer et al. 2021). The occurrence of hybrids, particularly when cryptic features may lead to taxonomic misclassification, can decrease the overall conservation value of the collection (Schmidt et al. 2015; Shirley et al. 2015). Therefore, identifying hybrid individuals in ex-situ facilities is crucial for conservation purposes, especially considering that the survival chances of hybrids in such facilities are higher compared to the wild (Modesto et al. 2018). For instance, anthropogenic hybridization (hybridization of species that are geographically isolated but not reproductively) between the African penguin (*Spheniscus demersus*) and the Humboldt penguin (*S. humboldti*) occurred in a penguin colony (Modesto et al. 2018).

Misidentification of species has happened in several cases. One of the examples is the case of olinguito (*Bassaricyon neblina*), as it was identified as olingo (*Bassaricyon* sp.), until the taxonomic revision of this genus (Helgen et al. 2013). Moreover, due to the genetic screening of the Philippine crocodiles (*Crocodylus mindorensis*), it was discovered that one individual was wrongly assigned to this species even though it was Western Nile crocodile (*C. niloticus*) (Hauswaldt et al. 2013). Most recently, the species' misclassification might have occurred in the case of a Zoo population of the southern tree hyraxes (*Dendrohyrax arboreus*) (Schneiderová et al. 2024). However, their vocal repertoire doesn't include typical components of southern tree hyraxes, therefore, Schneiderová et al. (2024) proposed the possibility, that this Zoo population might be misclassified and includes conspecifics from the uniquely vocalizing population of hyraxes from Taita Hills, Kenya (Rosti et al. 2020).

The situation in African dwarf crocodiles (*Osteolaemus* spp.) was more problematic. *O. tetraspis* is among the most commonly held crocodile species worldwide (Ziegler et al. 2017). Recent findings revealed three cryptic species within this genus (Eaton et al. 2009), with only slight differences in cranial characteristics (Schmidt et al. 2015). Subsequent studies focused on species determination and possible hybridization within this genus (Franke et al. 2013; Schmidt et al. 2015; Shirley et al. 2015). The study of Schmidt et al. (2015) underscored the importance of molecular genetics in zoological garden breeding programs, revealing that almost 30% of individuals within the European Association of Zoos and Aquaria (EAZA) and the Association of Zoos and Aquaria (AZA) institutions are hybrids. Comparison with genealogical records confirmed that

these hybrids were F1, F2, or backcrosses, as no wild-caught hybrid individuals (F0) were identified.

Hybridization of subspecies has also occurred in chimpanzees (*Pan troglodytes*) (Hvilsom et al. 2013). Hvilsom et al. (2013) confirmed that the majority of founders of the EAZA population originated from West Africa, with 40% of all genotyped individuals. However, they also discovered that 23% of individuals are hybrids with part of *P. t. verus* ancestry.

It is important to integrate phylogenetic relationships between species or subspecies into conservation breeding programs to prevent hybridization. Therefore, understanding the origin of specimens is crucial (Franke et al. 2013) to avoid mixing populations from different geographical locations, which could lead to outbreeding depression or hybridization. In the case of sugar gliders (*Petaurus breviceps* s.l.), where phylogenetic relationships and taxonomy are not fully understood, assessing their origin is particularly important.

In the USA, Campbell et al. (2019) conducted a study to determine the origin of captive sugar gliders in relation to illegal wildlife trade. The origin was confirmed to be from Indonesia, thus illegal activity was not substantiated. In opposite, the origin of sugar gliders kept across Europe remains uncertain. Moreover, assessing the origin of the European population is crucial, especially in light of recent findings, such as the discovery of new cryptic species (Cremona et al. 2021).

3. Aims of the Thesis

The aims of this study encompass two primary goals: first, to determine the species and the origin of captive-bred sugar gliders in Europe through the utilization of molecular genetics; second, to determine the species specificity of barking calls from available recordings from wild populations and to compare them with recordings from the captivity.

4. Methods

4.1. Sample Collection

Genetic samples were obtained from five zoological gardens and seven private breeders from four European countries. In total, 76 samples were collected, comprising 74 buccal swabs and two tissue samples, with 41 samples originating from zoological gardens and 35 from private breeders. The locations of all samples are visualized in Figure 3. All samples collected within this study are listed in Table 9 in Appendix 1.

For bioacoustic analysis, recordings were gathered from seven groups across sampled localities using Song Meter SM 4 and Song Meter Mini recorders (Wildlife Acoustics, Concord, Massachusetts, USA). The recorders were configured to record at a sampling rate of 48 kHz and 16-bit resolution for several days in each group (For detailed information see Table 1). The recorders were set up to record for 12 hours with 15-minute recording periods and a one-minute break according to the day/night light schedule of the respective studied group.

For comparative analysis with wild sugar gliders, 91 recordings were sourced from the iNaturalist.com website (https://www.inaturalist.org/) up to February 29th. Out of these, 71 recordings were analyzed and 20 were not used due to low quality, species misidentification, and copyright. Furthermore, the recordings were assigned to the Krefft's glider (*P. notatus*) or the sugar glider (*P. breviceps*) based on the location, where the recordings were recorded, and the proposed distribution of the species. The list of recordings, including their respective authors, is provided in Table 10 in Appendix 2. The locations of all recordings are visualized in Figure 4.

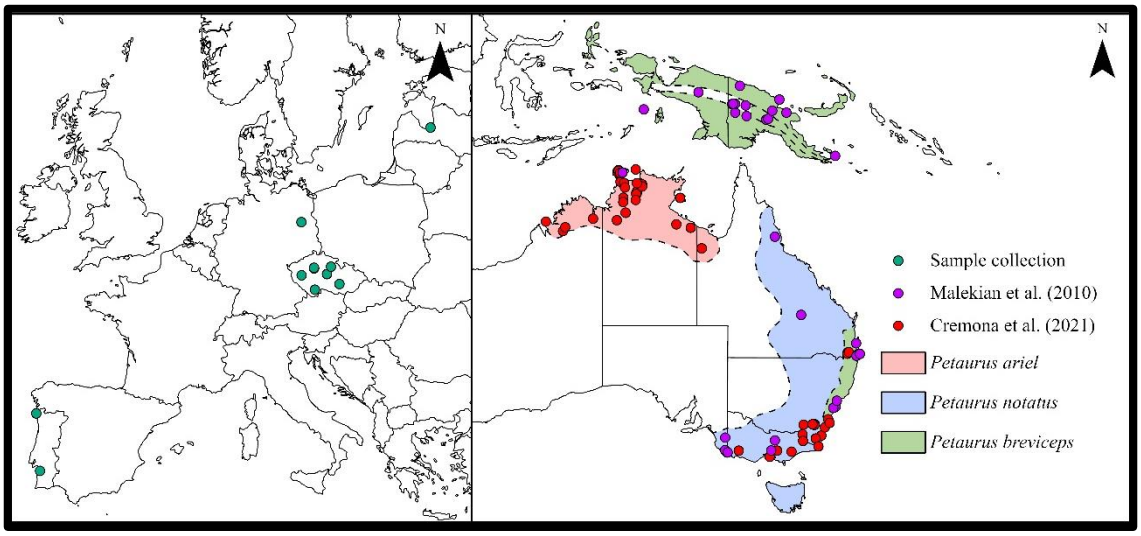

Figure 3: Map of sample collection, including locations of samples from wild specimens (Malekian et al. 2010; Cremona et al. 2021). Created in ArcGIS Pro software 3.0.2 (Esri 2022, 2023).

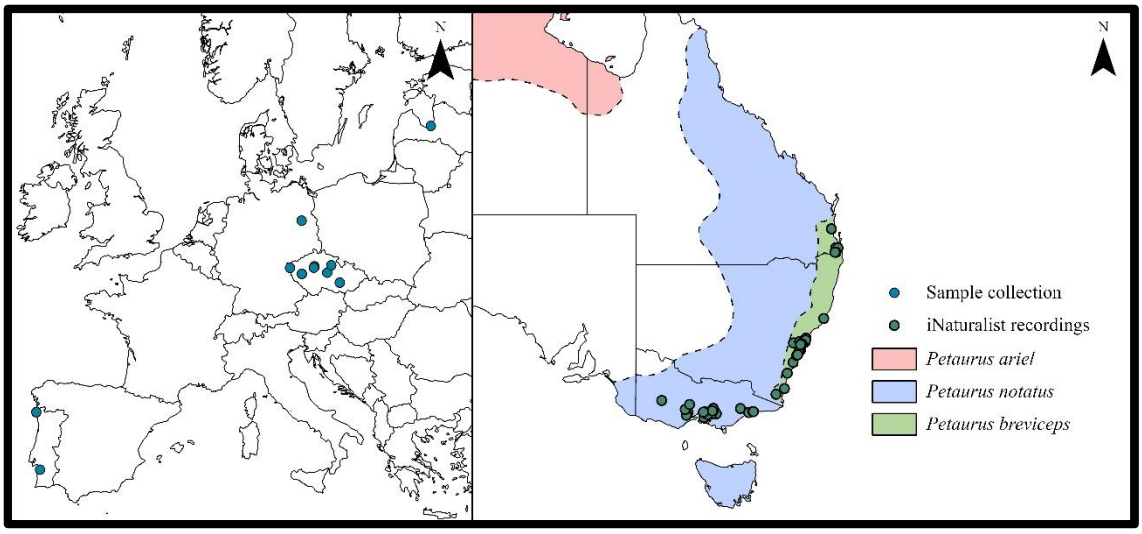

Figure 4: Map of recordings from Europe, including recordings from iNaturalist of the wild sugar glider (*Petaurus breviceps*) and Krefft's glider (*P. notatus*) from Australia. Created in ArcGIS Pro software 3.0.2 (Esri 2022, 2023).

Table 1: Detailed information about study groups of captive sugar gliders (*Petaurus breviceps*), including location and the size of each group; and recording, including the period and schedule of recording, and the total number of recorded recordings per each location with the number of detected barking calls.

Study group			Acoustic sampling			
Location	Species	N	Period	Schedule	Total	Detected
Berlin	*Petaurus breviceps*	10	23/10/2023-05/11/2023	9:00 - 21:00	624	33
	Petaurus breviceps	4	06/11/2023-07/11/2023	9:00 - 21:00	96	1
Brno	*Petaurus breviceps*	2	03/08/2023-18/08/2023	19:00 - 07:00	720	0
Melides	*Petaurus breviceps*	12	19/09/2023-03/10/2023	19:00 - 07:00	672	44
Pilsen	*Petaurus breviceps*	5	28/04/2023-04/05/2023	19:00 - 07:00	288	7
Prague	*Petaurus breviceps*	3	10/5/2023-01/06/2023	09:00 - 21:00	1065	118
Riga	*Petaurus breviceps*	15	04/11/2023-18/11/2023	10:00 - 22:00	672	35
Vila Praia de Ancora	*Petaurus breviceps*	5	22/09/2023-23/09/2023	19:00 - 07:00	76	10

4.2. Laboratory Work

4.2.1. Extraction of DNA

All genetic samples underwent processing at the Laboratory of Molecular Genetics (Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague), and genomic DNA was extracted using the Presto™ Buccal Swab gDNA Extraction Kit from Geneaid for buccal swabs and the DNeasy Blood & Tissue Kit from Qiagen for tissue samples. All procedures outlined in the attached protocols were followed. DNA extracted from buccal swabs was eluted into 30 µl of Elution buffer, while DNA from tissue samples was eluted into 100 µl of buffer AE.

Subsequently, DNA concentration was determined through spectrophotometric measurement using a NanoDrop One (Thermo Fisher Scientific™) at λ=260 nm, and the obtained DNA was stored in a freezer at -20°C.

4.2.2. Markers

The selected markers included two mitochondrial genes (NADH dehydrogenase subunit 2 - ND2; NADH dehydrogenase subunit 4 - ND4) and two nuclear genes (ω-globin, von Willebrand factor - vWF) were chosen based on the previous research focusing on sugar gliders (Malekian et al. 2010; Campbell et al. 2019; Cremona et al. 2021). All primers are listed in Table 2.

Table 2: List of markers used in this study.

