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The Intersection of Genetics and Conservation: A DNA-based Investigation of Wildlife Conservation Issues

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Zusammenfassung

Der weltweite Rückgang der Reptilienbestände ist zunehmend besorgniserregend, und Schätzungen zufolge ist die Zahl an Reptilien in den letzten fünfzig Jahren erheblich zurückgegangen. Habitatsverlust ist hierbei der treibende Faktor, jedoch spielen auch die Nutzung biologischer Ressourcen und Wilderei eine entscheidende Rolle. Aufzucht hat sich als mögliche Lösung zur Eindämmung der illegalen Wildentnahme herausgestellt. Diese birgt jedoch Probleme, da die Tierhaltenden nicht wissen, woher die Individuen stammen. Das Risiko der unauthorisierten Beschaffung steigt dadurch. Zur Bekämpfung des illegalen Handels mit Wildtieren sind DNA-Analysen zu einem wichtigen Instrument geworden, das Aufschluss über die Herkunft der Arten gibt und bei der Identifizierung hilft. In dieser Studie konzentriere ich mich auf die Hornviper (*Vipera ammodytes*), genauer gesagt auf eine kürzlich entdeckte Population in Winzendorf (Niederösterreich). Die Hypothese ist, dass diese Population durch illegale Auswilderungen von in Gefangenschaft gehaltenen Tieren entstanden ist. Meine Ergebnisse zeigen, dass in Winzendorf Individuen aus zwei verschiedenen mitochondrialen Haplogruppen vorkommen. Drei Individuen zeigen eine enge genetische Verwandtschaft mit Individuen aus Italien und Österreich (Kärnten) und eines mit Individuen aus Montenegro und Albanien. Die noch nicht erforschten Auswirkungen illegaler Freisetzungen in Winzendorf unterstreichen die Notwendigkeit von Monitoring und Schutzmaßnahmen. Diese Studie zeigt die Bedeutung von DNA-Analysen für das Verständnis der Verwandtschaftsbeziehungen und die Entwicklung von Erhaltungsstrategien angesichts des weltweiten Rückgangs der Reptilienpopulationen.

Abstract

The global decline of reptile populations has become an increasing concern, with estimates suggesting a substantial decrease in numbers over the last fifty years. While habitat loss is the primary factor contributing to extinction, biological resource use and poaching also play crucial roles in the decline of these populations. Captive breeding has emerged as a potential solution to mitigate sourcing from the wild; however, it presents challenges such as disconnecting pet owners from the species' origin and increasing the risk of illegal laundering. To combat illegal wildlife trafficking, DNA evidence has become an essential tool, offering insights into species origins, and aiding in species identification. In this study, I focus on the nose-horned viper (*Vipera ammodytes*), more precisely on a recently discovered population in Lower Austria (Winzendorf). The most likely explanation is that this population started with illegal releases to the wild of captive individuals (likely from pet trade). Indeed, my findings show the presence of individuals from two different mitochondrial haplogroups in Winzendorf. Three individuals showing a close genetic relationship with individuals from Italy and Austria (Carinthia) and one with individuals from Montenegro and Albania. The implications of unauthorized releases and the absence of adverse interactions in Winzendorf underscore the need for continued monitoring and conservation efforts. This study highlights the importance of DNA analysis in understanding species relationships and guiding conservation strategies in the face of global reptile population declines.

Introduction

A strong decline of reptile populations has been observed globally, with estimates indicating a decrease to half their population size in the last half-century (Nordstrom et al., 2022). Moreover, a fifth of these species are currently threatened with extinction. A significant factor contributing to this decline is the trade in reptiles (Marshall et al., 2020). Of the 10,272 known reptile species, less than 8% are subject to regulation by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the European Wildlife Trade Regulations (EWTR). However, the International Union for Conservation of Nature (IUCN) Red List has assessed 45% of the world's reptile species, revealing that at least 1,390 species are at risk due to "biological resource use". Of these, 355 species are intentionally targeted by collectors, including 194 species not listed under CITES (Auliya et al., 2016). Illegal reptile trade can also be hidden via document fraud, which may involve misrepresenting the species or its origin, laundering them as captive-bred or marking them as animals for “display only” (UNODC world WISE database). While captive breeding presents itself as a sustainable alternative to harvesting from the wild, it has the drawback of disconnecting pet owners from the species' origin. Even more so, there is an elevated risk of illegal laundering associated with captive breeding practices (Robinson et al., 2015). Given the vast number of reptile species, authorities may find it difficult to detect such fraud, which requires a high level of taxonomic expertise (Ogden, 2010). Furthermore, a growing number of pet owners overwhelmed with reptile care may release them into the wild, posing a threat to native populations (Episcopio-Sturgeon & Pienaar, 2020), as previously observed in Florida, concerning the rapid increase of *Phyton bivittatus* (Dorcas et al., 2012).

International Wildlife Trade (IWT), also termed wildlife and forest crime (UNODC; https://www.unodc.org/documents/data-and analysis/wildlife/2020/World_Wildlife_Report_2020_9July.pdf), is recognized as one of the most significant organized international crimes. About ten years ago it was estimated to generate 7 to 23 billion US Dollars annually and was projected to grow at a rate three times that of the global economy, approximately 15-21% per year (Sollund, 2016; Uhm, 2016). Wildlife and Forest Crime primarily involves poaching, smuggling, capture, and collection of protected or managed species (UNODC; https://www.unodc.org/e4j/en/wildlife-crime/module-3/key-issues/criminalization-of-wildlife-trafficking.html). A critical concern associated with illegal trade is the transmission of pathogens, with an estimated 75% of emerging pathogens believed to be zoonotic (Taylor et al., 2001). A major hurdle in combating IWT crimes is the problem of species identification (Gouda et al., 2020). Traditional species identification often relies on morphological traits, which requires high levels of taxonomic expertise (Iyengar, 2014;

Linacre & Tobe, 2011; Masters et al., 2019). Utilizing DNA methods, however, circumvents the reliance on morphological characteristics and offers crucial insights into the origin of species (Ogden & Linacre, 2015). A prominent method for genetic identification at the species level involves examining standardized markers located in the mitochondrial DNA, so called DNA barcodes (Hebert et al, 2003; Smart et al., 2021). This technique has been effectively applied in the past for snakeskin analysis (Dubey et al., 2011) and has proven to be a reliable indicator for *Vipera ammodytes*.

This study focuses on the nose-horned viper (*Vipera ammodytes*), a venomous snake, inhabiting dry, rocky hills with sparse vegetation in Southern Europe, including the Balkans, the Southern Alps (Tomovic, 2006). As a species it is listed as “Least Concern” on the International Union for Conservation of Nature (IUCN) Red List (Agasyan et al., 2008) and as “Critically Endangered” by the Austrian Umweltbundesamt (Gollmann, 2007). However, local populations such as isolated populations in the Alps (for example the Carinthian population), Serbia or Bulgaria are threatened mainly due to illegal poaching (Ursenbacher et al., 2008). In Austria this species is natively found in the Southern regions of Carinthia and in a smaller population in Styria (Cabela et al., 2001). The origin of the Lower Austrian (Winzendorf) population, *Vipera ammodytes ammodytes*, is thought to be non-native. However, some people favour the idea of an autochthonous, glacial refugial population. A study by Thanou et al. in 2023 specifically targeted temporal and spatial agents in the context of *V. ammodytes* speciation.

Investigating this matter, we examined various mitochondrial genetic lineages categorized into distinct clades in Ursenbacher et al., (2008). These clades include the Montenegrin clade, North-eastern clade, North-western clade, South-western clade, Cyclades clade, Peloponnese clade, and the South-eastern clade. The South-eastern clade further subdivides into the Turkish subclade, Eastern subclade, and, Southern subclade. This comprehensive classification system allows for a thorough investigation into the genetic diversity and distribution patterns (Ursenbacher et al., 2008). Examining the taxonomy of *V. ammodytes*, which is based on morphological features, the species can be categorized in six subspecies: *V. a. ammodytes* (Linnaeus, 1758), *V. a. montandoni* (Boulenger, 1904), *V. a. meridionalis* (Boulenger, 1903), *V. a. ruffoi* (Bruno, 1968), and *V. a. gregorwallneri* (Sochurek, 1974). The sixth subspecies, *V. a. transcaucasiana* is by some considered as a separate species (Baran and Atatür, 1998; Nilson. et al., 1999; Obst, 1983). In this thesis, I work with the assumption that *V. a. transcaucasiana* is a separate species.

In my master’s thesis, I am investigating the potential origins of the Winzendorf population. I aim to determine whether it is more closely related to the population in Carinthia or to other populations in Europe.

Methods

Sampling

Samples	Place of collection	Haplogroup	Barcodes	Barcodes for 16S DNA	Additional information
VT01	St. Veit, Kärnten	NW	33	73	tissue
VT02	1-4= NZ v. Eltern, Ulcinje, Insel Ata, Montenegro	MO	34	74	tissue
VT03	Dorf Milanovo, bei Svoge, Bulgarien	NE	35	75	tissue
VT04	SSW Radmani, 45°11'54"/13°40'49", (107m NN), Istrien, Kroatien	NW	36	76	tissue
VT05	Musi bei Passo di Tanamea, NE Tarcento, Friaul, Italien	NW	37	77	tissue
VT06	Slowenien	NW	38	78	tissue
VT07	Star Dojran, Dojranski-See, Mazedonien	S	39	79	tissue
VT08	Griffen, Kärnten	NW	40	80	tissue
VT09	Thesprotia, Mavroneri, (120m, 39°40'N/220°10'E), Griechenland	SW?	41	81	tissue
VT10	Ada Bojana, Montenegro	MO	42	82	tissue
VT11	Krk, ca. 170m, 44°59'N/14°38'E, Kroatien	NW	43	83	tissue
VT12	Pfaffen, Südtirol, Italien	NW	44	84	tissue
VS13	Winzendorf	?	45	85	saliva
VS14	Winzendorf	?	46	86	saliva
VH15	Winzendorf	?	47	87	skin
V16	Winzendorf	?	48	88	saliva
VP17	Austria	NW	49	89	plasma
VP18	Austria	NW	50	90	plasma
VP19	Italy	NW	51	91	plasma
VE20	Greece	SW/S	52	92	erythrocytes
VE21	Greece	SW/S	53	93	erythrocytes
VP22	Bulgaria	NE/E	54	94	plasma

VT23	Bulgaria	NE/E	55	95	tissue
V24	Bulgaria	NE/E	56	96	Extracted DNA from unknown origin

Table 1. The table presents the allocation of barcodes to respective samples, including place of collection, haplogroup, and specific barcodes assigned. Additionally, the type of sample is specified.

After receiving the Austrian legislation permit RU5-BE-64/023-2022, I participated in the capturing of two V. ammodytes specimens, which occurred in September 2023. The weather was optimal, being warm and sunny, which is ideal for this species. The samples were originally obtained for a different research project, working with transect data. During the excursion, we tactically surveyed the Winzendorf region, investigating areas that are known for *V. ammodytes* sightings. Two individuals were successfully collected using a bite-proof glove by HexArmor, saliva samples were taken with swaps that were stored in ethanol, followed by the release of the animals.

Figure 1. Vipera ammodytes sampled in Winzendorf in June 2021. Photo by Stefan Prost

Additionally, I conducted sampling from 13 ethanol-preserved specimens, which were provided by the herpetology collection of the Natural History Museum Vienna (by Dr. Silke Schweiger). Delicately, small fragments of muscle tissue were extracted from each specimen, ensuring the use of fresh blades for each individual sample. These individuals originate from countries marked in red on the map below (Figure 1) and were predominantly collected during the 20th century. Furthermore, nine supplementary samples were incorporated, provided by the Natural History Museum Vienna. These specimens were derived either from shed skin or previously extracted genetic material, thereby enhancing our molecular dataset. The two additional

samples from *V. ammodytes* from Winzendorf were collected during a trip in June 2021 by Dr. Silke Schweiger.

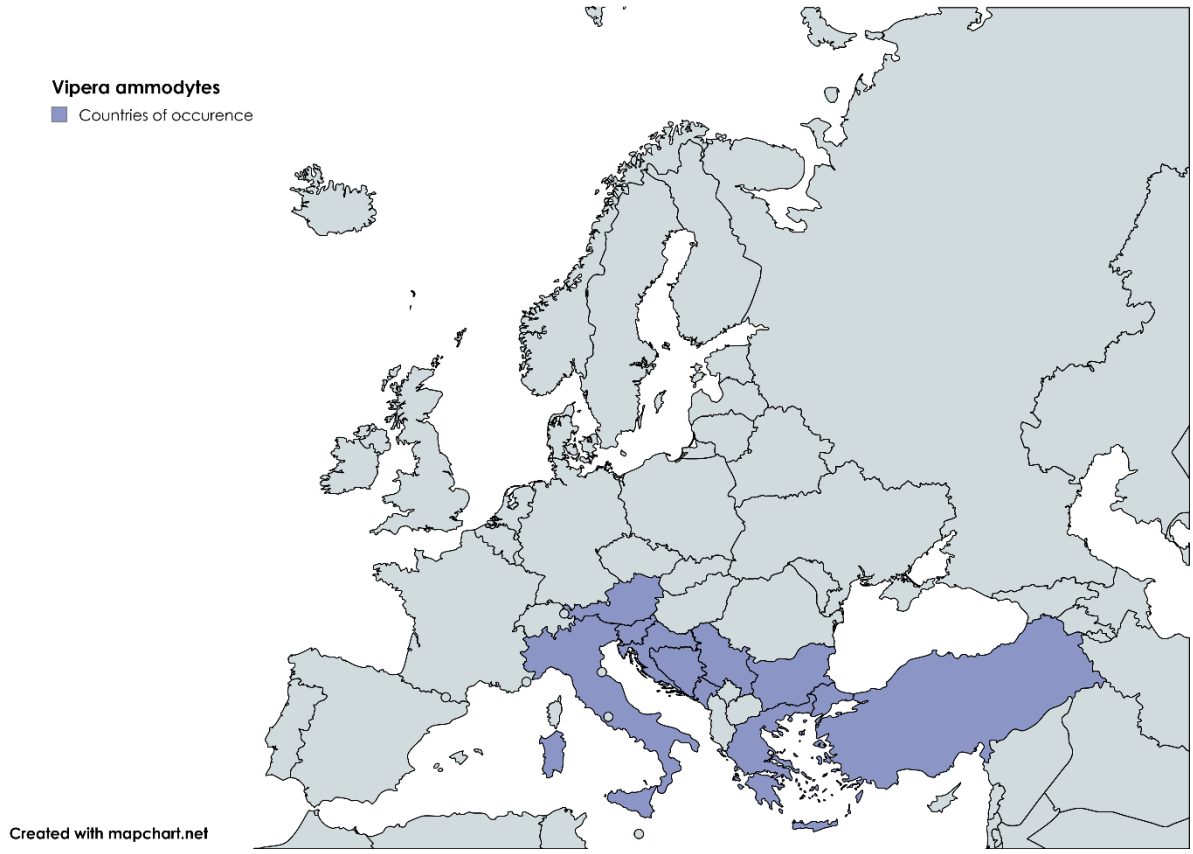

Figure 2. Map illustrating countries where V. ammodytes occurs (marked in blue). It is important to mention that V. ammodytes is only in small parts of Northern Italy, in some areas in the West of Türkiye, and distinct areas in Austria, not the entire countries, which is incorrectly portrayed by the map. The gray dots symbolize nations that were too small to be visualized.

DNA extraction and amplification

DNA analysis was conducted in accordance with the methodologies outlined in Pomerantz et al. 2022. For DNA extraction, I employed either automatic DNA extractions using the ThermoFisher KingFisher Apex DNA extraction robot (Appendix 1) or using the E.Z.N.A.® Tissue DNA Kit Protocol (see Appendix 2).

The DNA extraction was performed using the lysis buffer for the ThermoFisher KingFisher Apex DNA extraction, consisting of:

Amount	**Reagent**
100 mM	NaCl

50 mM	Tris
10 mM	EDTA

Table 2. lysis buffer for one sample.

Amount	**Reagent**
50000 µl	NaCl
2.5 ml	Tris
1 ml	EDTA

Table 3. respective lysis buffer mixture for all samples.

The tissue lysis was performed on 65 degrees Celsius.

The following solution was used for the DNA extraction on the ThermoFisher KingFisher Apex for tissue samples:

Amount	**Reagent**
21000 µl	Mag Max Core Lysis Solution
21000 µl	Mag Max Binding Solution
1200 µl	Mag Max Core Magnetic beads
43200 µl	total

Table 4. solution for DNA extraction.

The two saliva samples (VS 13 and VS 14), as well as the shed skin samples (VH 15), which we took about 1 cm^2 of, were treated using the E.Z.N.A.® Tissue DNA Kit Protocol (see Appendix 2):

1. 200 µl Tl Buffer were added to all of the samples.
2. 25 µl Proteinase K were added.
3. All samples were vortexed.
4. All tubes were brought to the shaker, which was set to 55°C. For pre-digestion the skin sample remained inside for 30 min.
5. VH15 was transferred to a petri dish and washed with 2 ml PBS buffer and cut into small pieces.
6. It was then transferred to a new tube, 200 µl TL Buffer and 25 µl Proteinase K were added and the samples was transferred back to the shaker at 55°C.
7. After two hours in the shaker at 55°C, VS13 and VS14 were centrifuged for 5 min at 13,000g.

8. The liquid content was then transferred to a 1,5 ml tube and 220 µl BL Buffer were added.
9. The samples were then incubated at 70°C for 10 min.
10. 220 µl EtOH (100%) were added and vortexed.
11. HiBind was added to a 2 ml tube, where the samples were added to.
12. The samples were centrifuged at 13,000 g for 1 min.
13. The filtrate was discarded, and the tube reused.
14. 500 µl HBC Buffer were added and centrifuged for 1 min, contrary to the protocol that suggested 30 sec.
15. A new tube was being used and a HiBind was added.
16. 700 µl DNA wash buffer was added, and the samples were centrifuged for one minute at 13,000g.
17. The filtrate was discarded, and step 16 was repeated.
18. The empty columns were centrifuged for 2 min at 13,000g.
19. The columns were transferred to 1,5 ml tubes each.
20. The elution buffer was heated to 70°C and 200 µl were added to each tube.
21. After 2 min waiting at room temperature, the samples were centrifuged at 13,000 g for 1 min.
22. Step 21 was repeated.
23. The columns, elutions and saliva tips were stored in the freezer.
24. While the protocol was performed for the saliva samples, the VH15 was still in the shaker at 55°C, 300 rpm. 25 µl Proteinase K from the Omega Kit and 25 µl DTT were added. Due to the skin still being not fully dissolved, the sample was transferred back to the shaker.
25. After 47h incubation time, VH15 was removed from the shaker and centrifuged at 13,000g for 5 min.
26. The liquid, without the still existing skin, was transferred to a new 1,5 ml tube (it could not be avoided to have tiny skin traces inside).
27. 220 µl BL Buffer were added.
28. The sample was incubated at 70°C for 10 min.
29. 220 µl EtOH (100%) were added and vortexed.
30. HiBind was added to a 2 ml tube and the sample transferred to it.
31. The sample was centrifuged at 13,000g for 1 min and the filtrate was discarded, the tube reused.
32. 500 µl HBC Buffer were added and centrifuged for 1 min, contrary to the protocol that suggested 30 sec.
33. The filtrate was discarded. A new 2 ml tube was being used.

34. 700 µl DNA wash buffer was added and the samples were centrifuged for one minute at 13,000g.
35. The filtrate was discarded, and step 34 was repeated.
36. The empty columns were centrifuged for 2 min at 13,000g.
37. The column was transferred to a 1,5 ml Eppendorf tube.
38. The elution buffer was heated to 70°C and 200 µl were added to each tube.
39. After 2 min waiting at room temperature, the samples were centrifuged at 13,000 g for 1 min.
40. Step 39 was repeated.
41. The tube with the DNA was then labelled as “VH15 DNA”, the column as “VH15 column”. The DNA extracts were stored in the freezer (-20C) and the columns in the fridge (+4C).

The amount of extracted DNA was quantified using the Qubit platform, which is quantifying nucleic acids by detecting incorporated fluorescent dyes. The Qubit quantification protocol was followed, using these reagents:

Amount	**Reagent**
15 µl	Qubit dsDNA HS Reagent
2985 µl	Qubit dsDNA HIS Buffer
10 µl	Standard 1 and 2*

*for each tube

Table 5. reagents for Qubit.

The mixture for the standards contained:

Amount	**Reagent**
190 µl	Qubit Mix
10 µl	Standard

Table 6. mixture for standards.

The mixture for the samples contained:

Amount	**Reagent**
199 µl	Qubit Mix
1 µl	Standard

Table 7. mixture for samples.

DNA amplification

After the isolation of DNA, the amplification of DNA barcodes was achieved through polymerase chain reaction (PCR). The PCR protocol was executed as follows:

Amount*	**Reagent**
0.6 µl	BSA
6.4 µl	H_2O
12.5 µl	Master Mix
1.75 µl	f Primer
1.75 µl	r Primer
2 µl	Sample

*amounts were used per sample

Table 8. PCR protocol.

Repetitions	**Time**	**Temperature**	**Step**
	15 min	95°C	
34	30 sec	95°C	Denaturation
34	1 min	X°C	Annealing
34	1 min	72°C	Extension
	10 min	72°C	Final Extension
		Cool down to 8°C	

Table 9. Temperature setting for PCR.

I utilized different primers:

Primer forward		**Primer reverse**		**Amplicon size**	**DNA Indices**	**Publication**
amCytB -1f	*TAC AAA ACT CAC ATG CCA TC*	amCytB -1r	*CCG AAG AAG GCT GTT G*	*154 bp*	CytB	unpublished
amCytB -2f	*TAA ACG ACC CAG AAA ATT TC*	amCytB -2r	*CAG GTT TAA TGT GTT GTG GG*	*63 bp*	CytB	unpublished
amCytB -1f	*TAC AAA ACT CAC ATG CCA TC*	amCytB -2r	*CAG GTT TAA TGT GTT GTG GG*	*576 bp*	CytB	unpublished
amND5 -1f	*TTT ACC TCC TCA GCA CTT TTC TG*	amND5 -1r	*AAG TGT TCT CGT GAG TGG GTT G*	*341 bp*	ND5	unpublished
LS14841	*GGATCAAACATTTCA ACTTGATG*	HCYTV_L	*AGGCTCCAGCAAC CCATTAGG*	*	CytB	Ursenbacher et al. 2008

16AS	*GTATCCTAACCGTG CAAAG*	H-3056	*CCGGTCTGAACTC AGATCACG*	*463 bp*	16S	Ursenbacher et al. 2008

*Both laboratory and in silico amplification (www.ncbi.nlm.nih.gov/tools/primer-blast) did not result in a DNA fragment, indicating an issue in the published primers.