Name	Targeted genes	Primer sequence (5'→3')	Author
mmND2.1	ND2	GCACCATTCCACTTYTGAGT	Osborne & Christidis (2001)
mrND2c	ND2	GATTTGCGTTCGAATGTAGC AAG	Osborne & Christidis (2001)
mt10812H	ND4	TGACTACCAAAAGCTCATGT AGAAGC	Arevalo et al. (1994)
mt11769L	ND4	TTTTACTTGGATTTGCACCA	Arevalo et al. (1994)
G807	vWF	GACTTGGCYTTYCTSYTGGA TGG	Amrine-Madsen et al. (2003)
G2526	vWF	TTGATCTCATCSGTRGCRGG ATTGC	Amrine-Madsen et al. (2003)
G314	ω-globin	GGAATCATGGCAAGAAGGTG	Wheeler et al. (2001)
G2526	ω-globin	CCGGAGGTGTTYAGTGGTAT TTTC	Arevalo et al. (1994)

4.2.3. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was employed to amplify specific DNA regions based on the selected primers. For each reaction, a 25 µl reaction mixture was prepared, consisting of 12.5 µl PPP Master Mix (Top-Bio), 8.5 µl of RNase-free water, 1 µl of forward primer, 1 µl of reverse primer, and 2 µl of extracted DNA. The T100 Thermal Cycler (BIO-RAD) was utilized following adjusted protocols from previous studies (Malekian et al. 2010; Campbell et al. 2019; Cremona et al. 2021) according to the markers. The protocol details are outlined in Table 3.

To validate the PCR results, gel electrophoresis was conducted using a 1% agarose gel. Gel electrophoresis is a technique for visualizing DNA on a gel by applying an electric current and utilizing fluorescent dyes for detection.

Table 3: PCR protocol used in this study.

Step	Temperature (°C)	Time (min)	Marker
1.	95	9	All
2.	94	0:45	All
3.	50	0:45	ND2
	54	0:45	ND4
	60	0:45	ω-globin
4.	72	0:45	All
5.	Go to step 2., 34x		
6.	72	10	All
7.	12	Forever	All

4.2.4. Purification

The successfully amplified fragments were purified using the protocols provided with the Gel/PCR DNA Fragments Extraction Kit (Geneaid). Subsequently, the concentrations of the PCR products were measured using the NanoDrop One (Thermo Fisher Scientific™).

4.2.5. Sequencing

The successfully amplified and purified DNA fragments were prepared for sequencing. Sequences were generated by the Sanger sequencing method with the respective forward primers for each marker (ND2 - mmND2.1; ND4 - mt10812H; ω-globin - G314). The sequencing process took place in the service laboratory at the Faculty of Science, Charles University.

4.3. Data Processing and Analysis

4.3.1. Genetic Data

The sequences were manually edited and aligned using Geneious Prime 2023.1.2 software (www.geneious.com). For the sequence alignment, we have applied the Clustal Omega 1.2.2 (Sievers et al. 2011; Sievers & Higgins 2018; Sievers et al. 2020). In addition, sequences of wild (Malekian et al. 2010; Cremona et al. 2021) and captive (Campbell et al. 2019) sugar gliders were downloaded from NCBI GenBank. All sequences used in this study are listed in Table 11 in Appendix 3.

Geneious Prime 2023.1.2 software (www.geneious.com) was also used in order to perform phylogenetic analysis for the ND2 gene and also for a combined dataset of all markers. Therefore, the MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001) plugin was used. Based on Malekian et al. (2010) *Petaurus abidi*, the closest sister group, was used as the outgroup. The substitution model was determined by jModelTest (Guindon & Gascuel 2003; Darriba et al. 2012). The TIM2+I+G was determined as the most suitable, however, due to the unavailability of this model in the Geneious Prime 2023.1.2 software the GTR substitution model was selected, as it was the second most suitable model. Lastly, gamma variation, 1.1 million chain length, 200 subsampling frequency, and burn-in length of

100,000 were chosen for the construction of trees. The FigTree v1.4.4 (Rambaut 2018) was used for editing and visualization of the phylogenetic trees.

For each captive individual, sequences of ND2 and ND4 genes were concatenated to a total length of 1465 bp, and the assessment of the diversity of captive populations was conducted via DnaSp6 v6.12.03 (Rozas et al. 2017), where the number of haplotypes (N_h), haplotype diversity (H_d), nucleotide diversity (Pi), and standard deviation of haplotype diversity were calculated.

The haplotype network was created by TCS v1.23 software (Clement et al. 2000). For the creation of the network the alignment of sequences of ND2 from Europe, USA, Indonesia, and Papua New Guinea was used. The sequences originating from Australia were excluded as well as those that were missing a larger part of the sequence. The number of mutation steps was set to 45. The haplotype network was visualized using the TCS Beautifier (Múrias Dos Santos et al. 2016).

4.3.2. Bioacoustic Data

For the acoustic analysis, our focus was on the barking call. This vocalization was selected, because it was proposed to serve as an alarm call, which is in other species, like ground squirrels (Schneiderová & Policht 2012), stable and species-specific call. The barking call was successfully recorded in all localities, except for Brno, where no sugar glider vocalization was detected. In total, 4213 recordings were obtained from 8 groups, with the barking call detected in 248 of them. Private breeders provided 7 recordings of barking calls from 3 sampled groups, and another 2 recordings from 2 groups not sampled for genetic samples were added to this study.

All recordings underwent analysis using Avisoft SASLab Pro 5.3.2 software (Avisoft Bioacoustics, Berlin, Germany). Recordings were imported into Avisoft SASLab Pro, converted to mono format if needed and the sampling frequency was reduced from 48 kHz to 12 kHz. In some cases, the "Change Volume" function was applied to amplify the barking calls. All obtained recordings were carefully checked, and every barking call was labeled. The labeled barking calls were visualized using oscillogram, power spectrum, and spectrogram using the following settings: FFT length 1024, frame size 25%, and Hamming window. These settings were chosen as they resulted in the most suitable resolution.

Eight parameters were selected for measuring: the total number of barks in a call (Nb), duration of the call (Dur), duration of individual barks (Dur_b), the interval between barks (Int), peak frequency (Peak_f), peak fundamental frequency (Peak_f0), and maximum fundamental frequency (Max_f0). All measured parameters with descriptions can be found in Table 4. The parameters were measured in all recordings manually. The recordings from captivity were averaged by the weighted mean of all recordings per group. Therefore, in total 12 recordings of captive sugar gliders were included in this study together with 38 recordings of wild sugar gliders and 33 recordings of Krefft's glider. Figure 5 illustrates the spectrogram with measured parameters for visual reference. Finally, the barking rate (Rate) was calculated as the number of barks divided by the duration of the whole call.

Table 4: The list of acoustic parameters measured from barking calls.

Call parameter	Description
Dur (s)	Duration of the call
Dur_b (s)	Duration of individual barks
Rate	Number of barks per second
Nb	The total number of barks in a call
Int (s)	Interval between barks
Peak_f (Hz)	Peak frequency
Peak_f0 (Hz)	Peak fundamental frequency
Max_f0 (Hz)	Maximum fundamental frequency

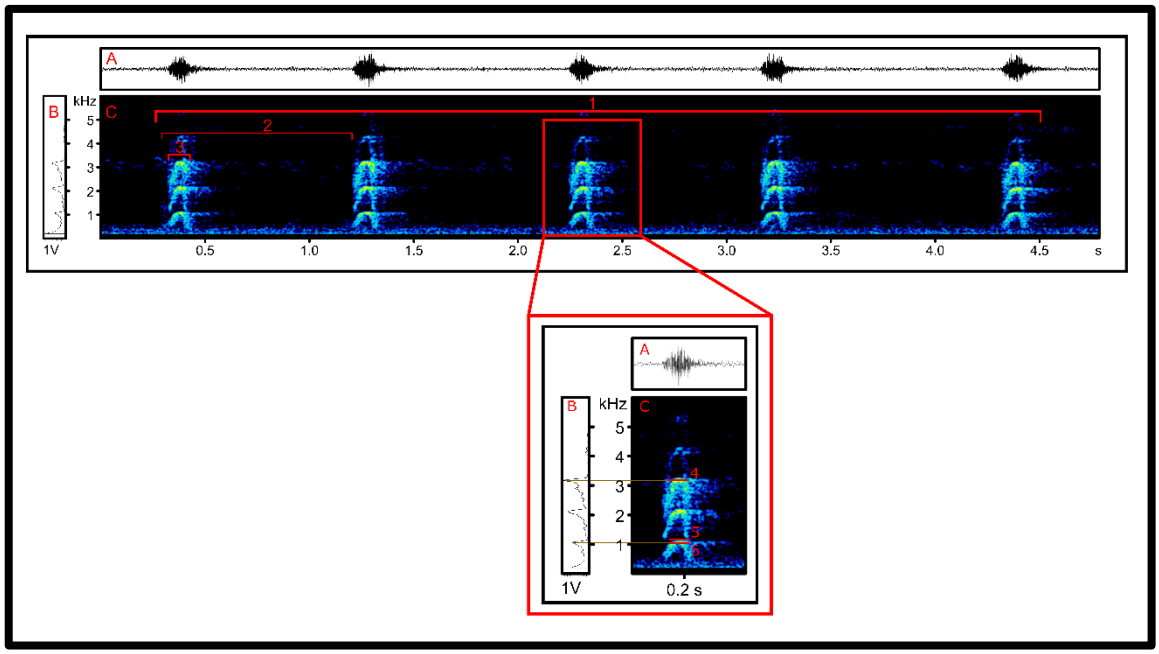

Figure 5: Visualization of the vocalization with measured parameters. The letters are assigned to three forms of sound visualization: A – oscillogram, B – power spectrum, and C – spectrogram. The parameters are labeled with numbers: 1 – call duration, 2 – interval, 3 – bark duration, 4 – peak frequency, 5 – maximum of fundamental frequency, and 6 – peak fundamental frequency.

All descriptive and statistical analyses were performed with the use of the R 4.3.3 software (R Core Team 2024) and 'AICcmodavg' (Mazerolle 2023), 'broom' (Robinson et al. 2023), 'car' (Fox & Weisberg 2019), 'carData' (Fox et al. 2022), 'corrplot' (Wei & Simko 2021), 'factoextra' (Kassambara & Mundt 2020), 'ggcorrplot' (Kassambara 2023a), 'ggfortify' (Tang et al. 2016; Horikoshi & Tang 2018), 'ggplot2' (Wickham 2016), 'ggpubr' (Kassambara 2023b), 'loadings' (Yamamoto 2023), 'magrittr' (Bache & Wickham 2022), 'readxl' (Wickham & Bryan 2023), 'rstatix' (Kassambara 2023c) and 'tidyverse' (Wickham et al. 2019) packages. For each measured and calculated acoustic parameter, mean, maximum, minimum, and standard deviation were calculated. Two measured acoustic parameters, the number of barks (Nb) and duration of the call (Dur), were excluded from subsequent analyses as the recordings from iNaturalist might not include the whole vocalization, and these parameters are thus not entirely reliable.

To compare the barking calls of three groups of sugar gliders from which the recordings were available (captive European population recorded in this study and Krefft´s glider (*Petaurus notatus*), and sugar glider (*P. breviceps*) from iNaturalist), one-way ANOVA was performed for each of the remaining variables (Rate, Dur_b, Int, Peak_f, Peak_f0, and Max_f0). All these variables were evaluated and tested for

assumptions of normal distribution and equal variance with the “Q-Q Plot” and “Residuals vs Fitted” Plot and the Shapiro-Wilks and Levene's tests; those variables that did not meet these assumptions were log-transformed. Therefore, the logarithmic transformation was applied to the Int and Peak_f parameters, but despite that, the Peak_f did not meet the normality. However, as the ANOVA is quite robust against violations of the normality assumption (Knief & Forstmeier 2021) this was not an obstacle to the application of this test.

The principal component analysis (PCA) was used to reduce the six measured and correlated acoustic parameters into uncorrelated principal components. The first two principal components were used to create a scatterplot in order to further visualize potential differences between the three groups of sugar gliders. Prior to running the PCA, variables were standardized by subtracting their means and dividing by their standard deviations. Hierarchical cluster analysis (CLU) was performed and a dendrogram was created for additional demonstration of the acoustic similarity of barking calls of the three studied groups of sugar gliders. Principal components with eigenvalues > 1 entered this analysis based on the unweighted pair group method with arithmetic mean (UPGMA) in which the Euclidean distances were used.

5. Results

5.1. Genetic Analysis

Sequences of the ND2 and ND4 mitochondrial genes were successfully obtained from all 76 samples. After sequence alignment and editing, a total of 666 bp of ND2 and 657 bp of ND4 were used for further analyses. In the case of ω-globin, due to low variability, only 23 individuals from seven out of the twelve different groups were sequenced, and 639 bp were used after editing. Amplification of the von Willebrand factor was unsuccessful in this study.

In the Bayesian phylogenetic trees, the proposed clades consisting of Papua New Guinea (PNG), Indonesia, and Australia by Malekian et al. (2010) were confirmed (Figure 7). The distinction between the PNG (brown) and Indonesian (orange) clades is supported by Bayesian high posterior probabilities (1) as well as the separation of the Australian clades (0.98). The differentiation between the newly described species, *Petaurus ariel* (red), *P. notatus* (blue), and *P. breviceps* (green), proposed by Cremona et al. (2021) was also confirmed (Figure 6) with high support (0.86 - 1). Our samples clustered together with the samples of the captive individuals from the USA and together they formed a monophyletic clade (yellow in Figure 6 and Figure 7), which is closely related to the Indonesian island Kai Besar Island, which is however geographically close to PNG. Australian genotypes are not represented within the captive populations nor the PNG/Indonesian clades. Therefore, it suggests the Indonesian/PNG origin of both captive populations.

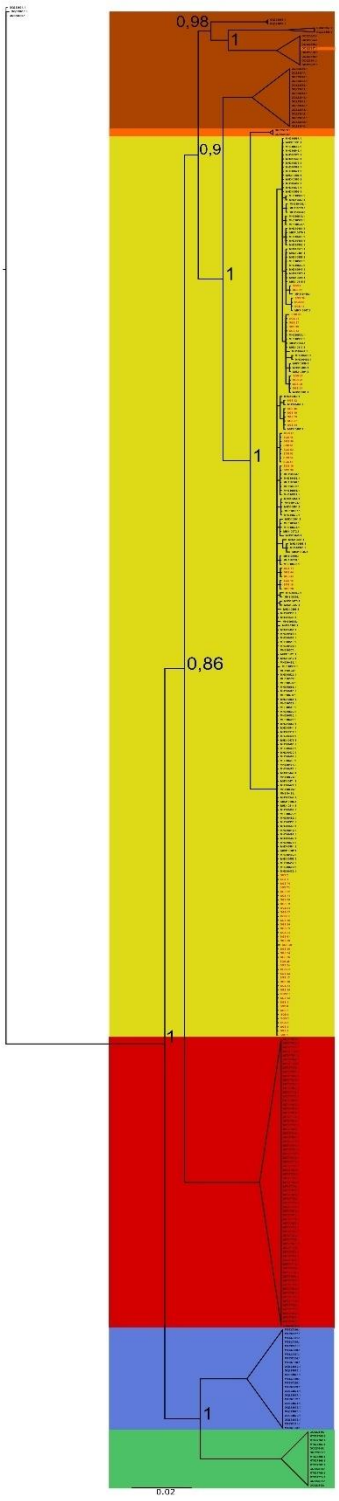

Figure 6: Bayesian phylogenetic tree based on 362 sequences of ND2 gene. The tree demonstrates the relationships among the captive populations (yellow) and wild populations of a sugar glider (*Petaurus breviceps*) from Indonesia (orange), Papua New Guinea (brown), and Australia (green), other species were assigned to the following color: the savannah glider (*P. ariel*) – red, and the Krefft's glider (*P. notatus*) - blue. The outgroup used to root the phylogeny was *P. abidi*. Bayesian posterior probabilities are indicated on the branch nodes.

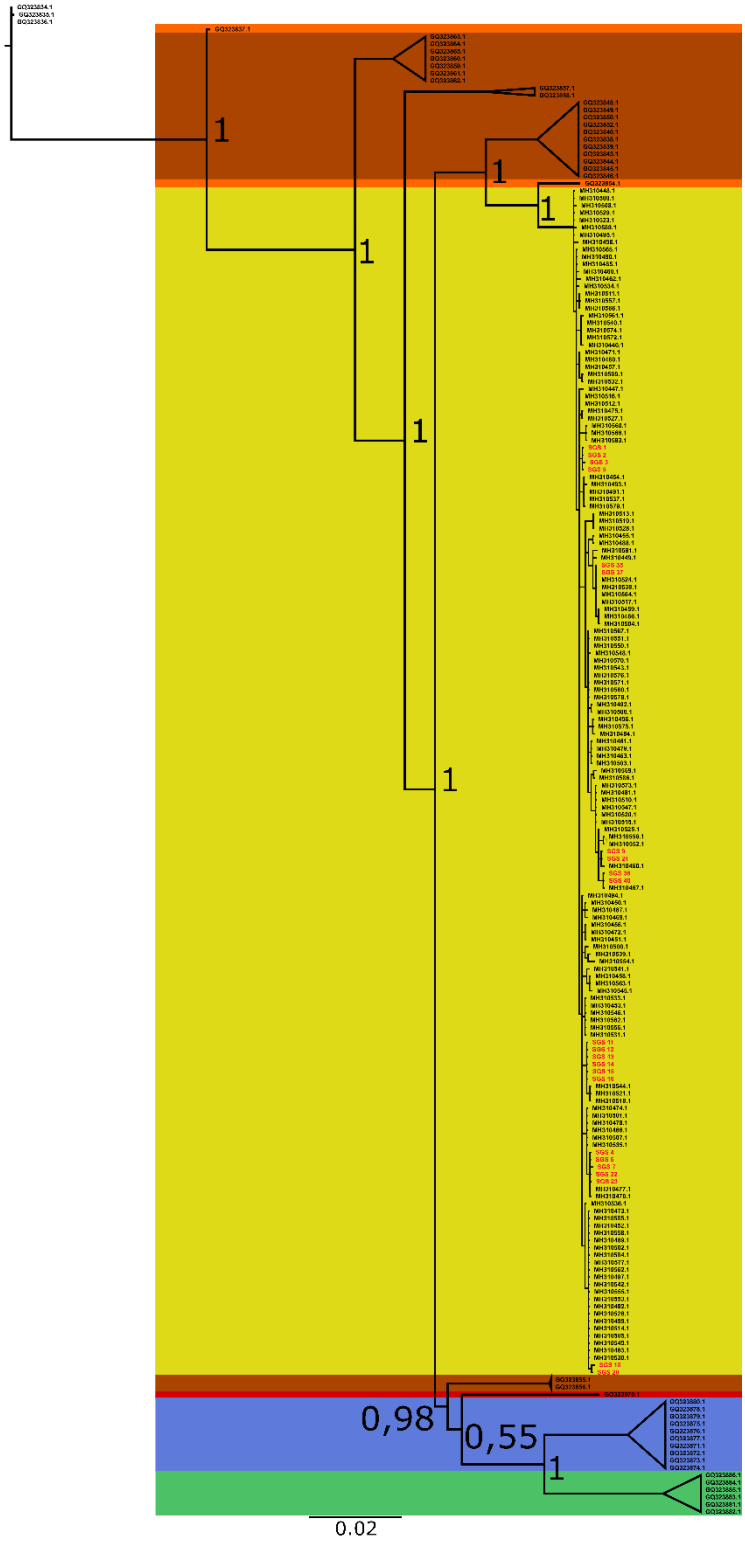

Figure 7: Bayesian consensus tree using 206 concatenated sequences of ND2, ND4, and ω-globin genes. The tree demonstrates the phylogenetic relationships between the captive populations (yellow) and wild populations of sugar gliders (*Petaurus breviceps*).

Genetic diversity analysis in the DnaSP found 45 and 17 haplotypes within the datasets of two captive populations from the USA and Europe, respectively. High values of haplotype diversity (H_d) were observed in both populations with nucleotide diversity (Pi) slightly higher in the USA population (Table 5).

Table 5: The summary statistics of two captive populations of *Petaurus breviceps*. N - number of individuals, N_h - number of haplotypes, H_d - haplotype diversity, Pi - nucleotide diversity, and Sd - standard deviation of haplotype diversity were calculated for the combined datasets of two mitochondrial genes ND2 and ND4.

Location	N	N_h	Hd	Pi	Sd
USA	141	45	0.955	0.00556	0.008
Europe	76	17	0.907	0.00473	0.017

For the haplotype network (Figure 8), 245 sequences of the length of 525 bp were used. Overall, 52 haplotypes were observed within the haplotype network, out of which 31 belonged to the captive populations from the USA and Europe (visualized with yellow and dark yellow color in Figure 8). The captive population differed by 17 mutation steps from the Indonesian population (orange color in Figure 8), where the sequences from Kai Besar Island are located. The closest haplotypes from PNG (brown color in Figure 8) were 33 mutation steps away from the haplotypes of both captive populations.

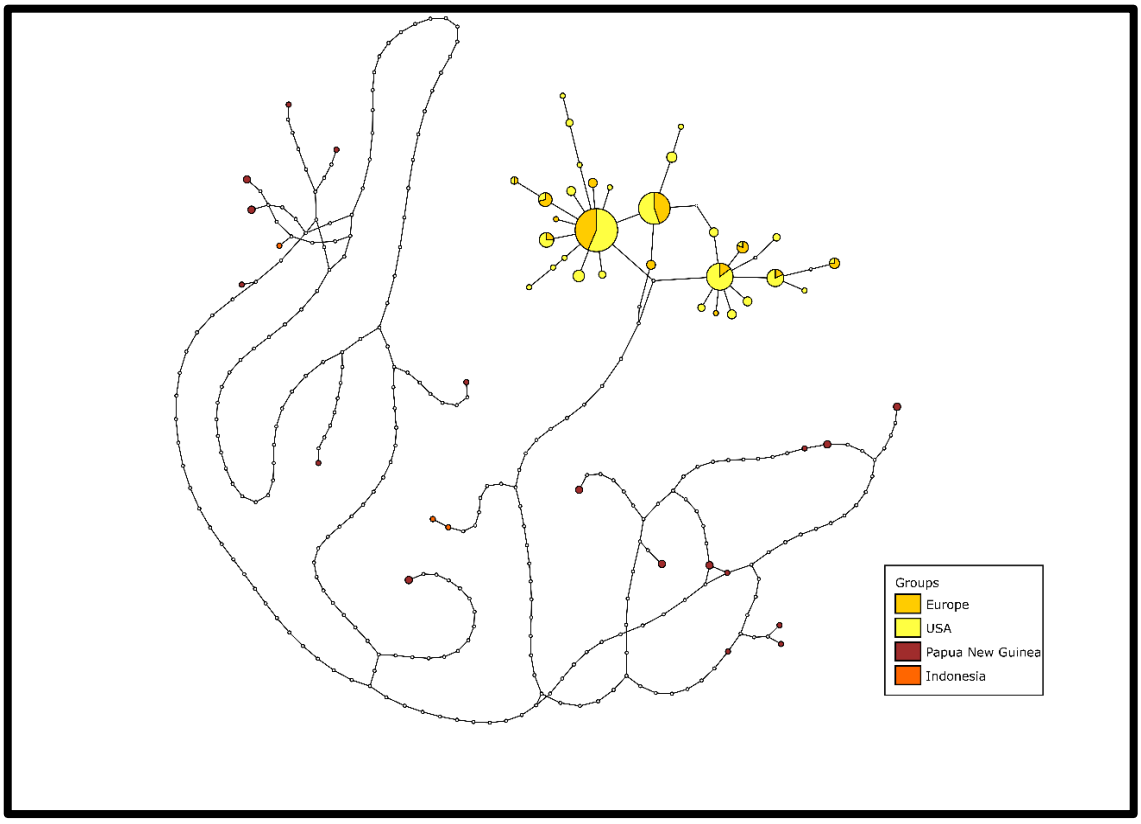

Figure 8: TCS haplotype network using ND2 gene including 76 sequences from Europe, 140 from the USA (Campbell et al. 2019), and 29 sequences from Indonesia and Papua New Guinea (Malekian et al. 2010). The circles represent haplotypes and are proportional to their frequency. Mutation steps are visualized with empty circles. Color codes of the circles represent the origin of the sequences.

5.2. Bioacoustic analysis

The descriptive statistics were conducted for each group separately. The descriptive statistics indicated similar values for all of the measured acoustic parameters. The barking call consisted of a maximum of 127 barks and a minimum of 2 barks with the longest call lasting 155 seconds and the shortest only 4 seconds. The highest number of barks per second was 1.58 and the lowest was 0.045. The longest duration per bark was 0.177 seconds and the shortest was 0.062 seconds. Moreover, the longest interval between barks was 3.11 seconds, while the shortest interval was 0.642 seconds. The highest peak frequency was emitted at 2432 Hz, while the lowest was 611 Hz. The maximum fundamental frequency was 1125 Hz along with 1066 Hz which was the peak of the fundamental frequency. On the other hand, the lowest maximum fundamental frequency was 670 Hz, and the lowest peak was 583 Hz. The descriptive statistics of each group are shown in Table 6.

Table 6: Descriptive statistics of all measured and calculated parameters from the barking call of wild Sugar glider (*Petaurus breviceps*), Krefft's glider (*P. notatus*), and the European captive population of Sugar glider.