Table 10. list of primers used for this research project.

By running those primer combinations through primer-blast, the suitable annealing temperature and fragment length are presented.

For the PCR conducted to test the primer pairs, we employed the following:

Amount	**Reagent**
0.6 µl	BSA
6.4 µl	H_2O
12.5 µl	Master Mix
1.75 µl	CytB 1f Primer
1.75 µl	CytB 1r Primer
2 µl	Sample*

*per tube

Table 11. list of ingredients for PCR.

In total, 13.5 µl of solution, including the sample, were inside the Eppendorf tubes.

After the mixture was prepared, I vortexed it. If the samples did not have the same volume as others, I adjusted the amount with water. A 2% agarose gel was prepared with TBE Buffer and 2.5 µl midori green.

Repetitions	**Time**	**Temperature**	**Step**
	15 min	95°C	
34	30 sec	95°C	Denaturation
34	1 min	X°C	Annealing
34	1 min	72°C	Extension
	10 min	72°C	Final Extension
		Cool down to 8°C	

Table 12. temperature settings for PCR.

I prepared a master mix for 16 samples, whereas 9.75 µl were allocated to each sample:

Amount	**Reagent**
5.4 µl	BSA

57.6 µl	H_2O
112.5 µl	Master Mix

Table 13. master mix for 16 samples.

The samples were vortexed and briefly centrifuged for 2 sec in the MiniStar.

The PCR machine was prepared under the following set-up:

Annealing Temperature	**Spot in PCR machine**	**Sample**	**Mixture**	**Primer**	**Primer**
647°C	1.1	2 µl	9.75 µl	0.875 µl CytB 1f	0.875 µl CytB 1r
	1.2	2 µl	9.75 µl	0.875 µl CytB 2f	0.875 µl CytB 2r
	1.3	2 µl	9.75 µl	0.875 µl CytB 1f	0.875 µl CytB 2r
	1.4	2 µl	9.75 µl	0.875 µl amND5 1f	0.875 µl amND5 1r
59.3°C	2.1	2 µl	9.75 µl	0.875 µl CytB 1f	0.875 µl CytB 1r
	2.2	2 µl	9.75 µl	0.875 µl CytB 2f	0.875 µl CytB 2r
	2.3	2 µl	9.75 µl	0.875 µl CytB 1f	0.875 µl CytB 2r
	2.4	2 µl	9.75 µl	0.875 µl amND5 1f	0.875 µl amND5 1r
52.3°C	3.1	2 µl	9.75 µl	0.875 µl CytB 1f	0.875 µl CytB 1r
	3.2	2 µl	9.75 µl	0.875 µl CytB 2f	0.875 µl CytB 2r
	3.3	2 µl	9.75 µl	0.875 µl CytB 1f	0.875 µl CytB 2r
	3.4	2 µl	9.75 µl	0.875 µl amND5 1f	0.875 µl amND5 1r
49°C	4.1	2 µl	9.75 µl	0.875 µl CytB 1f	0.875 µl CytB 1r
	4.2	2 µl	9.75 µl	0.875 µl CytB 2f	0.875 µl CytB 2r
	4.3	2 µl	9.75 µl	0.875 µl CytB 1f	0.875 µl CytB 2r
	4.4	2 µl	9.75 µl	0.875 µl amND5 1f	0.875 µl amND5 1r

Table 14. settings for gradient PCR.

In total 13.5 µl liquid were added per tube.

The gradient PCR assessed various annealing temperatures for the primer pairs to determine which one performs best.

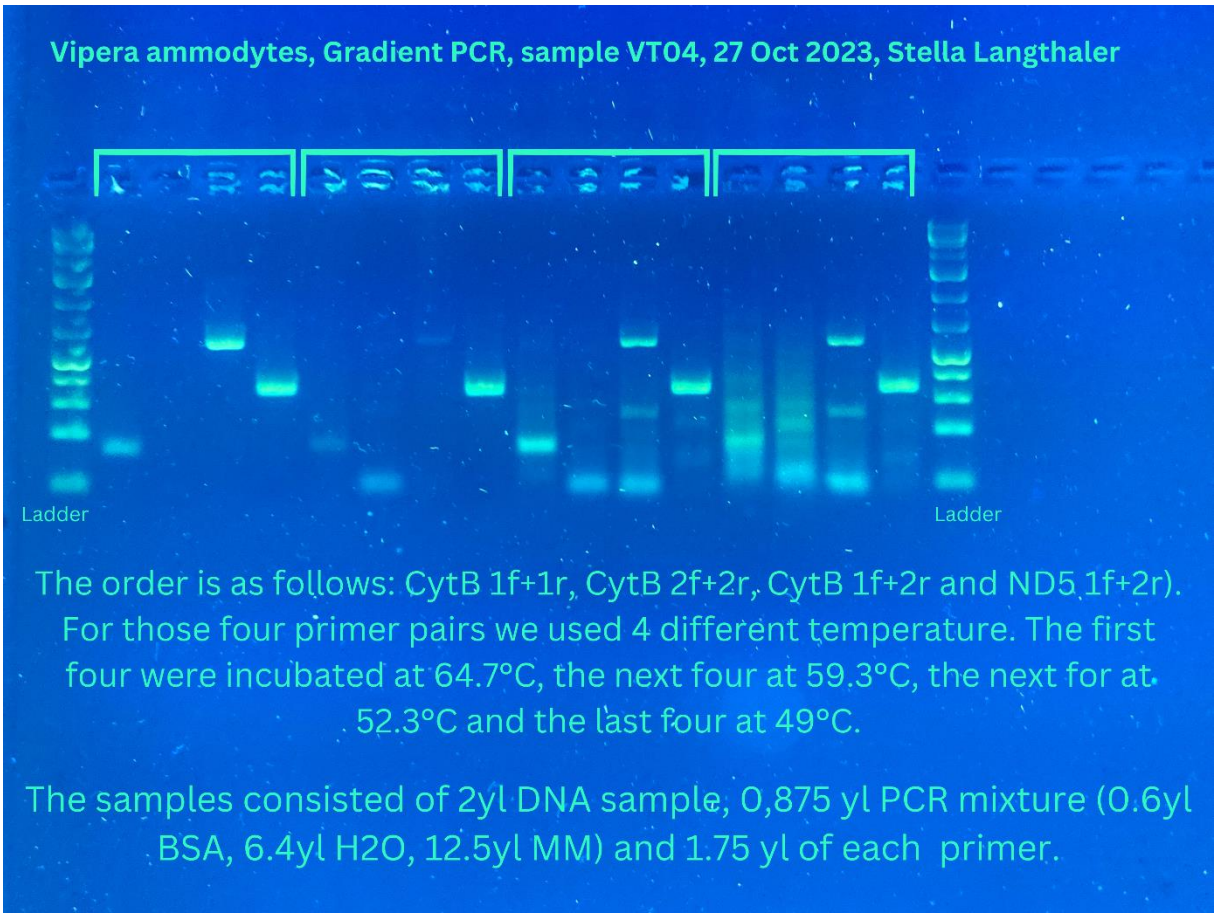

Figure 3. Visualization of the gradient PCR.

The samples provided by the Natural History Museum Vienna comprised plasma, erythrocytes, and pre-subtracted DNA. For the plasma cells, I processed them using the E.Z.N.A.® Tissue DNA Kit with the Cultured Cells Protocol (refer to Appendix 2), while for muscle and erythrocytes, I utilized the E.Z.N.A.® Tissue DNA Kit Protocol (refer to Appendix 2).

Due to the erythrocytes containing very little sample, I added 25 µl DTT to the samples and vortexed them. Afterwards they were put in the incubator at 55°C, with 300 rpm, for 2h.

For the plasma samples I transferred 25 µl plasma into a 1.5 ml Eppendorf tube. I centrifuged them for one minute at 13,000g, but no pellets formed. Therefore, I added 200 µl PBS and 25 µl Proteinase K and vortexed them. The optional step from the original protocol was skipped.

From this point onwards I followed the original protocol with minor adaptions which are documented in the following protocol:

1. 220 µl instead of 200 µl BL Buffer were added to all the samples.
2. The 25 µl Proteinase K were not added at this step, because it was already inside.
3. The samples were put into the incubator at 70°C for 10 minutes.
4. 220 µl Ethanol were added.

5. All samples were vortexed.
6. Samples were transferred into HiBind column.
7. Centrifuged for 1 min at 13,000g. Filtrate discarded.
8. 500 µl HBC Buffer were added.
9. Centrifuged for 30 sec and 13,000g.
10. The filtrate and collection tube were discarded.
11. 700 µl DNA wash buffer was added and the samples were centrifuged for 30 sec at 13,000g.
12. 70°C-warm 200 µl Elution buffer were added to each sample, we waited 2 min and centrifuged them at full speed for 30 sec.
13. Elution Buffer (200 µl) was added again, we centrifuged the samples for 2 min.
14. I stored the samples in the freezer (-20C) and the columns in the fridge (+4C).

For the other samples we followed and E.Z.N.A.® Tissue DNA Kit Protocol (see Appendix 2), which was performed the following:

1. After two hours in the shaker at 300 rpm, 55°C, the three samples were centrifuged for 5 min at 13,000 g.
2. The samples were transferred to 1.5 ml tubes, excluding the insoluables.
3. 220 µl BL Buffer were added to all the samples.
4. All samples were vortexed.
5. All tubes were brought to the incubator, which was set to 70°C for 10 min.
6. 220 µl 100% EtOH were added, and samples were vortexed.
7. HiBinds were inserted into tubes.
8. Samples were centrifuged for 1 min at full speed and filtrate was discarded.
9. 500 µl HBC Buffer were added and centrifuged for 30 sec and full speed.
10. A new collection tube was used.
11. 700 µl DNA wash Buffer was added, samples were centrifuged at full speed for 30 sec.
12. Step 11 was repeated.
13. Empty columns were centrifuged for 2 min at 13,000 g.
14. 200 µl Elution Buffer were heated to 70°C and added to the samples. Follow waiting time of protocol and centrifuge at full speed for 1 min.
15. Step 14 was repeated.
16. DNA was stored on -20C.

PCR for all *V. ammodytes* samples:

Amount	Reagent
0.6 µl	BSA
6.4 µl	H_2O
12.5 µl	Master Mix
1.75 µl	CytB 1f Primer
1.75 µl	CytB 1r Primer
2 µl	Sample*

*per tube

Table 15. solution for PCR.

In total, 13.5 µl of solution, including the sample, were inside the Eppendorf tubes. The concentrations of ingredients were chosen based on the previous results.

After the mixture was prepared, I vortexed it. If the samples did not have the same volume as others, I adjusted the amount with water. A 2% agarose gel was prepared with TBE Buffer and 2,5 µl midori green.