Parameters	Sugar glider (*Petaurus breviceps*)					Krefft's glider (*P. notatus*)					Sugar glider - Europe (*P. breviceps*)				
	N	Max	Min	Mean	Sd	N	Max	Min	Mean	Sd	N	Max	Min	Mean	Sd
Dur (s)	38	62	5.36	22.3	12.7	33	74.6	4	24.8	17.5	12	155	4.17	71.4	48.1
Nb	38	78	6	21	15.9	33	66	3	17.9	13.9	12	127	2	48.7	42
Rate	38	1.58	0.422	0.926	0.332	33	1.25	0.443	0.749	0.213	12	1.08	0.045	0.652	0.285
Dur_b (s)	38	0.177	0.065	0.101	0.024	33	0.167	0.062	0.109	0.027	12	0.139	0.087	0.115	0.02
Int (s)	38	2.71	0.642	1.33	0.572	33	2.84	0.942	1.55	0.485	12	3.11	0.931	1.63	0.653
Peak_f (Hz)	38	2069	638	1278	442	33	2062	611	1298	408	12	2432	644	1634	592
Peak_f0 (Hz)	38	1066	636	823	116	33	1052	583	806	101	12	1038	612	860	130
Max_f0 (Hz)	38	1125	665	884	117	33	1100	670	871	98,4	12	1090	964	943	107

For most of the tested acoustic parameters, the one-way ANOVA did not find a significant difference between groups. The only parameters that differed significantly were Rate and Interval (Int). However, when comparing these parameters between the studied groups by Tukey post-hoc test it revealed that Interval is not significantly different among the groups. On the other hand, there was a significant difference in Rate. The parameter was significantly different between the recordings of wild sugar gliders and Krefft's gliders (see SG/KG in Table 7) and recordings of wild and captive sugar gliders (see SG/SGE in Table 7). The difference between Kreft's glider and the captive sugar glider is not significant (see SGE/KG in Table 7). These results suggest that the wild sugar gliders bark more rapidly in comparison to the two other groups (Figure 9).

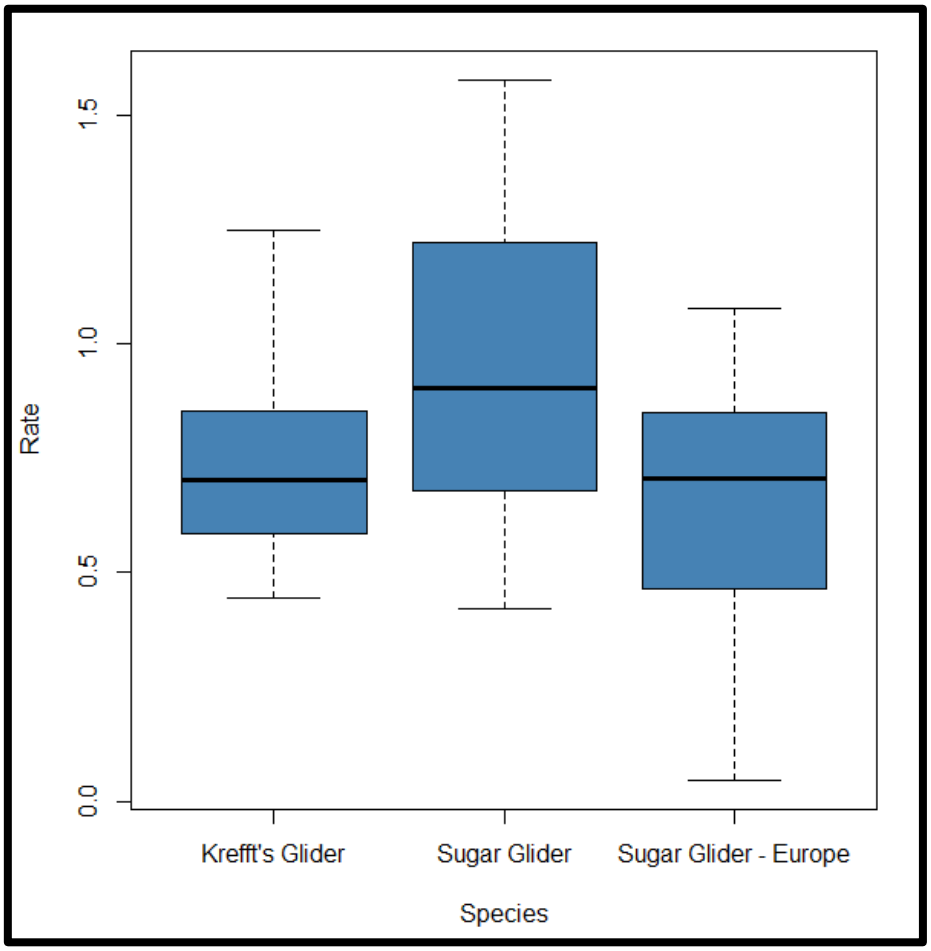

Figure 9: The boxplot graph demonstrating the differences among groups in the Rate parameter.

Table 7: Comparisons of the three studied groups using one-way ANOVA and Tukey post-hoc test for all parameters.

Call parameter	ANOVA			
	p-value (α=0.05)	SG/KG	SGE/KG	SGE/SG
Rate	0.00447	0.0278615	0.5676451	0.012455
Dur_b	0.158	-	-	-
Int	0.0418	0.0670745	0.9594987	0.1439394
Peak_f	0.14	-	-	-
Peak_f0	0.358	-	-	-
Max_f0	0.144	-	-	-

The correlations of the measured acoustic parameters are shown in Figure 10. The PCA extracted two principal components with eigenvalues > 1, which accounted for more than 70 % of the variability in the data. The first principal component was most strongly correlated with frequency parameters measured from the fundamental harmonic (Peak f0 and Max f0), while the second principal component was most strongly correlated with parameters corresponding to the call rate (Rate and Int; Table 6). A Scatterplot showing the position of each call within the dimension of these two principal components is shown in Figure 11. There are no clearly separated clusters formed by the three groups, thus, this plot also indicates that there are no remarkable differences in the barking calls of the three groups. Additionally, a dendrogram (Figure 12) based on hierarchical clustering did not show separated clusters that would correspond to the three studied groups of sugar gliders.

Table 8: Factor loadings of the first two principal components.

Parameters	PC1	PC2
Rate	-0.5073772	-0.7845980
Int	0.5208523	0.7997119
Dur_b	0.3956861	0.2818297
Peak_f	0.5752771	-0.4492381
Peak_f0	0.8567107	-0.4061585
Max_f0	0.8828756	-0.3621556

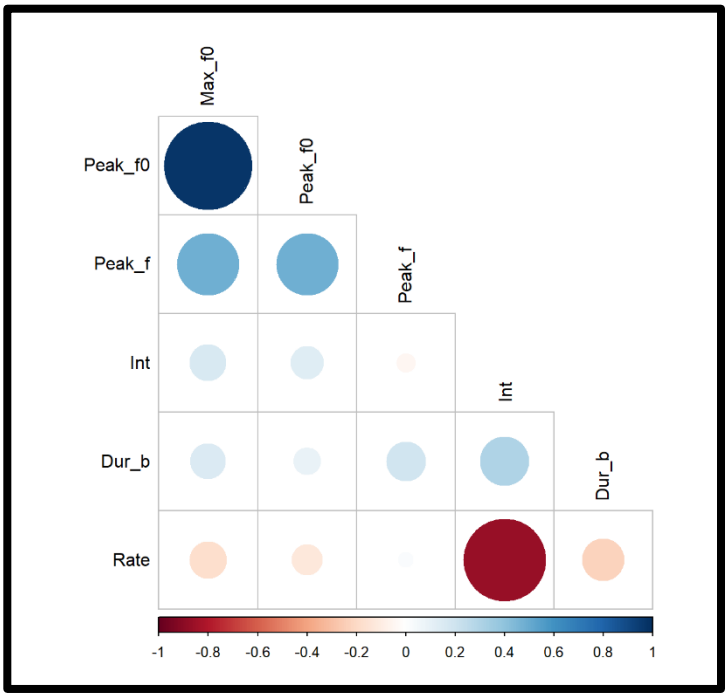

Figure 10: Correlation of the measured acoustic parameters.

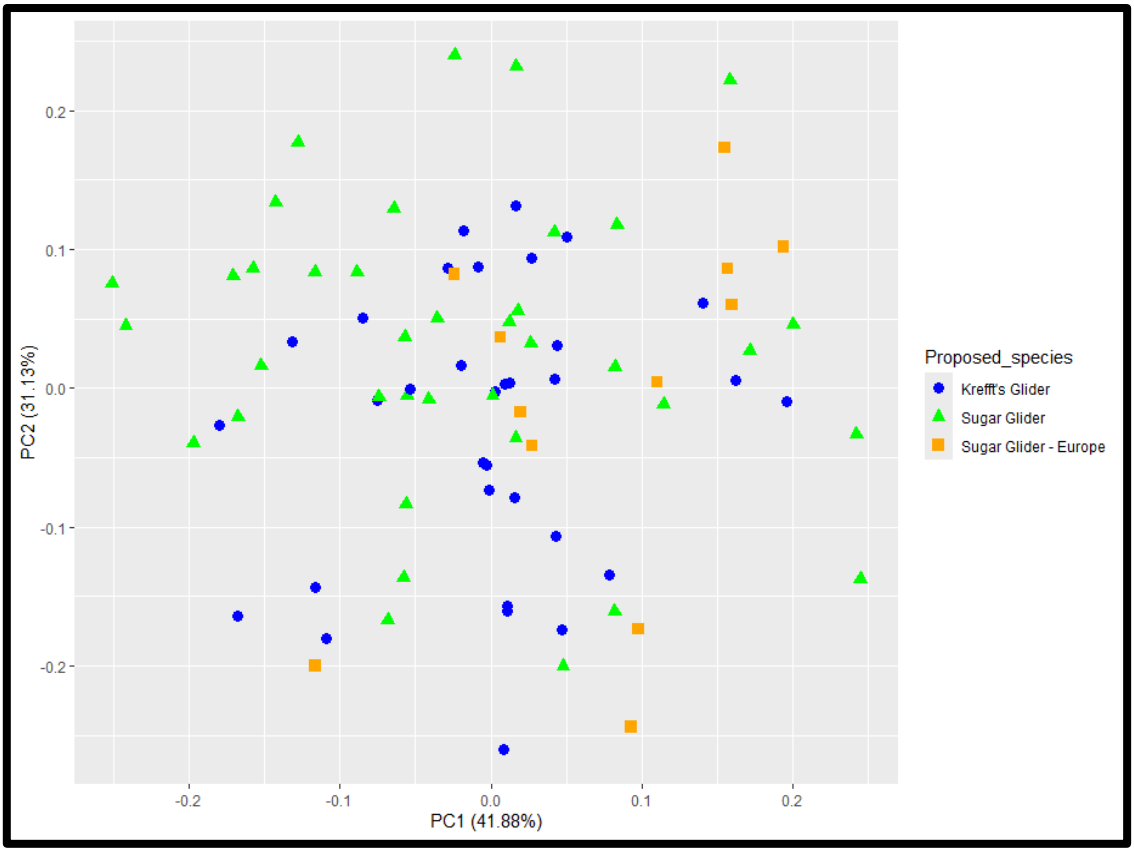

Figure 11: Scatterplot showing the position of each call within the dimension of the two principal components.

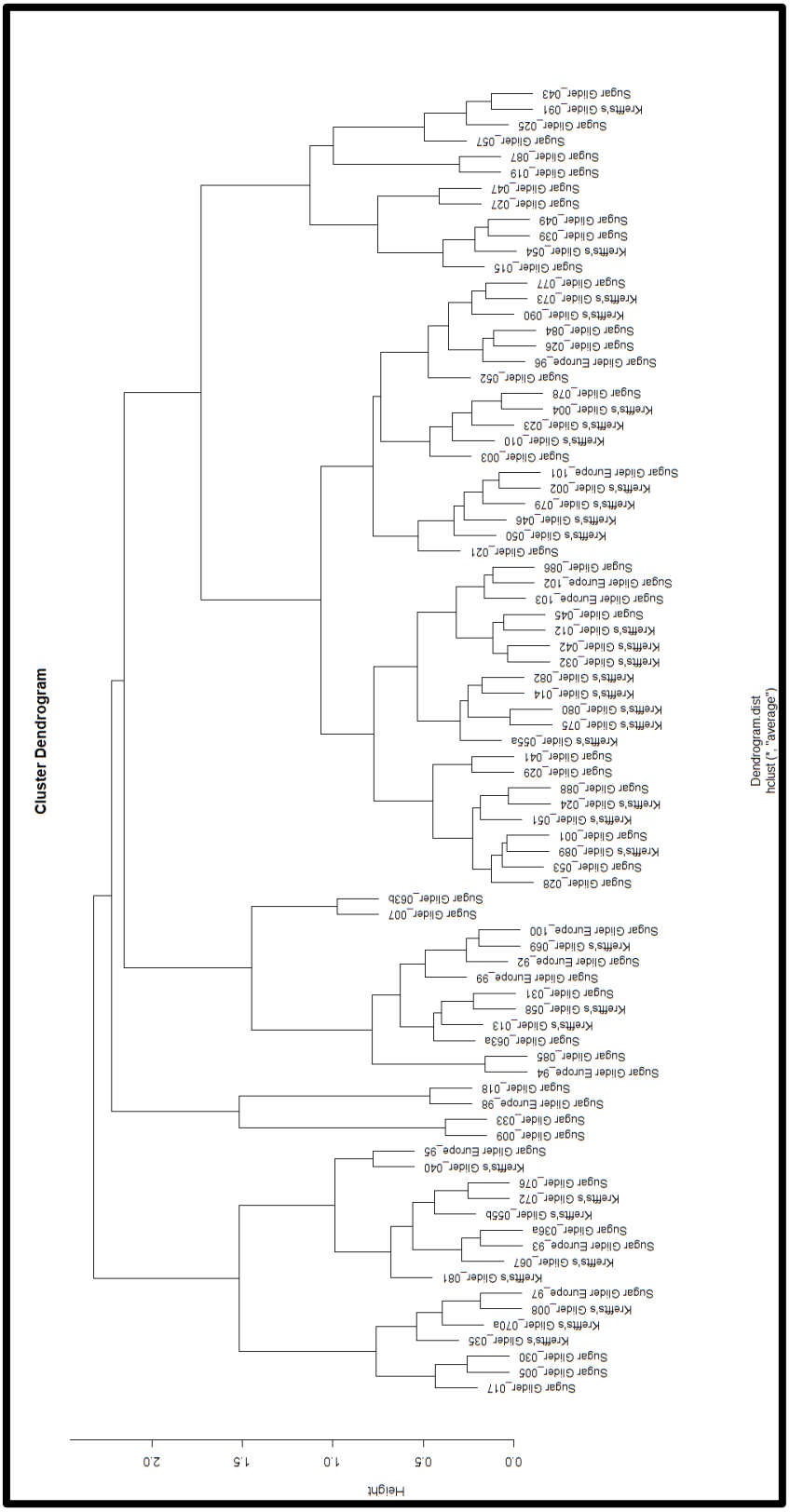

Figure 12: Dendrogram showing relationships among recordings.

6. Discussion

Correct species determination is crucial for the proper ex-situ management, and it is necessary to avoid admixture among differentiated clades or species, as was for example described in chimpanzees (*Pan troglodytes*) where subspecies were mixed, and it led to severe consequences in the captive population management (Hvilsom et al. 2013). Another case of species' misidentification and subsequent hybridization was reported in the African dwarf crocodiles (*Osteolaemus* spp.) (Franke et al. 2013; Schmidt et al. 2015; Shirley et al. 2015), which led to changes in the captive breeding management in order to prevent hybridization cases. Sugar gliders (*Petaurus breviceps* s.l.) recently underwent dramatic taxonomic revisions in the continental Australia, therefore, it was necessary to evaluate the origin and correct species assignment of the ex-situ populations because sugar gliders are very popular and very common pet animals.

The captive populations from Europe and the USA showed a strong affiliation in the phylogenetic trees, where these two populations formed a single cluster. The haplotype network also supported these findings because haplotypes are shared between the captive populations. Such a result indicates that the origin of both captive populations is very similar. Based on phylogenetic relationships, the specimens from Europe and the USA are closely related to the samples collected on Kai Besar Island which belongs to Indonesia and is in close proximity to New Guinea. However, the close relatedness of specimens from captivity to the individuals from Kai Besar Island could be caused due to the low number of samples from this particular locality (n=2). Moreover, the lack of samples from Indonesia and PNG could result in incorrect detection of the origin of the captive populations as none of the haplotypes from the captivity matches the haplotypes from the wild. Therefore, large-scale sample collection across the islands should be performed in order to find the source population of the captive populations.

The Indonesian/PNG origin is consistent with the findings of Campbell et al. (2019). There is no evidence of the captive populations originating in Australia, as the Australian species proposed by Cremona et al. (2021) are phylogenetically distinct with high posterior probabilities. Southeast Asia belongs to the wildlife trade hotspots as described by Nijman (2010). Campbell et al. (2019) hypothesize that the source location

of the USA population is Sorong, Indonesia, and based on our results, it is likely that the European population originates in the same locality.

Lack of knowledge about the origin of the founders of captive populations is very common (Cosson et al. 2007; Witzenberger & Hochkirch 2011), thus genetic methods are necessary to determine it. The origin of maternal lineage was possible to detect for example in binturong (*Arctictis binturong*) (Cosson et al. 2007), Angolan Colobus monkey (*Colobus angolensis*) (McDonald et al. 2023), or Galapágos tortoise (*Geochelone nigra*) (Burns et al. 2003). However, successful determination requires a good and full comparative database from the wild, otherwise, even genetic tests may fail to determine the origin (Pastorini et al. 2015).

In our study, high haplotype diversity was observed in both captive populations based on the mitochondrial ND2 and ND4 genes, with values of 0.955 for the USA population and 0.907 for the European population. On the other hand, the nucleotide diversity was relatively low, with slightly higher diversity in the USA population. In natural populations, this pattern often implies a recent demographic expansion of a population, when new haplotypes emerge rapidly without significant changes at the nucleotide level. However, in our case, it could be a result of repeated imports of animals from several source localities within a close geographical range.

Many captive populations are based on the low number of founders (Witzenberger & Hochkirch 2011), and together with the bottleneck effect and genetic drift, it often results in low genetic diversity which is considered as a threat to the survival of the population. This was observed for example in the Matschie's tree kangaroos (*Dendrolagus matschiei*), where only two mitochondrial control region haplotypes were found in the captive population most likely due to low founder diversity (McGreevy et al. 2009). Low haplotype diversity was also observed in Grevy's zebra (*Equus grevyi*) (Ito et al. 2017). Moreover, if the population is small and composed of related animals, the effect of inbreeding can rapidly decrease the viability of the captive population, as in the case of Asian lions (*Panthera leo leo*) (Atkinson et al. 2018). On the other hand, novel haplotypes were described by Farré et al. (2022) in the population of François' langur (*Trachypithecus francoisi*).

In the case of the European captive sugar gliders, there are no signs of decreased genetic variation, however, within each breeding group, most of the individuals shared

the same haplotype. Therefore, the exchange of individuals is highly recommended in order to sustain a genetically viable population.

The barking call is a very common sound produced by the species. It is a loud call and was proposed to serve as an alarm call (Smith 1973; Goldingay 1994; Raftery 2015). However, there is still uncertainty about its function. In our study, it was not possible to enhance the knowledge about the functions of the barking call given that the recording was done via automatic recording units, therefore without any monitoring of the animal's behavior during the vocalization.

The barking call is a loud stereotypical call, which is emitted in frequencies up to 6 kHz. The main characteristic is the repetition of uniform syllables - barks. The repeated syllables are harmonic and very variable, with a peak of fundamental frequency starting at 612 - 1066 Hz. The high variability is in all measured parameters and was demonstrated by the descriptive statistics. For example, the length of the vocalization differed from 4 to 155 seconds, with the length of a single bark lasting 0.062 to 0.177 seconds, and the range of barking rate was 0.045 to 1.58 barks per second. There was a relatively big discrepancy between the length of the call and the number of barks between the recordings from captivity and wild. It could have been caused due to the nature of the recording, as the recordings from iNaturalist are used in order to determine the observed (recorded) species, therefore the recordings are much shorter.

Loud (advertisement) and alarm calls are commonly used for communication over long distances in many nocturnal mammals (e.g. galagos) (Zimmermann 1990; Butynski et al. 2006; Masters et al. 2017; Bettridge et al. 2019), but also in some diurnal species (e.g. ground squirrels) (Matrosova et al. 2012; Schneiderová & Policht 2012; McRae 2020; Diggins 2021). In many species, they are stereotypic and species-specific, therefore they can be used in the species-determination process. This is commonly used for example in Galagos, where the species have been divided into five groups based on the type of their advertisement call (Masters et al. 2017). In some species, the loud calls differ mainly in the frequencies at which they are emitted (Schneiderová & Policht 2012), but also in intervals of the syllables (Zimmermann 1990; Bettridge et al. 2019). The species-specificity of these calls is easily recognizable, which is not the case of sugar gliders.

Even though there are big genetic differences between the sugar glider and Krefft's glider, the barking call sounds very similar, and there are barely any differences

even between the captive and wild animals. Further analysis performed within this study found that there is no significant distinction in the barking call among the species. The only parameter that showed a significant difference among groups was Rate. Therefore, the wild sugar glider barks more rapidly than other groups included in our study. This could be consistent with interspecific differentiation between *Galago crassicaudatus* and *G. garnetti*, whose vocalization significantly differed in shorter intervals between syllables within their loud calls (Zimmermann 1990). A similar case was discovered between the subspecies *Otolemur garnettii panganiensis* and *O. g. kikuyuensis* (Bettridge et al. 2019).

Moreover, several other studies described the distinction in vocalization between cryptic species. Such cases have happened for example in bats (Taylor et al. 2018), mouse lemurs (*Microcebus* spp.) (Hasiniaina et al. 2020), tree shrews (Esser et al. 2008), galagos (Zimmermann 1990; Bettridge et al. 2019; Pozzi et al. 2019, 2020), tarsiers (Burton & Nietsch 2010), and tree hyraxes (*Dendrohyrax* sp.) (Oates et al. 2022). Hasiniaina et al. (2020) described species-specific Tsak calls in eight species of the *Microcebus* genus. This speciation was proposed to be a result of genetic drift without any environmental influence. Similarly, Pozzi et al. 2020 described loud calls of Galago's (*Paragalago* spp.). The loud call of *P. zanzibaricus* had a different structure in comparison to *P. granti* and *P. cocos* who shared a similar call structure. The similar vocalization was explained by possible convergent evolution or retention of the ancestral state especially as they were distinguished as sister taxa.

Our results suggest that there is no distinction in the barking call between the groups, as there were almost no significant differences in time and frequency parameters of the call as shown by results of statistical tests and PCA. One of the reasons could be low selection pressure, therefore the call might not be important in the interspecific recognition nor the finding of a potential mate. Therefore, it is possible, that chemical communication is more important in the case of this species. Secondly, Cremona et al. (2021) proposed that the distinction between the sugar glider and Krefft's glider occurred around 1 mya, therefore the species-specificity might have not occurred yet. Moreover, due to big uncertainty about the function of the barking call, future studies should focus on resolving the potential meaning behind this vocalization and thus it could be assessed if the call is suitable for species identification.

7. Conclusions

We have found a strong relationship between the European and the USA captive population which was supported by the phylogenetic trees as well as the haplotype network. These populations exhibited close clustering with samples from Kai Besar Island. Nonetheless, to address taxonomic uncertainties within local taxa and to determine the source population for the pet trade, future studies should incorporate extensive sampling across Papua New Guinea and Indonesia.

Despite the captive populations forming a single cluster, we observed relatively high haplotype diversity, which suggests high rates of imports from source localities. Although the genetic diversity seems high, it is highly recommended for breeders to engage in animal exchanges to maintain a genetically diverse and viable population.

The barking call was described, but we were not able to determine the real function behind it, as the behavioral monitoring was not included in our research. Our study confirms that this vocalization exhibits high variability, and it is not species-specific, as revealed by statistical analysis revealing no distinct differentiation. The only parameter exhibiting significant variance among groups was the barking rate, which was notably higher in wild sugar gliders compared to the captive groups. Therefore, it suggests that the wild sugar gliders are barking more rapidly compared to the other two groups.

Given the absence of recordings for the savannah glider (*Petaurus ariel*), future studies should aim to record the barking call of this species. Additionally, considering the high variability and the uncertain function of this vocalization, subsequent research should focus on determining its function and evaluating its efficacy as a tool for species identification.

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Appendices

List of the Appendices:

Appendix 1: Samples collected within this study.

Appendix 2: List of recordings from iNaturalist with their authors.

Appendix 3: Sequences used within this study.

Appendix 1: Samples collected within this study.