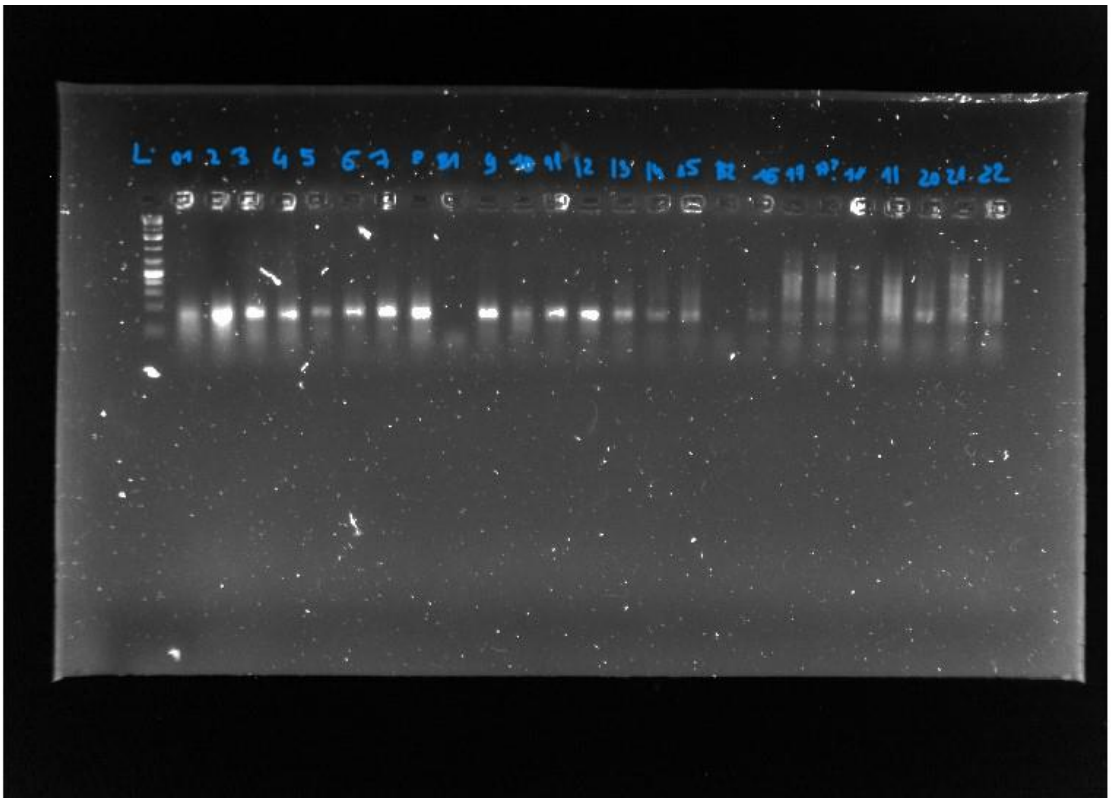

Figure 4. Results of the PCR (1-22, relating to the sample nominication system), annealing temperature set to 59°C, using CytB 1f and CytB 1r primers. The annealing temperature was based on the results of the gradient PCR and can vary.

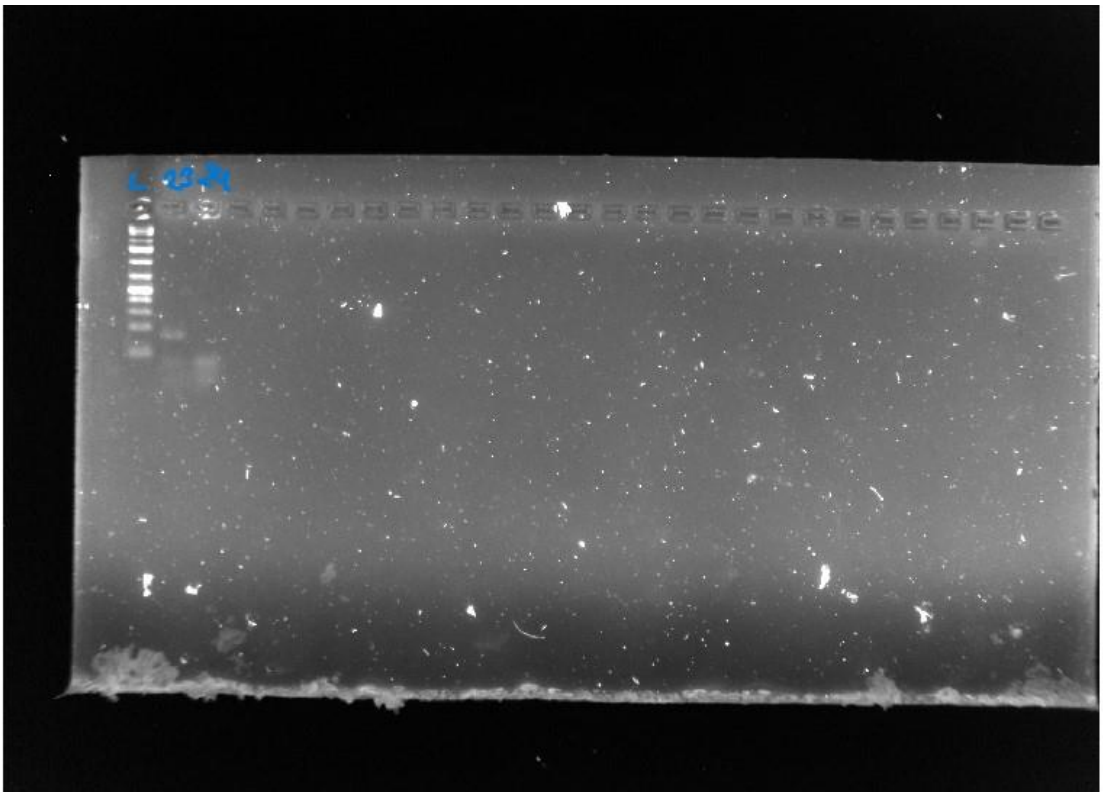

Figure 5. Results of the PCR (23 and 24, relating to the sample nominication system), annealing temperature set to 59°C, using CytB 1f and CytB 1r primers. The annealing temperature was based on the results of the gradient PCR and can vary.

The PCR was later also performed for the following primer pairs:

CytB 1f – 2f, CytB 2f-2r, amND5 1f – 1r

As before, the following was used:

Amount	**Reagent**
0.6 µl	BSA
6.4 µl	H_2O
12.5 µl	Master Mix
1.75 µl	CytB 1f Primer
1.75 µl	CytB 1r Primer
2 µl	DNA extract*

*per tube

Table 16. solution for PCR.

Which, excluding the sample, had to be multiplied by 39, since 75 reactions were performed.

Preparation of the gel:

Amount	Reagent
100 ml	0.5 x TBE
2 g	Agrar Powder
4 µl	Midori Green

Table 17. list of ingredients for the gel.

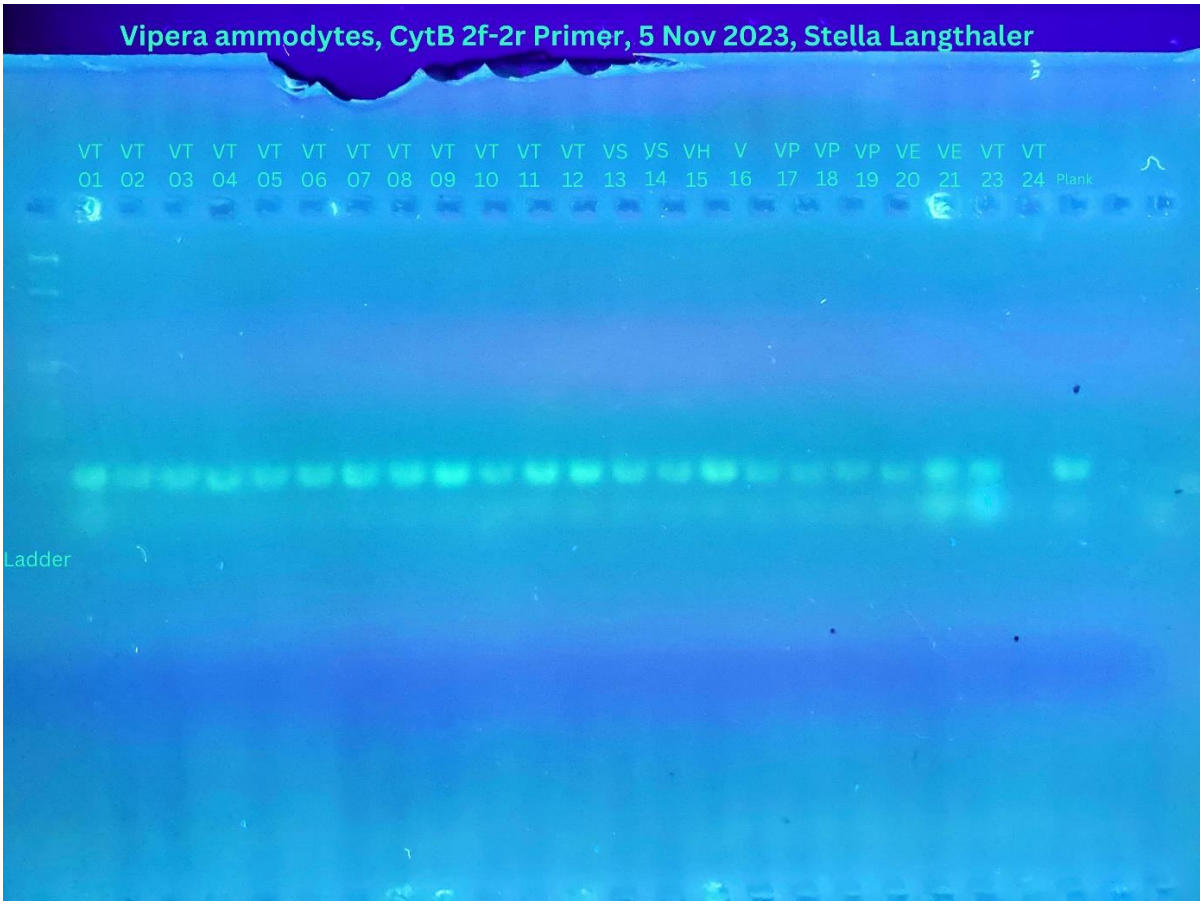

Figure 6. The PCR was conducted with CytB 2f-2r primer pairs for all samples.

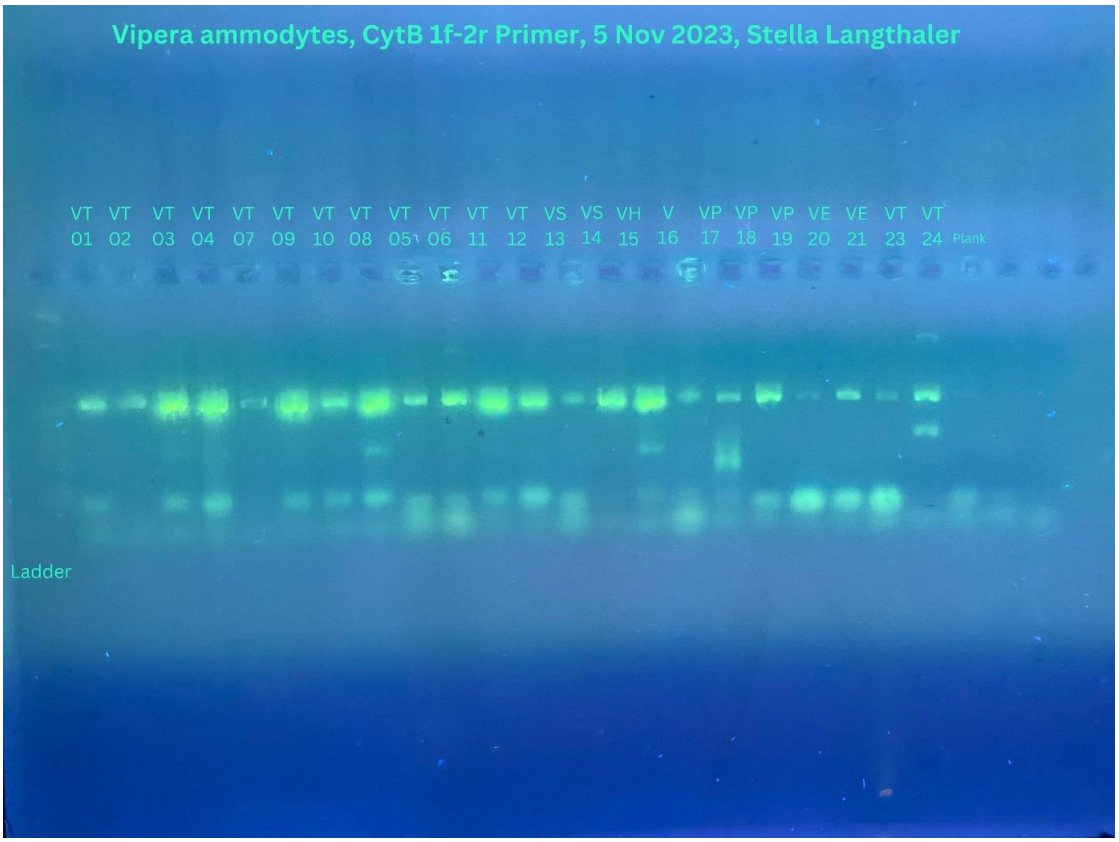

Figure 7. The PCR was conducted with CytB 1f – 2f primer pairs for all samples.