Table 9: The list of samples collected during this study. X stands for sequenced samples for certain markers.

Sample ID	Location	Type	ND2	ND4	Omega-globin
SGT1	Pilsen	Tissue	X	X	
SGT2	Pilsen	Tissue	X	X	
SGS1	Pilsen	Buccal swab	X	X	X
SGS2	Pilsen	Buccal swab	X	X	X
SGS3	Pilsen	Buccal swab	X	X	X
SGS4	Pilsen	Buccal swab	X	X	X
SGS5	Pilsen	Buccal swab	X	X	X
SGS6	Prague	Buccal swab	X	X	X
SGS7	Prague	Buccal swab	X	X	X
SGS8	Prague	Buccal swab	X	X	
SGS9	Brno	Buccal swab	X	X	X
SGS10	Brno	Buccal swab	X	X	
SGS11	Roztoky	Buccal swab	X	X	X
SGS12	Roztoky	Buccal swab	X	X	X
SGS13	Roztoky	Buccal swab	X	X	X
SGS14	Roztoky	Buccal swab	X	X	X
SGS15	Roztoky	Buccal swab	X	X	X
SGS16	Roztoky	Buccal swab	X	X	X
SGS17	Roztoky	Buccal swab	X	X	
SGS18	Golčův Jeníkov	Buccal swab	X	X	X
SGS19	Golčův Jeníkov	Buccal swab	X	X	
SGS20	Golčův Jeníkov	Buccal swab	X	X	X
SGS21	Hradec Králové	Buccal swab	X	X	X
SGS22	Hradec Králové	Buccal swab	X	X	X
SGS23	Hradec Králové	Buccal swab	X	X	X
SGS24	Hradec Králové	Buccal swab	X	X	
SGS25	České Budějovice	Buccal swab	X	X	
SGS26	České Budějovice	Buccal swab	X	X	
SGS27	Vila Praia de Ancora	Buccal swab	X	X	
SGS28	Vila Praia de Ancora	Buccal swab	X	X	
SGS29	Vila Praia de Ancora	Buccal swab	X	X	
SGS30	Vila Praia de Ancora	Buccal swab	X	X	
SGS31	Vila Praia de Ancora	Buccal swab	X	X	

Sample ID	Location	Type	ND2	ND4	Omega-globin
SGS32	Viana do Castelo	Buccal swab	X	X	
SGS33	Viana do Castelo	Buccal swab	X	X	
SGS34	Melides	Buccal swab	X	X	
SGS35	Melides	Buccal swab	X	X	X
SGS36	Melides	Buccal swab	X	X	
SGS37	Melides	Buccal swab	X	X	X
SGS38	Melides	Buccal swab	X	X	X
SGS39	Melides	Buccal swab	X	X	
SGS40	Melides	Buccal swab	X	X	X
SGS41	Melides	Buccal swab	X	X	
SGS42	Melides	Buccal swab	X	X	
SGS43	Melides	Buccal swab	X	X	
SGS44	Melides	Buccal swab	X	X	
SGS45	Melides	Buccal swab	X	X	
SGS46	Berlin	Buccal swab	X	X	
SGS47	Berlin	Buccal swab	X	X	
SGS48	Berlin	Buccal swab	X	X	
SGS49	Berlin	Buccal swab	X	X	
SGS50	Berlin	Buccal swab	X	X	
SGS51	Berlin	Buccal swab	X	X	
SGS52	Berlin	Buccal swab	X	X	
SGS53	Berlin	Buccal swab	X	X	
SGS54	Berlin	Buccal swab	X	X	
SGS55	Berlin	Buccal swab	X	X	
SGS56	Berlin	Buccal swab	X	X	
SGS57	Berlin	Buccal swab	X	X	
SGS58	Berlin	Buccal swab	X	X	
SGS59	Berlin	Buccal swab	X	X	
SGS60	Riga	Buccal swab	X	X	
SGS61	Riga	Buccal swab	X	X	
SGS62	Riga	Buccal swab	X	X	
SGS63	Riga	Buccal swab	X	X	
SGS64	Riga	Buccal swab	X	X	
SGS65	Riga	Buccal swab	X	X	
SGS66	Riga	Buccal swab	X	X	
SGS67	Riga	Buccal swab	X	X	

Sample ID	Location	Type	ND2	ND4	Omega-globin
SGS68	Riga	Buccal swab	X	X	
SGS69	Riga	Buccal swab	X	X	
SGS70	Riga	Buccal swab	X	X	
SGS71	Riga	Buccal swab	X	X	
SGS72	Riga	Buccal swab	X	X	
SGS73	Riga	Buccal swab	X	X	
SGS74	Riga	Buccal swab	X	X	

Appendix 2: List of recordings from iNaturalist with their authors.

Table 10: List of recordings from iNaturalist including information on author, location, year of collection, and link to the recording. Numbers correspond to those used in the study.

Number	Author	Year	Territory	Latitude	Longitude	Link
1	vinci1000	2023	New South Wales	−34.392152	150.847898	https://www.inaturalist.org/observations/176241139
2	Reiner Richter	2020	Victoria	−37.88	145.39	https://www.inaturalist.org/observations/61469067
3	Joel Poyitt	2020	New South Wales	−34.140221	151.021482	https://www.inaturalist.org/observations/48751690
4	Jess Roberts	2021	Victoria	−37.879406	147.864605	https://www.inaturalist.org/observations/73090234
5	prossington	2021	New South Wales	−33.693493	150.596895	https://www.inaturalist.org/observations/97080032
7	ecoem22	2023	New South Wales	−31.864755	151.404781	https://www.inaturalist.org/observations/174244813
8	Mononymous	2023	Victoria	−37.152754	142.561662	https://www.inaturalist.org/observations/159621790
9	mirv	2022	New South Wales	−34.856246	150.58129	https://www.inaturalist.org/observations/164176541
10	Jess Roberts	2021	Victoria	−37.879406	147.864605	https://www.inaturalist.org/observations/72994316
12	afisch80	2022	Victoria	−37.915608	145.568654	https://www.inaturalist.org/observations/142284748
13	gggpellas	2023	Victoria	−37.823076	148.135034	https://www.inaturalist.org/observations/176065548
14	ron_willemsen	2023	Victoria	−37.949747	145.896898	https://www.inaturalist.org/observations/162270977
15	takesa	2023	New South Wales	−35.506984	150.22772	https://www.inaturalist.org/observations/188125744
17	Colin Trainor	2023	Queensland	−28.20745	153.191026	https://www.inaturalist.org/observations/181333471
18	Donald Hobern	2018	New South Wales	−36.787041	149.54128	https://www.inaturalist.org/observations/11984489
19	Josh Magro	2023	New South Wales	−33.607796	150.972719	https://www.inaturalist.org/observations/192631065
21	christinerand	2023	New South Wales	−32.224673	152.453852	https://www.inaturalist.org/observations/173316259
23	koru	2022	Victoria	−37.745692	145.73065	https://www.inaturalist.org/observations/107032634

Number	Author	Year	Territory	Latitude	Longitude	Link
24	Mononymous	2020	Victoria	−37.388941	144.267417	https://www.inaturalist.org/observations/65485862
25	Michael Tervo	2022	Queensland	−26.8256	152.9136	https://www.inaturalist.org/observations/191741003
26	bpalmerau	2020	New South Wales	−33.714225	151.087872	https://www.inaturalist.org/observations/39642885
27	Colin Trainor	2023	Queensland	−28.233457	153.198466	https://www.inaturalist.org/observations/187490917
28	lynchyyywildlife11	2023	New South Wales	−34.090249	150.989437	https://www.inaturalist.org/observations/183818916
29	vinci1000	2023	New South Wales	−34.237251	150.920049	https://www.inaturalist.org/observations/189757148
30	Colin Trainor	2023	Queensland	−28.207741	153.191071	https://www.inaturalist.org/observations/181333469
31	Jason Brown	2022	New South Wales	−33.406046	151.367961	https://www.inaturalist.org/observations/117283989
32	Nimzee	2021	Victoria	−37.864223	145.32056	https://www.inaturalist.org/observations/81286855
33	vinci1000	2023	New South Wales	−34.409094	150.85002	https://www.inaturalist.org/observations/156388482
35	Mononymous	2020	Victoria	−37.391974	144.262974	https://www.inaturalist.org/observations/66488951
36	marlonnewling	2023	New South Wales	−33.639794	151.323802	https://www.inaturalist.org/observations/167342570
39	Louis Backstrom	2021	Queensland	−27.969158	153.18471	https://www.inaturalist.org/observations/103495705
40	suecee	2022	Victoria	−38.175427	145.132317	https://www.inaturalist.org/observations/110834475
41	Colin Trainor	2023	Queensland	−27.976024	153.317171	https://www.inaturalist.org/observations/150803255
42	rikef	2022	Victoria	−37.873998	145.307761	https://www.inaturalist.org/observations/105155458
43	Colin Trainor	2023	Queensland	−28.218954	153.195256	https://www.inaturalist.org/observations/181333466
45	Louis O'Neill	2023	New South Wales	−33.548436	151.30258	https://www.inaturalist.org/observations/150044172
46	Reiner Richter	2021	Victoria	−37.867126	144.165778	https://www.inaturalist.org/observations/72673901
47	vinci1000	2023	New South Wales	−34.441378	150.800761	https://www.inaturalist.org/observations/156014673
49	Colin Trainor	2023	Queensland	−28.232257	153.137494	https://www.inaturalist.org/observations/158013696
50	Louis O'Neill	2022	Victoria	−37.823016	148.160442	https://www.inaturalist.org/observations/115887228

Number	Author	Year	Territory	Latitude	Longitude	Link
51	ron_willemsen	2023	Victoria	−37.987861	145.481369	https://www.inaturalist.org/observations/163274517
52	vinci1000	2023	New South Wales	−34.400909	150.852992	https://www.inaturalist.org/observations/174834007
53	fw_bouddi	2023	New South Wales	−33.527799	151.345722	https://www.inaturalist.org/observations/187002027
54	ron_willemsen	2023	Victoria	−37.943616	145.240602	https://www.inaturalist.org/observations/194347882
55a,b	rikef	2023	Victoria	−37.840757	145.227171	https://www.inaturalist.org/observations/194015574
57	twan3253	2019	New South Wales	−33.759051	151.143038	https://inaturalist.org/observations/29118476
58	regnans	2023	Victoria	−37.910841	145.212145	https://www.inaturalist.org/observations/181343088
63a,b	grace1066	2023	New South Wales	−33.794686	151.038285	https://www.inaturalist.org/observations/186671043
67	archie_xyz	2023	Victoria	−37.965191	145.607698	https://www.inaturalist.org/observations/146447764
69	koru	2023	Victoria	−37.693017	145.742628	https://www.inaturalist.org/observations/161576801
70	koru	2022	Victoria	−37.626874	145.716246	https://www.inaturalist.org/observations/135100055
72	rikef	2022	Victoria	−37.930213	145.272385	https://www.inaturalist.org/observations/133197488
73	rikef	2023	Victoria	−37.993196	145.37144	https://www.inaturalist.org/observations/170424265
75	janineduffy	2023	Victoria	−38.046519	144.063038	https://www.inaturalist.org/observations/194949511
76	vinci1000	2024	New South Wales	−34.406594	150.853974	https://www.inaturalist.org/observations/195455520
77	vinci1000	2024	New South Wales	−34.4046	150.859709	https://www.inaturalist.org/observations/196266437
78	vinci1000	2024	New South Wales	−34.40225	150.860734	https://www.inaturalist.org/observations/196266637
79	melvinxu	2024	Victoria	−37.771822	145.651505	https://www.inaturalist.org/observations/197964053
80	graemelunt	2024	Victoria	−37.689553	143.996914	https://www.inaturalist.org/observations/198498972
81	caliginous	2024	Victoria	−37.855953	145.104199	https://www.inaturalist.org/observations/198639821
82	graemelunt	2024	Victoria	−37.689204	143.997228	https://www.inaturalist.org/observations/198992937
84	bluebowerstudio	2024	New South Wales	−36.4382	150.056635	https://www.inaturalist.org/observations/199885817

Number	Author	Year	Territory	Latitude	Longitude	Link
85	bluebowerstudio	2024	New South Wales	−36.438216	150.056706	https://www.inaturalist.org/observations/199885886
86	bluebowerstudio	2024	New South Wales	−36.438319	150.056431	https://www.inaturalist.org/observations/199886205
87	bluebowerstudio	2024	New South Wales	−36.438315	150.056764	https://www.inaturalist.org/observations/200078108
88	bluebowerstudio	2024	New South Wales	−36.438294	150.0568	https://www.inaturalist.org/observations/200078163
89	porkytama	2024	Victoria	−37.644749	147.362592	https://www.inaturalist.org/observations/200161833
90	caliginous	2024	Victoria	−37.855517	145.105697	https://www.inaturalist.org/observations/200265293
91	mononymous	2024	Victoria	−37.393973	144.263354	https://www.inaturalist.org/observations/200315664

Appendix 3: Sequences used within this study.