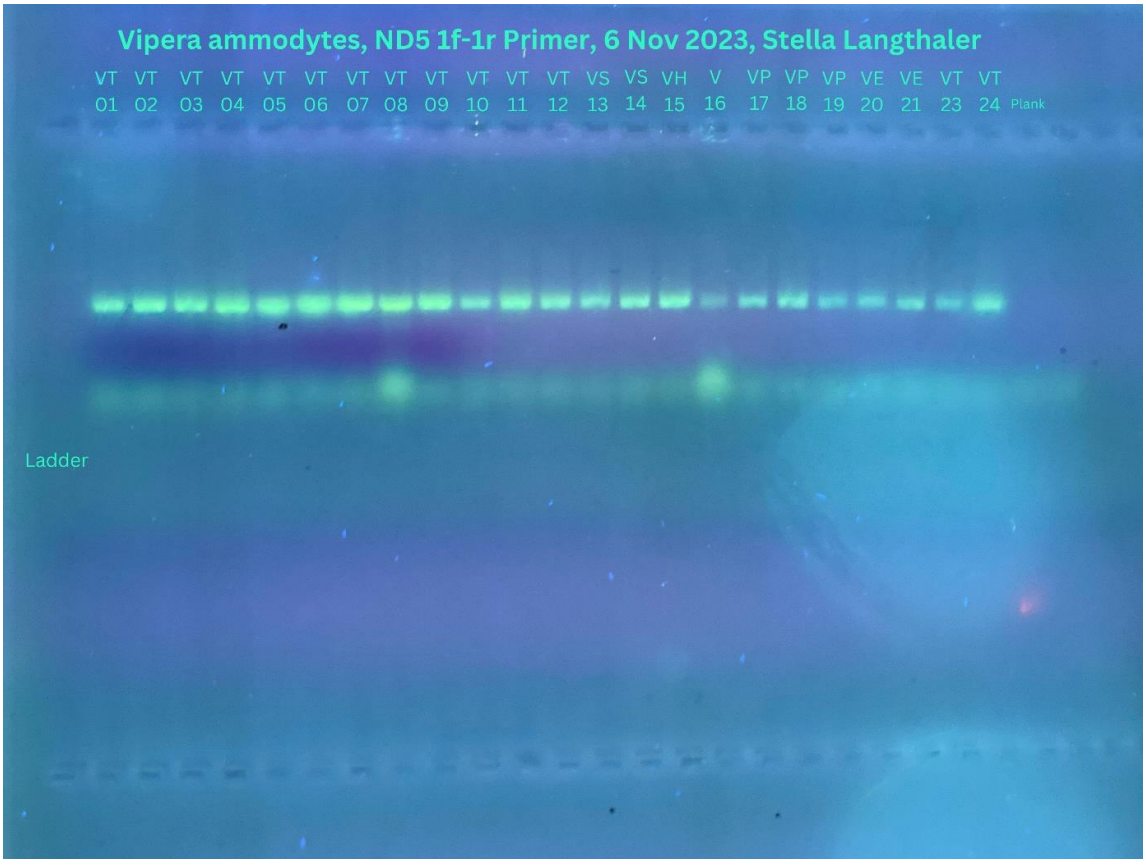

Figure 8. The PCR was conducted with amND5 1f – 1r primer pairs for all samples.

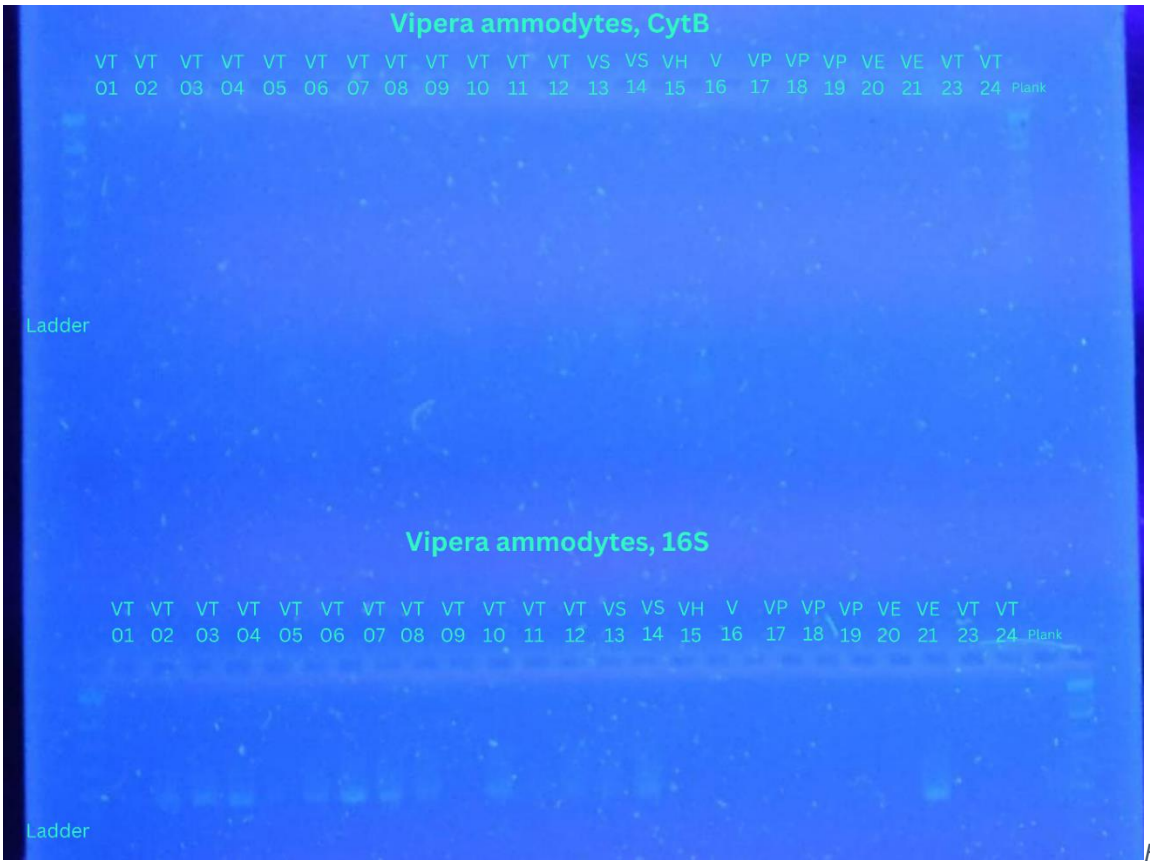

Figure 9. PCR amplification using the CytB and 16S primers from Ursenbacher et al. 2008. Note that the CytB failed to amplify for all samples.

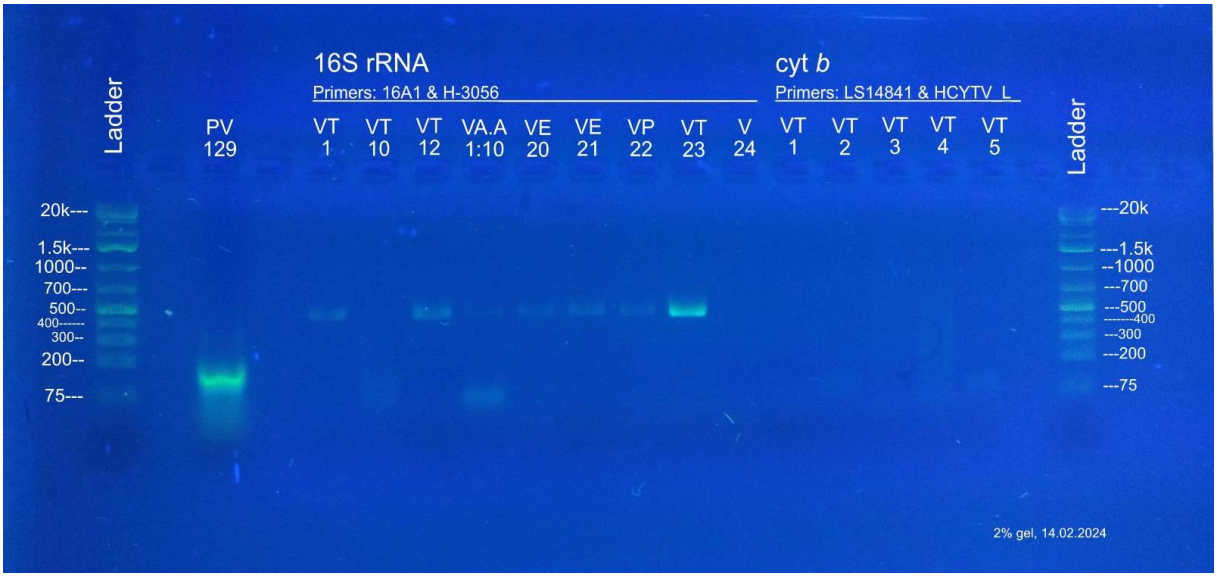

Figure 10. PCR amplification using the CytB and 16S primers from Ursenbacher et al. 2008. This is the second run, due to the unclear results from the first run (see Figure 9).

Sequencing Library Preparation

The preparation of the sequencing library was conducted according to the instruction manual for the Native Barcoding Kit 96 V14 by Oxford Nanopore Technologies (ONT) (see Appendix 3). This process spanned approximately one working day, not including the time allocated for the sequencing process itself.

For the library preparation I allocated indices (ONT barcodes) to each of the samples.

Run1				Run2			
Sample	**Indices**	**Sample**	**Indices**	**Sample (16S)**	**Indices (16S)**	**Sample (16S)**	**Indices (16S)**
VT01	33	VS13	45	VT01	73	VS13	85
VT02	34	VS14	46	VT02	74	VS14	86
VT03	35	VH15	47	VT03	75	VH15	87
VT04	36	V16	48	VT04	76	V16	88
VT05	37	VP17	49	VT05	77	VP17	89
VT06	38	VP18	50	VT06	78	VP18	90
VT07	39	VP19	51	VT07	79	VP19	91
VT08	40	VE20	52	VT08	80	VE20	92
VT09	41	VE21	53	VT09	81	VE21	93
VT10	42	VP22	54	VT10	82	VP22	94
VT11	43	VT23	55	VT11	83	VT23	95
VT12	44	V24	56	VT12	84	V24	96

Table 18. Assignment of Indices to samples.