Table 11: NCBI sequences used within this study. NG stands for New Guinea, PNG stands for Papua New Guinea.

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
Petaurus breviceps	NG	−5.25	142.70	GQ323839*	GQ323906*	GQ324009*
P. breviceps	NG	−5.25	142.70	GQ323840*	GQ323907*	GQ324010*
P. breviceps	NG	−5.25	142.70	GQ323841*	GQ323908*	-
P. breviceps	NG	−5.25	142.70	GQ323842*	GQ323909*	-
P. breviceps	NG	−5.11	141.41	GQ323843*	GQ323910*	GQ324022*
P. breviceps	NG	−5.11	141.41	GQ323844*	GQ323911*	GQ324023*
P. breviceps	NG	−6.25	142.78	GQ323838*	GQ323905*	GQ324008*
P. breviceps	NG	−4.7	145.91	GQ323845*	GQ323912*	GQ324006*
P. breviceps	NG	−4.7	145.91	GQ323846*	GQ323913*	GQ324004*
P. breviceps	NG	−6.58	144.65	GQ323847*	GQ323914*	-
P. breviceps	NG	−6.58	144.65	GQ323849*	GQ323916*	GQ324011*
P. breviceps	NG	−6.50	144.85	GQ323848*	GQ323915*	GQ324016*
P. breviceps	NG	−6.50	144.85	GQ323852*	GQ323919*	GQ324012*
P. breviceps	NG	−6.50	144.85	GQ323850*	GQ323917*	GQ324013*
P. breviceps	NG	−6.50	144.85	GQ323851*	GQ323918*	-
P. breviceps	NG	−10.00	151.25	GQ323856*	GQ323923*	GQ324020*
P. breviceps	NG	−10.00	151.25	GQ323855*	GQ323922*	GQ324005*
P. breviceps	NG	−3.41	142.15	GQ323857*	GQ323924*	GQ324019*
P. breviceps	NG	−3.41	142.15	GQ323858*	GQ323925*	GQ324021*
P. breviceps	NG	−5.75	145.23	GQ323864*	GQ323931*	GQ324018*
P. breviceps	NG	−5.75	145.23	GQ323863*	GQ323930*	GQ324014*
P. breviceps	NG	−5.93	146.6	GQ323865*	GQ323932*	GQ324017*
P. breviceps	NG	−5.93	141.7	GQ323859*	GQ323926*	GQ323990*
P. breviceps	NG	−5.93	141.7	GQ323861*	GQ323928*	GQ323991*
P. breviceps	NG	−5.1	141.7	GQ323862*	GQ323929*	GQ323989*
P. breviceps	NG	−5.08	141.5	GQ323860*	GQ323927*	GQ324015*
P. breviceps	Indonesia	−4.00	138.2	GQ323837*	GQ323904*	GQ324024*
P. breviceps	Indonesia	−5.62	132.97	GQ323853*	GQ323920*	-
P. breviceps	Indonesia	−5.62	132.97	GQ323854*	GQ323921*	GQ324003*
P. breviceps	Australia	−33.63	151.28	GQ323882*	GQ323949*	GQ323982*

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
P. breviceps	Australia	−33.72	151.07	GQ323883*	GQ323950*	GQ323981*
P. breviceps	Australia	−28.82	153.28	GQ323886*	GQ323953*	GQ323986*
P. breviceps	Australia	−33.05	151.40	GQ323881*	GQ323948*	GQ323984*
P. breviceps	Australia	−28.63	153.61	GQ323884*	GQ323951*	GQ323980*
P. breviceps	Australia	−27.63	153.25	GQ323885*	GQ323952*	GQ323983*
P. breviceps	Australia	−24.973	147.993	GQ323880*	GQ323947*	GQ324002*
P. breviceps	Australia	−17.61	145.50	GQ323878*	GQ323945*	GQ323987*
P. breviceps	Australia	−17.61	145.50	GQ323879*	GQ323946*	GQ323988*
P. breviceps	Australia	−37.93	140.93	GQ323873*	GQ323940*	GQ323998*
P. breviceps	Australia	−36.52	140.74	GQ323871*	GQ323938*	GQ323997*
P. breviceps	Australia	−36.52	140.74	GQ323872*	GQ323939*	GQ323996*
P. breviceps	Australia	−37.70	140.73	GQ323876*	GQ323943*	GQ323992*
P. breviceps	Australia	−37.90	140.99	GQ323875*	GQ323942*	GQ323993*
P. breviceps	Australia	−36.77	145.50	GQ323874*	GQ323941*	GQ323994*
P. breviceps	Australia	−37.73	145.14	GQ323877*	GQ323944*	GQ323995*
P. breviceps	Australia	−11.558	130.933	GQ323870*	GQ323937*	GQ324007*
P. abidi	PNG	−3.42	142.1	GQ323836*	GQ323903*	GQ323973*
P. abidi	PNG	−3.42	142.1	GQ323835*	GQ323902*	GQ323971*
P. abidi	PNG	−3.42	142.1	GQ323834*	GQ323901*	GQ323970*
P. ariel	Australia	-	-	MT537830**	-	-
P. ariel	Australia	-	-	MT537766**	-	-
P. ariel	Australia	-	-	MT537775**	-	-
P. ariel	Australia	-	-	MT537861**	-	-
P. ariel	Australia	-	-	MT537786**	-	-
P. ariel	Australia	-	-	MT537846**	-	-
P. ariel	Australia	-	-	MT537811**	-	-
P. ariel	Australia	−12.833	132.817	MT537757**	-	-
P. ariel	Australia	−16.183	123.617	MT537795**	-	-
P. ariel	Australia	−13.485	132.250	MT537865**	-	-
P. ariel	Australia	−13.485	132.250	MT537779**	-	-
P. ariel	Australia	−12.559	130.977	MT537835**	-	-
P. ariel	Australia	−12.558	130.973	MT537825**	-	-
P. ariel	Australia	−12.558	130.972	MT537843**	-	-
P. ariel	Australia	−12.559	130.973	MT537860**	-	-
P. ariel	Australia	−11.267	132.199	MT537840**	-	-

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
P. ariel	Australia	−11.267	132.199	MT537745**	-	-
P. ariel	Australia	−15.905	128.128	MT537796**	-	-
P. ariel	Australia	−15.905	128.128	MT537850**	-	-
P. ariel	Australia	−13.487	132.248	MT537750**	-	-
P. ariel	Australia	−13.557	132.287	MT537863**	-	-
P. ariel	Australia	−13.558	132.291	MT537792**	-	-
P. ariel	Australia	−13.941	131.016	MT537770**	-	-
P. ariel	Australia	−14.333	131.012	MT537866**	-	-
P. ariel	Australia	−16.051	130.402	MT537837**	-	-
P. ariel	Australia	−16.050	130.399	MT537800**	-	-
P. ariel	Australia	−17.069	125.247	MT537832**	-	-
P. ariel	Australia	−12.679	132.813	MT537746**	-	-
P. ariel	Australia	−12.679	132.813	MT537763**	-	-
P. ariel	Australia	−12.928	132.538	MT537793**	-	-
P. ariel	Australia	−12.928	132.538	MT537749**	-	-
P. ariel	Australia	−12.928	132.538	MT537772**	-	-
P. ariel	Australia	−12.928	132.538	MT537858**	-	-
P. ariel	Australia	−13.256	132.370	MT537782**	-	-
P. ariel	Australia	−13.256	132.370	MT537785**	-	-
P. ariel	Australia	−12.864	132.809	MT537862**	-	-
P. ariel	Australia	−15.350	131.244	MT537788**	-	-
P. ariel	Australia	−15.340	131.226	MT537753**	-	-
P. ariel	Australia	−15.339	131.226	MT537844**	-	-
P. ariel	Australia	−15.350	131.244	MT537851**	-	-
P. ariel	Australia	−15.340	131.226	MT537755**	-	-
P. ariel	Australia	−13.576	132.255	MT537809**	-	-
P. ariel	Australia	−11.759	130.634	MT537805**	-	-
P. ariel	Australia	−12.439	130.744	MT537816**	-	-
P. ariel	Australia	−12.544	131.080	MT537807**	-	-
P. ariel	Australia	−11.501	130.447	MT537842**	-	-
P. ariel	Australia	−14.148	132.194	MT537787**	-	-
P. ariel	Australia	−14.149	132.194	MT537848**	-	-
P. ariel	Australia	−16.758	137.447	MT537833**	-	-
P. ariel	Australia	−11.403	130.575	MT537744**	-	-
P. ariel	Australia	−11.406	130.566	MT537791**	-	-

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
P. ariel	Australia	−11.407	130.566	MT537747**	-	-
P. ariel	Australia	−11.402	130.576	MT537773**	-	-
P. ariel	Australia	−11.406	130.566	MT537827**	-	-
P. ariel	Australia	−11.341	130.546	MT537794**	-	-
P. ariel	Australia	−11.363	130.535	MT537790**	-	-
P. ariel	Australia	−11.408	130.566	MT537764**	-	-
P. ariel	Australia	−11.363	130.535	MT537815**	-	-
P. ariel	Australia	−11.363	130.535	MT537804**	-	-
P. ariel	Australia	−11.408	130.566	MT537776**	-	-
P. ariel	Australia	−11.408	130.566	MT537768**	-	-
P. ariel	Australia	−11.408	130.566	MT537759**	-	-
P. ariel	Australia	−11.403	130.574	MT537847**	-	-
P. ariel	Australia	−11.403	130.575	MT537856**	-	-
P. ariel	Australia	−16.444	136.075	MT537784**	-	-
P. ariel	Australia	−12.961	131.165	MT537752**	-	-
P. ariel	Australia	−18.695	138.492	MT537859**	-	-
P. ariel	Australia	−16.716	125.461	MT537839**	-	-
P. ariel	Australia	−12.566	132.319	MT537813**	-	-
P. ariel	Australia	−11.600	130.700	MT537761**	-	-
P. breviceps	Australia	−28.500	152.433	MT537765**	-	-
P. breviceps	Australia	−28.617	152.417	MT537762**	-	-
P. breviceps	Australia	−36.317	149.917	MT537808**	-	-
P. breviceps	Australia	−28.467	152.550	MT537803**	-	-
P. breviceps	Australia	−36.600	149.383	MT537806**	-	-
P. breviceps	Australia	−34.783	150.583	MT537760**	-	-
P. breviceps	Australia	−35.550	150.267	MT537767**	-	-
P. breviceps	Australia	−35.133	150.717	MT537774**	-	-
P. notatus	Australia	-	-	MT537831**	-	-
P. notatus	Australia	-	-	MT537838**	-	-
P. notatus	Australia	−35.300	148.217	MT537777**	-	-
P. notatus	Australia	−37.850	147.083	MT537828**	-	-
P. notatus	Australia	−35.289	149.150	MT537836**	-	-
P. notatus	Australia	−35.250	149.167	MT537857**	-	-
P. notatus	Australia	−35.250	149.283	MT537801**	-	-
P. notatus	Australia	−36.217	148.133	MT537789**	-	-