Sequencing

The sequencing library of the pooled samples were sequenced on ONT's MinION Mk1C sequencing platform. Run 1 included amplicons for (CytB1f-r, CytB2f-r,CytB1f-2r and ND5) and run 2 all the 16S amplicons. The sequencing process was conducted over a 24-hour period, after which the data were retrieved (see Appendix 3).

Bioinformatics

Basecalling, the conversion of raw sequencing information into the human-readable fastq format, and demultiplexing were performed using ONT's Dorado software (https://github.com/nanoporetech/dorado). Next, the quality of the sequencing reads was assessed using NanoPlot (https://github.com/wdecoster/NanoPlot). Docker served as the platform for data preparation. Two data processing pipelines were used. For the 16S data, consensus sequences were generated using NGSpeciesID (Sahlin et al. 2021). Finally, the sequencing data were polished using Racon (https://github.com/rvaser/spoa) (Dubey et al., 2011). The consensus sequences were then combined with the 16S data from Ursenbacher et al. 2008, aligned with MAFFT v 7.507 (http://mafft.cbrc.jp/alignment/software/) and primer sequences removed using AliView (https://github.com/AliView). For the CytB1f-r, CytB2f-r,CytB1f-2r and ND5 data, first primers were removed using cutadapt (Martin 2011). The reads were then mapped against the *Vipera berus* reference mitochondrial genome (NC_036956.1) using BWA version 0.7.17 (Li and Durbin 2009) and samtools (Li et al. 2009). Consensus sequences were then generated using ANGSD version 0.940 (Korneliussen et al. 2014; using -doFasta 2 and -doCounts 1) and the regions extracted using bedtools version 2.30.0 (Quinlan and Hall 2010). *CytB* sequences were then combined with the data from Ursenbacher et al. 2008 and Thanou et al. 2023 and *ND5* sequences with the data from Ursenbacher et al. 2008. Alignments were performed using MAFFT v 7.507 (http://mafft.cbrc.jp/alignment/software/).

Phylogenetic relationships were then analysed and visualized using median-joining haplotype networks implemented in the software PopArt (Leigh and Bryant 2015).

Results

I obtained PCR amplicons for all primer pairs for all samples, except for the 16S fragment for samples VP22 and VT23, as these were mixed by accident. Even the longer fragments (e.g. 16S with 463 bp) successfully amplified for the plasma samples, which are known to contain very little and very fragmented DNA. However, one of the Greek samples (VT9) was omitted from the 16S haplotype network (Figure 11) due to incorrect sequence identification. Therefore, Figure 11 portrays the genetic relationships among my 21 samples and the data from Ursenbacher et al. 2008 and Thanou et al. 2023.

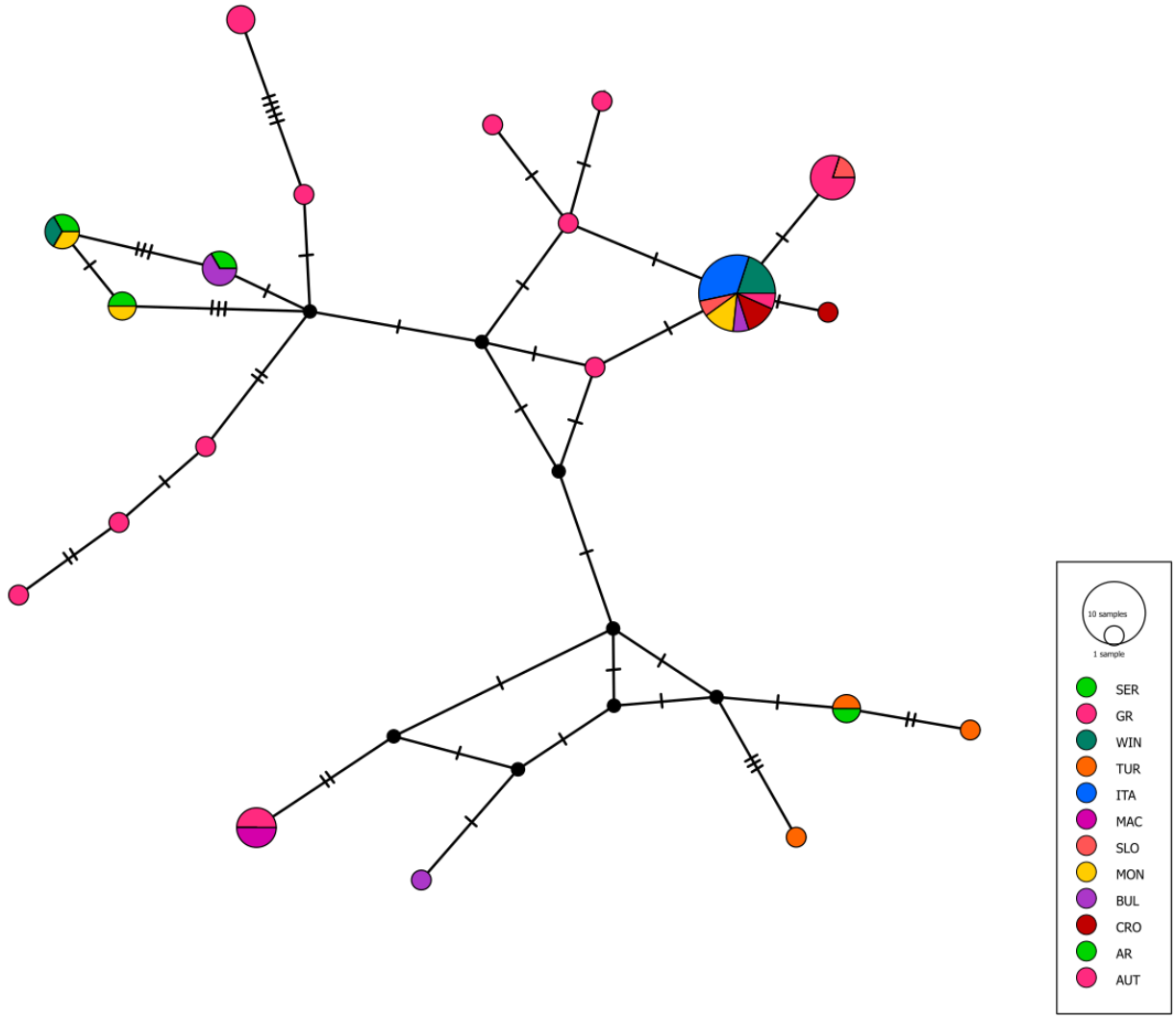

Figure 11. Median-joining haplotype network using CytB amplicons, depicting the genetic relationships among various individuals, including published data from Ursenbacher, 2008. The abbreviations correspond to different geographical locations: SER for Serbia (light green), GR for Greece (pink), WIN for Winzendorf in Austria (dark green), TUR for Türkiye (orange), ITA for Italy (blue), MAC for Macedonia (pink-purple), SLO for Slovenia (pink-orange), MON for Montenegro (yellow), BUL for Bulgaria (purple), CRO for Croatia (dark red), AR for Armenia (light green), and AUT for Austria (pink).

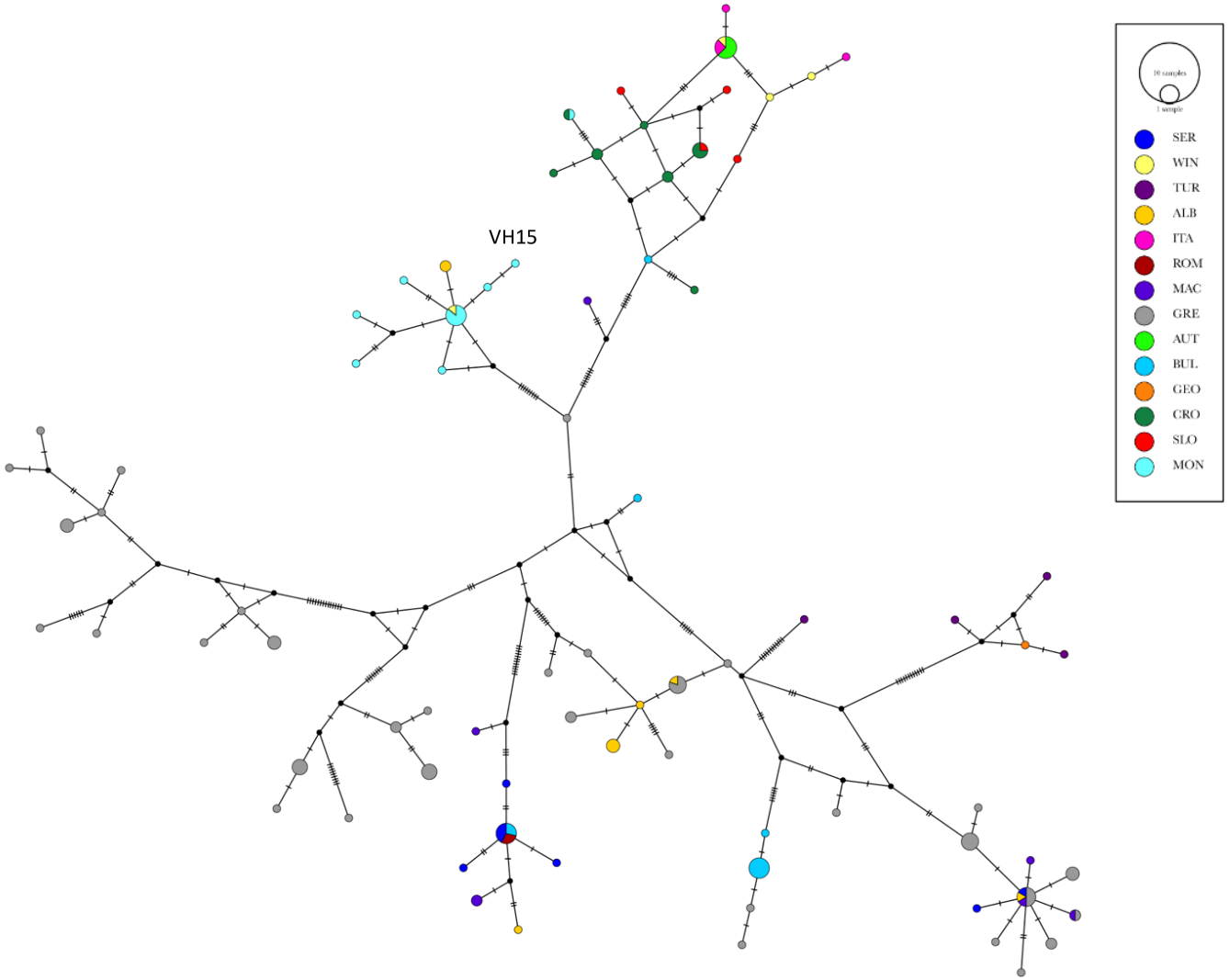

Figure 12. Median-joining haplotype network for the CytB amplicons, including my sequences and the data from 118 individuals sourced from Ursenbacher et al. in 2008 and Thanou et al. 2023. The abbreviations correspond to different geographical locations: SER for Serbia (dark blue), GRE for Greece (gray), WIN for Winzendorf in Austria (yellow), TUR for Türkiye (purple), ITA for Italy (pink), MAC for Macedonia (dark blue-purple), SLO for Slovenia (red), MON for Montenegro (turquoise), BUL for Bulgaria (light blue), CRO for Croatia (dark green), GEO for Georgia (orange), ROM for Romania (dark red), ALB for Albania (dark yellow), and AUT for Austria (bright green).

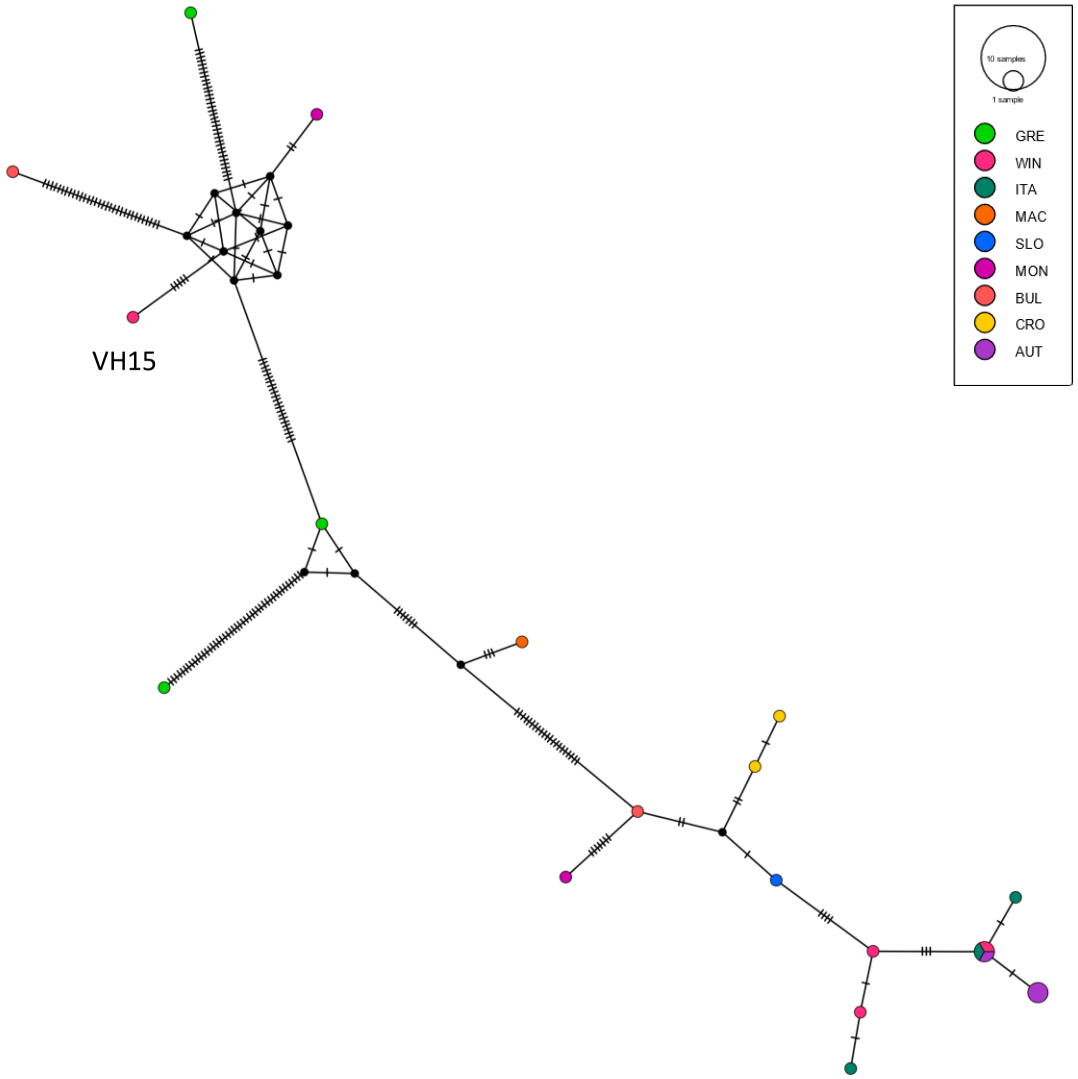

Figure 13. Median-joining haplotype network for the combined CytB and ND5 amplicons from this study. The abbreviations correspond to different geographical locations: GRE for Greece (light green), WIN for Winzendorf in Austria (pink), ITA for Italy (dark green), MAC for Macedonia (orange), SLO for Slovenia (blue), MON for Montenegro (pink-purple), BUL for Bulgaria (pink-orange), CRO for Croatia (yellow), and AUT for Austria (purple).

The presented results reveal that the samples obtained from Winzendorf belong to two distinct haplogroups (in all three analyses, see Figure 11, 12, 13). While VH15 clusters closely to samples from Montenegro, all other Winzendorf samples, show a close relationship to samples from Italy and Austria. Furthermore, one sample (VS14) shows the same haplotype as one sample from Italy and one from Carinthia (Austria). In a separate study, Ursenbacher et al. 2008 show that Italian, Carinthian, Croatian, Slovenian and Bosnia and Herzegovina populations belong to the same "North-Western" clade, as defined in the publication (Ursenbacher et al., 2008). Consistent with expectations, the Turkish, and Armenian, as well as Greek, and Macedonian populations display closely related haplotypes to each other.

Furthermore, a cluster can be detected in the Montenegrin population, displaying close genetic ties with an individual from Albania, as well as with one individual from Winzendorf. Similarly, the Serbian population shows a connection with populations from Albania, Macedonia, and Romania. In contrast, Greek individuals are dispersed across the haplotype network (refer to Figure 11).

Figure 12 depicts a notable association among populations from Winzendorf, Italy, and Carinthia (Austria). Meanwhile, like the Greek population, the Bulgarian population demonstrates a dispersed distribution throughout the haplotype network.

Discussion

Possible origins of the Winzendorf individuals

The findings suggest that the population in Winzendorf likely originated from two or more source populations, as it includes genetic haplotypes found in Italy/Austria and in Montenegro (see Ursenbacher et al. 2008). The result supports the hypothesis of unauthorized releases, most likely via the pet trade. Indeed, Filz et al. 2018 assessed the invasion risk of several commonly traded reptiles and found a very high establishment risk for *V. ammodytes* in Central Europe. These illegal releases pose high risks to local fauna and flora, yet in the case of *V. ammodytes* in Winzendorf*,* no adverse interactions have been observed. The reptiles' habitat in Winzendorf closely resembles its native habitat, facilitating good chances for survival for this species. Indeed, it is hypothesized that *V. ammodytes* has been present in Winzendorf for a while, although the first verified sighting took place in May 2020 (Silke Schweiger, personal communication). Thus far, no negative impacts attributed to *V. ammodytes* have been documented. Currently, the Winzendorf population is monitored to assess population stability (Wallner, 2023). Additionally, monitoring other reptile species in the region could shed light on potential prey-pressure or competition factors influencing their existence.

The data obtained does not show clear evidence that the population from Winzendorf is not closely related to the population from Carinthia. However, there are indicators of a stronger connection with the North-Western clade. One reason to suspect an unauthorized release in Winzendorf is the initial sighting of these individuals in 2020, although it is suspected that the species has been present for around 20 years. This, coupled with their proximity to the population in southern Austria, raises concerns of illicit introduction. Additionally, considering the possibility of inter-population mating in captivity, it is plausible that such interactions could obscure the clarity of the results obtained. Additional research is needed to investigate the relationship between the Winzendorf population and especially the ones from Carinthia and Italy. The utilization of nuclear markers could potentially provide higher resolution for the distinction of the Carinthian and the Italian population.

As the glacial retreat proceeded, the territorial expansion of *V. ammodytes* from southern to northern territories became apparent. Through a Templeton, Crandall, and Sing (TCS) analysis, the likelihood of the reptile's migration along two distinct pathways, originating from the central region of Dalmatia, became distinct (Ursenbacher et al., 2008). One plausible route suggests a journey across Croatia's offshore islands, proceeding through Istria, and eventually extending into Slovenia. Meanwhile, the alternative route proposes a movement across the expanse of the Adriatic basin, ultimately leading to the northeastern reaches of Italy

(Ursenbacher et al., 2008). Compared to this study, these tendencies can be seen in Figure 11, although the genetic divergence is not as evident.

Ursenbacher et al. 2008 showed that the genetic diversity among *V. ammodytes* populations in the Balkan is notably high, likely stemming from distinct refugia during past glaciations. Similar genetic variations have been observed in this region among various species, including plants and lizards (Magri et al., 2006; Sotripoulos et al. 2007; Wallis and Arntzen, 1989). Interestingly, the genetic findings do not support the difference in morphological traits. Those are likely attributed due to differentiating environmental conditions, as this phenomenon has been shown in different snake species (Castellano et al., 1994).

In 2023, Thanou conducted research that echoed similar discoveries concerning the genetic divergence observed in the Balkan, theorized to have originated from allopatric speciation. Their study investigated thousands of single nucleotide polymorphisms (SNPs) and identified several hybrid zones. Intriguingly, two sympatric sub-populations emerged within a roughly 50-kilometer radius in a region of Serbia, yet they displayed no signs of hybridization. Comparing the data presented in Figure 12 reveals a notable genetic divergence among individuals from Serbia. This observation aligns with the conclusions drawn by Thanou in 2023, further supporting their findings regarding the diverse origins of populations in the region.

It is probable that *V. ammodytes* populations in the Balkan underwent bottlenecks, which could have contributed to the increased genetic diversity between them (Ursenbacher, 2008). Similarly, my own findings indicate significant genetic distance among individuals from this region (refer to Figures 11 and 13). Notably, VH15, one of the individuals from Winzendorf, is associated with a different haplogroup and showing a remarkably close affiliation with individuals from the Balkan (as depicted in Figure 11 and 13). This unexpected result could suggest several unauthorized releases in the same region, potentially even leading to the mating of individuals from different origins.

Unauthorized releases can destroy ecosystems

Unauthorized releases pose a threat to native ecosystems. Especially when invasive species adapted well adapted to the local conditions, they have the potential to spread uncontrollably. In 2000 the abundance of the non-native, invasive Burmese python (*Phyton bivittatus*) in the Everglades, Florida, increased drastically. Consequently, native mammals and birds have begun to disappear from the area. Raccoons have experienced declines exceeding 99%, closely trailed by equally severe drops in bobcat and opossum populations. Following the increase of Burmese python, rabbits have become so rare, failing to be detected (Dorcas et

al., 2012). This is one of the possible dangers of an unauthorized release in a habitat that enables the invasive species to spread uncontrollably, without any natural predators. However, future studies could continue monitoring the presence of *V. ammodytes* and behaviour to ascertain if any changes occur over time.

It is crucial to underscore that not all alien species are harmful, as many fail to survive in new regions. Even if they establish themselves, they may become a stable player in the ecosystem. Only a small fraction, approximately 14% of all alien vertebrates, become invasive (https://www.ipbes.net/IASmediarelease). A study by the University of Nebraska – Lincoln, reflects on the effect on non-native species in Florida, the epicentre of newly-introduced organisms. It suggests that few of the alien species resulted in economic or ecological impacts, while still having noted problematic introductions, as demonstrated by *P. bivittatus* (Dorcas et al., 2012). This and other studies encourage responsible pet ownership and to identify the species with the greatest risk for native populations when released into the wild (e.g. Hardin, 2007).