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
P. notatus	Australia	−35.317	149.250	MT537799**	-	-
P. notatus	Australia	−35.258	149.083	MT537754**	-	-
P. notatus	Australia	−37.344	149.650	MT537864**	-	-
P. notatus	Australia	−38.330	145.000	MT537853**	-	-
P. notatus	Australia	−37.750	145.700	MT537780**	-	-
P. notatus	Australia	−37.750	142.030	MT537852**	-	-
P. notatus	Australia	−36.830	148.170	MT537751**	-	-
P. breviceps	USA	47.717	−122.2	MH310446***	MH310590***	MH247781***
P. breviceps	USA	47.717	−122.2	MH310447***	MH310591***	MH247782***
P. breviceps	USA	47.717	−122.2	MH310582***	MH310724***	MH247918***
P. breviceps	USA	47.717	−122.2	MH310583***	MH310725***	MH247919***
P. breviceps	USA	40.085	−74.215	MH310448***	MH310592***	MH247783***
P. breviceps	USA	42.124	−77.035	MH310449***	MH310593***	MH247784***
P. breviceps	USA	39.384	−76.658	MH310450***	MH310594***	MH247785***
P. breviceps	USA	39.384	−76.658	MH310451***	MH310595***	MH247786***
P. breviceps	USA	29.639	−95.618	MH310452***	MH310596***	MH247787***
P. breviceps	USA	29.639	−95.618	MH310453***	MH310597***	MH247788***
P. breviceps	USA	29.639	−95.618	MH310454***	MH310598***	MH247789***
P. breviceps	USA	29.639	−95.618	MH310455***	MH310599***	MH247790***
P. breviceps	USA	29.639	−95.618	MH310456***	MH310600***	MH247791***
P. breviceps	USA	29.639	−95.618	MH310457***	MH310601***	MH247792***
P. breviceps	USA	29.639	−95.618	MH310458***	MH310602***	MH247793***
P. breviceps	USA	29.639	−95.618	MH310459***	MH310603***	MH247794***
P. breviceps	USA	29.639	−95.618	MH310460***	MH310604***	MH247795***
P. breviceps	USA	29.639	−95.618	MH310461***	MH310605***	MH247796***
P. breviceps	USA	29.639	−95.618	MH310462***	MH310606***	MH247797***
P. breviceps	USA	29.639	−95.618	MH310463***	MH310607***	MH247798***
P. breviceps	USA	29.639	−95.618	MH310464***	-	MH247799***
P. breviceps	USA	29.639	−95.618	MH310465***	-	MH247800***
P. breviceps	USA	29.639	−95.618	MH310466***	MH310608***	MH247801***
P. breviceps	USA	29.639	−95.618	MH310467***	MH310609***	MH247802***
P. breviceps	USA	29.639	−95.618	MH310468***	MH310610***	MH247803***
P. breviceps	USA	29.639	−95.618	MH310469***	MH310611***	MH247804***
P. breviceps	USA	29.639	−95.618	MH310470***	MH310612***	MH247805***
P. breviceps	USA	29.639	−95.618	MH310471***	MH310613***	MH247806***

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
P. breviceps	USA	29.639	−95.618	MH310472***	MH310614***	MH247807***
P. breviceps	USA	29.639	−95.618	MH310473***	MH310615***	MH247808***
P. breviceps	USA	29.639	−95.618	MH310474***	MH310616***	MH247809***
P. breviceps	USA	29.639	−95.618	MH310475***	MH310617***	MH247810***
P. breviceps	USA	29.639	−95.618	MH310476***	-	MH247811***
P. breviceps	USA	29.639	−95.618	MH310477***	MH310618***	MH247812***
P. breviceps	USA	29.639	−95.618	MH310478***	MH310619***	MH247813***
P. breviceps	USA	29.639	−95.618	MH310479***	MH310620***	MH247814***
P. breviceps	USA	29.639	−95.618	MH310480***	MH310621***	MH247815***
P. breviceps	USA	29.639	−95.618	MH310481***	MH310622***	MH247816***
P. breviceps	USA	29.639	−95.618	MH310482***	MH310623***	MH247817***
P. breviceps	USA	29.639	−95.618	MH310483***	MH310624***	MH247818***
P. breviceps	USA	29.639	−95.618	MH310484***	MH310625***	MH247819***
P. breviceps	USA	29.639	−95.618	MH310485***	MH310626***	MH247820***
P. breviceps	USA	29.639	−95.618	MH310486***	MH310627***	MH247821***
P. breviceps	USA	29.639	−95.618	MH310487***	MH310628***	MH247822***
P. breviceps	USA	29.639	−95.618	MH310488***	MH310629***	MH247823***
P. breviceps	USA	29.639	−95.618	MH310489***	MH310630***	MH247824***
P. breviceps	USA	29.639	−95.618	MH310490***	MH310631***	MH247825***
P. breviceps	USA	29.639	−95.618	MH310491***	MH310632***	MH247826***
P. breviceps	USA	29.639	−95.618	MH310492***	MH310633***	MH247827***
P. breviceps	USA	29.639	−95.618	MH310493***	MH310634***	MH247828***
P. breviceps	USA	29.639	−95.618	MH310494***	MH310635***	MH247829***
P. breviceps	USA	29.639	−95.618	MH310495***	MH310636***	MH247830***
P. breviceps	USA	29.639	−95.618	MH310496***	MH310637***	MH247831***
P. breviceps	USA	29.639	−95.618	MH310497***	MH310638***	MH247832***
P. breviceps	USA	29.639	−95.618	MH310498***	MH310639***	MH247833***
P. breviceps	USA	29.639	−95.618	MH310499***	MH310640***	MH247834***
P. breviceps	USA	29.639	−95.618	MH310500***	MH310641***	MH247835***
P. breviceps	USA	29.639	−95.618	MH310501***	MH310642***	MH247836***
P. breviceps	USA	29.639	−95.618	MH310502***	MH310643***	MH247837***
P. breviceps	USA	29.639	−95.618	MH310503***	MH310644***	MH247838***
P. breviceps	USA	29.639	−95.618	MH310504***	MH310645***	MH247839***
P. breviceps	USA	29.639	−95.618	MH310505***	MH310646***	MH247840***
P. breviceps	USA	29.639	−95.618	MH310506***	MH310647***	MH247841***

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
P. breviceps	USA	29.639	−95.618	MH310507***	MH310648***	MH247842***
P. breviceps	USA	29.639	−95.618	MH310508***	MH310649***	MH247843***
P. breviceps	USA	29.639	−95.618	MH310509***	MH310650***	MH247844***
P. breviceps	USA	29.639	−95.618	MH310510***	MH310651***	MH247845***
P. breviceps	USA	29.639	−95.618	MH310511***	MH310652***	MH247846***
P. breviceps	USA	29.639	−95.618	MH310512***	MH310653***	MH247847***
P. breviceps	USA	29.639	−95.618	MH310513***	MH310654***	MH247848***
P. breviceps	USA	29.639	−95.618	MH310514***	MH310655***	MH247849***
P. breviceps	USA	29.639	−95.618	MH310515***	MH310656***	MH247850***
P. breviceps	USA	29.639	−95.618	MH310516***	MH310657***	MH247851***
P. breviceps	USA	29.639	−95.618	MH310517***	MH310658***	MH247852***
P. breviceps	USA	29.639	−95.618	MH310518***	MH310659***	MH247853***
P. breviceps	USA	29.639	−95.618	MH310519***	MH310660***	MH247854***
P. breviceps	USA	29.639	−95.618	MH310520***	MH310661***	MH247855***
P. breviceps	USA	29.639	−95.618	MH310521***	MH310662***	MH247856***
P. breviceps	USA	29.639	−95.618	MH310522***	MH310663***	MH247857***
P. breviceps	USA	29.639	−95.618	MH310523***	MH310664***	MH247858***
P. breviceps	USA	29.639	−95.618	MH310524***	MH310665***	MH247859***
P. breviceps	USA	29.639	−95.618	MH310525***	MH310666***	MH247860***
P. breviceps	USA	29.639	−95.618	MH310526***	MH310667***	MH247861***
P. breviceps	USA	29.639	−95.618	MH310527***	MH310668***	MH247862***
P. breviceps	USA	29.639	−95.618	MH310528***	MH310669***	MH247863***
P. breviceps	USA	29.639	−95.618	MH310529***	MH310670***	MH247864***
P. breviceps	USA	29.639	−95.618	MH310530***	MH310671***	MH247865***
P. breviceps	USA	29.639	−95.618	MH310531***	MH310672***	MH247866***
P. breviceps	USA	29.639	−95.618	MH310532***	MH310673***	MH247867***
P. breviceps	USA	32.742	−96.824	MH310536***	MH310678***	MH247872***
P. breviceps	USA	29.639	−95.618	MH310537***	MH310679***	MH247873***
P. breviceps	USA	29.639	−95.618	MH310538***	MH310680***	MH247874***
P. breviceps	USA	29.639	−95.618	MH310539***	MH310681***	MH247875***
P. breviceps	USA	29.639	−95.618	MH310540***	MH310682***	MH247876***
P. breviceps	USA	29.639	−95.618	MH310541***	MH310683***	MH247877***
P. breviceps	USA	29.639	−95.618	MH310542***	MH310684***	MH247878***
P. breviceps	USA	29.639	−95.618	MH310543***	MH310685***	MH247879***
P. breviceps	USA	29.639	−95.618	MH310544***	MH310686***	MH247880***

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
P. breviceps	USA	29.639	−95.618	MH310545***	MH310687***	MH247881***
P. breviceps	USA	29.639	−95.618	MH310547***	MH310689***	MH247883***
P. breviceps	USA	29.639	−95.618	MH310548***	MH310690***	MH247884***
P. breviceps	USA	29.639	−95.618	MH310549***	MH310691***	MH247885***
P. breviceps	USA	29.639	−95.618	MH310550***	MH310692***	MH247886***
P. breviceps	USA	29.639	−95.618	MH310551***	MH310693***	MH247887***
P. breviceps	USA	29.639	−95.618	MH310552***	MH310694***	MH247888***
P. breviceps	USA	29.639	−95.618	MH310553***	MH310695***	MH247889***
P. breviceps	USA	29.639	−95.618	MH310554***	MH310696***	MH247890***
P. breviceps	USA	29.639	−95.618	MH310555***	MH310697***	MH247891***
P. breviceps	USA	29.639	−95.618	MH310556***	MH310698***	MH247892***
P. breviceps	USA	29.639	−95.618	MH310557***	MH310699***	MH247893***
P. breviceps	USA	29.639	−95.618	MH310558***	MH310700***	MH247894***
P. breviceps	USA	29.639	−95.618	MH310559***	MH310701***	MH247895***
P. breviceps	USA	29.639	−95.618	MH310560***	MH310702***	MH247896***
P. breviceps	USA	29.639	−95.618	MH310561***	MH310703***	MH247897***
P. breviceps	USA	29.639	−95.618	MH310562***	MH310704***	MH247898***
P. breviceps	USA	29.639	−95.618	MH310563***	MH310705***	MH247899***
P. breviceps	USA	29.639	−95.618	MH310564***	MH310706***	MH247900***
P. breviceps	USA	29.639	−95.618	MH310546***	MH310688***	MH247882***
P. breviceps	USA	29.639	−95.618	MH310565***	MH310707***	MH247901***
P. breviceps	USA	29.639	−95.618	MH310566***	MH310708***	MH247902***
P. breviceps	USA	29.639	−95.618	MH310567***	MH310709***	MH247903***
P. breviceps	USA	29.639	−95.618	MH310568***	MH310710***	MH247904***
P. breviceps	USA	29.639	−95.618	MH310569***	MH310711***	MH247905***
P. breviceps	USA	29.639	−95.618	MH310570***	MH310712***	MH247906***
P. breviceps	USA	29.639	−95.618	MH310571***	MH310713***	MH247907***
P. breviceps	USA	29.639	−95.618	MH310572***	MH310714***	MH247908***
P. breviceps	USA	29.639	−95.618	MH310573***	MH310715***	MH247909***
P. breviceps	USA	29.639	−95.618	MH310574***	MH310716***	MH247910***
P. breviceps	USA	29.639	−95.618	MH310575***	MH310717***	MH247911***
P. breviceps	USA	29.639	−95.618	MH310576***	MH310718***	MH247912***
P. breviceps	USA	29.639	−95.618	MH310577***	MH310719***	MH247913***
P. breviceps	USA	29.639	−95.618	MH310578***	MH310720***	MH247914***
P. breviceps	USA	29.639	−95.618	MH310579***	MH310721***	MH247915***
P. breviceps	USA	29.639	−95.618	MH310580***	MH310722***	MH247916***

Species	Region	Latitude	Longitude	GenBank accession		
				ND2	ND4	ω-globin
P. breviceps	USA	29.639	−95.618	MH310581***	MH310723***	MH247917***
P. breviceps	USA	-	-	MH310533***	MH310675***	MH247869***
P. breviceps	USA	-	-	MH310534***	MH310676***	MH247870***
P. breviceps	USA	-	-	MH310535***	MH310677***	MH247871***
P. breviceps	USA	45.085	−93.221	MH310584***	MH310726***	MH247920***
P. breviceps	USA	45.085	−93.221	MH310585***	MH310727***	MH247921***
P. breviceps	USA	45.085	−93.221	MH310586***	MH310728***	MH247922***
P. breviceps	USA	45.085	−93.221	MH310587***	MH310729***	MH247923***
P. breviceps	USA	44.268	−105.501	MH310588***	MH310730***	MH247924***
P. breviceps	USA	44.268	−105.501	MH310589***	MH310731***	MH247925***

* Malekian et al. (2010) ** Cremona et al. (2021) *** Campbell et al. (2019)