Ethical concerns

While there are numerous accounts of invasive species impacting ecosystems negatively, there are instances where the opposite occurs. In Hawaii, for example, rainforests partially rely on birds for seed dispersal. However, the number of native bird species on the islands has declined by over two-thirds (Case, 2021). Over time, non-native pet birds were illegally released and have spread throughout the islands. These are now filling the ecological roles of the extinct ones, enabling the respective plants to reproduce (Foster & Robinson, 2007).

Vipera ammodytes has not raised concerns regarding its potential negative impact on the ecosystem.

Using DNA barcoding to fight wildlife crime

Gouda et al. (2020) shed light on the significant challenge of species identification in illicit wildlife trafficking, revealing that 70% of wildlife crime cases face prosecution hurdles due to inaccurate identification. DNA barcoding emerges as a promising solution, not only enabling precise species identification but also providing insights into their geographical origins. This advancement allows authorities not only to identify the species involved but also to pinpoint poaching hotspots. Consequently, effective prosecution becomes feasible, thereby impeding organized illicit trafficking networks.

A study conducted by Wasser et al. in 2015 demonstrated success in identifying poaching hotspots in the ivory trade using microsatellite DNA loci with a high mutation rate. Through the application of the "Smoothed Continuous Assignment Technique" (SCAT) (Wasser et al., 2004), the researchers were able to create a detailed map of the origin of the elephants involved in the trade. This information has helped inform authorities, enabling them to identify patterns and concentrate law enforcement efforts at its source. The forensic techniques in this study have demonstrated accuracy in species identification, particularly in tracing specimens back to their geographic sources. The establishment of a comprehensive database is essential to streamline conservation efforts (Iyengar, 2014). In the realm of wildlife crime, the emphasis shifts from individual genetic identification to uncovering the origins and affiliations of specimens within subpopulations.

The markers applied in this study can also be used to investigate (illegal) trade in *V. ammodytes*. Filippi and Luiselli, 2000, investigated threats to the Italian snake fauna and concluded that the Italian population of *V. ammodytes* is threatened by trade. Furthermore, overcollection of individuals for venom extraction poses a strong risk for *V. ammodytes*, e.g. in Serbia (Ajtić, 2008) or Montenegro (Ajyić et al. 2005). Similar to the investigation on the possible origins of the Winzendorf individuals (for my thesis), the markers can be applied to source potential origins of illegally collected and traded *V. ammodytes* individuals.

References

Agasyan, A., Avci, A., Tuniyev, B., Isailovic, J. C., Lymberakis, P., Andrén, C., Cogalniceanu, D., Wilkinson, J., Ananjeva, N., Üzüm, N., Orlov, N., Podloucky, R., Tuniyev, S., Kaya, U., Sindaco, R., Böhme, W., Ajtic, R., Tok, V., … Jelić, D. (2008). IUCN Red List of Threatened Species: Vipera ammodytes. IUCN Red List of Threatened Species. https://www.iucnredlist.org/en

Ajtić, R. (2009). Nose-horned viper (Vipera ammodytes): Conservation problems in Serbia. Zaštita prirode, 60(1-2), 319-326.

Auliya, M., Altherr, S., Ariano-Sanchez, D., Baard, E. H., Brown, C., Brown, R. M., Cantu, J.-C., Gentile, G., Gildenhuys, P., Henningheim, E., Hintzmann, J., Kanari, K., Krvavac, M., Lettink, M., Lippert, J., Luiselli, L., Nilson, G., Nguyen, T. Q., Nijman, V., … Ziegler, T. (2016). Trade in live reptiles, its impact on wild populations, and the role of the European market. Biological Conservation, 204, 103–119. https://doi.org/10.1016/j.biocon.2016.05.017

Baran, I., Atatür, M.K., 1998. Turkish herpetofauna (amphibians and reptiles), Ankara.

Biological identifications through DNA barcodes | Proceedings of the Royal Society of London. Series B: Biological Sciences. (o. J.). Abgerufen 31. März 2024, von https://royalsocietypublishing.org/doi/abs/10.1098/rspb.2002.2218

Boulenger, G.A., 1903. On the geographical variations of the sand-viper, Vipera ammodytes. Proc. Zool. Soc. Lond. 1, 185–186.

Boulenger, G.A., 1904. On the sand viper of Romania (Vipera ammodytes, var. montandoni).

Ann. Mag. Nat. Hist. 7, 134–135.

Bruno, S., 1968. Sulla Vipera ammodytes (Linnaeus, 1758) in Italia. Mem. Mus. Civ. Stor. Nat. 15, 289–336.

Cabela, A., Grillitsch, H., & Tiedemann, F. (2001). Atlas zur Verbreitung und Ökologie der Amphibien und Reptilien in Österreich. Umweltbundesamt, Wien, 1, 880.

Case, 2021, https://www.scientificamerican.com/article/invasive-species-can-sometimes-help-an-ecosystem/.

Castellano, S., Malhorta, A., Thorpe, R.S., 1994. Within-island geo graphic variation of the dangerous Taiwanese snake, Trimeresurus stejnegeri, in relation to ecology. Biol. J. Linnean Soc. 52, 365–375.

Dubey, B., Meganathan, P. R., & Haque, I. (2011). DNA mini-barcoding: An approach for forensic identification of some endangered Indian snake species. Forensic Science International: Genetics, 5(3), 181–184. https://doi.org/10.1016/j.fsigen.2010.03.001

Episcopio-Sturgeon, D. J., & Pienaar, E. F. (2020). Investigating Support for Management of the Pet Trade Invasion Risk. The Journal of Wildlife Management, 84(6), 1196–1209. https://doi.org/10.1002/jwmg.21867

Filippi, E., & Luiselli, L. (2000). Status of the Italian snake fauna and assessment of conservation threats. Biological conservation, 93(2), 219-225.

Filz, K. J., Bohr, A., & Lötters, S. (2018). Abandoned Foreigners: is the stage set for exotic pet reptiles to invade Central Europe?. Biodiversity and Conservation, 27, 417-435.

Gollmann, G. (2007): Rote Liste der in Österreich gefährdeten Lurche (Amphibia) und Kriechtiere (Reptilia). Rote Liste gefährdeter Tiere Österreichs. Checklisten, Gefährdungsanalysen, Handlungsbedarf. Teil 2: Kriechtiere, Lurche, Fische, Nachtfalter, Weichtiere. (Red. K. P. Zulka), pp. 37-60. Böhlau, Wien.

Gouda, S., Kerry, R. G., Das, A., & Chauhan, N. S. (2020). Wildlife forensics: A boon for species identification and conservation implications. Forensic Science International, 317, 110530. https://doi.org/10.1016/j.forsciint.2020.110530

Hebert, P. D., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(1512), 313-321.

Iyengar, A. (2014). Forensic DNA analysis for animal protection and biodiversity conservation: A review. Journal for Nature Conservation, 22(3), 195–205. https://doi.org/10.1016/j.jnc.2013.12.001

Korneliussen, T. S., Albrechtsen, A., & Nielsen, R. (2014). ANGSD: analysis of next generation sequencing data. BMC bioinformatics, 15, 1-13.

Linacre, A., & Tobe, S. S. (2011). An overview to the investigative approach to species testing in wildlife forensic science. Investigative Genetics, 2(1), 2. https://doi.org/10.1186/2041-2223-2-2

Linnaeus, 1758. Systema aturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis, tenth ed. Stockholm: Laurentii Salvius.

Marshall, B. M., Strine, C., & Hughes, A. C. (2020). Thousands of reptile species threatened by under-regulated global trade. Nature Communications, 11(1), 4738. https://doi.org/10.1038/s41467-020-18523-4

Masters, A., Ogden, R., Wetton, J. H., & Dawnay, N. (2019). Defining end user requirements for a field-based molecular detection system for wildlife forensic investigations. Forensic Science International, 301, 231–239. https://doi.org/10.1016/j.forsciint.2019.05.041

Nilson, G., Tuniyev, B., Andre´n, C., Orlov, N.L., 1999. Vipers of Caucasus: taxomonic considerations. In: Joger, U. (Ed.), Kaupia, Phylogeny and Systematics of the Viperidae. Hessisches Landesmuse- um Darmstadt/Technische Universita¨t Darmstadt, Darmstadt, pp. 103–106.

Nordstrom, B., Mitchell, N., Byrne, M., & Jarman, S. (2022). A review of applications of environmental DNA for reptile conservation and management. Ecology and Evolution

Obst, F.J., 1983. Zur Kenntnis der Schlangengattung Vipera (Reptilia, Serpentes, Viperidae). Zool. Abh. Staatl. Mus. Tier. Dresden 38, 229– 235.

Ogden, R. (2010). Forensic science, genetics and wildlife biology: Getting the right mix for a wildlife DNA forensics lab. Forensic Science, Medicine, and Pathology, 6(3), 172–179. https://doi.org/10.1007/s12024-010-9178-5

Ogden, R., & Linacre, A. (2015). Wildlife forensic science: A review of genetic geographic origin assignment. Forensic Science International: Genetics, 18, 152–159. https://doi.org/10.1016/j.fsigen.2015.02.008

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics, 26(6), 841-842.

Risk factors for human disease emergence | Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences. (o. J.). Abgerufen 7. Februar 2024, von https://royalsocietypublishing.org/doi/abs/10.1098/rstb.2001.0888

Robinson, J. E., Griffiths, R. A., St. John, F. A. V., & Roberts, D. L. (2015). Dynamics of the global trade in live reptiles: Shifting trends in production and consequences for sustainability. Biological Conservation, 184, 42–50. https://doi.org/10.1016/j.biocon.2014.12.019

Sahlin, K., Lim, M. C., & Prost, S. (2021). NGSpeciesID: DNA barcode and amplicon consensus generation from long-read sequencing data. Ecology and evolution, 11(3), 1392-1398.

Smart, U., Cihlar, J. C., & Budowle, B. (2021). International Wildlife Trafficking: A perspective on the challenges and potential forensic genetics solutions. Forensic Science International: Genetics, 54, 102551. https://doi.org/10.1016/j.fsigen.2021.102551

Sochurek, E., 1974. Vipera ammodytes gregorwallneri n. ssp.. Herpet. Blätt. 1, 1–4.

Sollund, R. (2016). Wildlife Trafficking in a Globalized World: An Example of Motivations and Modus Operandi from a Norwegian Case Study. In F. M. Angelici (Hrsg.), Problematic Wildlife: A Cross-Disciplinary Approach (S. 553–570). Springer International Publishing. https://doi.org/10.1007/978-3-319-22246-2_25

Sotiropoulos, K., Eleftherakos, K., Dz˘ukic´, G., et al., 2007. Phylogeny and biogeography of the alpine newt Mesotriton alpestris (Salaman dridae, Caudata), inferred from mtDNA sequences. Mol. Phylogenet. Evol. 45, 211–226.

Thanou, E., Jablonski, D., & Kornilios, P. (2023). Genome-wide single nucleotide polymorphisms reveal recurrent waves of speciation in niche-pockets, in Europe's most venomous snake. Molecular Ecology, 32(13), 3624–3640. https://doi.org/10.1111/mec.16944

Taylor, L. H., Latham, S. M., & Woolhouse, M. E. (2001). Risk factors for human disease emergence. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 356(1411), 983-989.

Tomovic, L. (2006). Systematics of the nose-horned viper (Vipera ammodytes, Linnaeus, 1758). The Herpetological Journal, 16(2), 191-201.

Uhm, D. P. van. (2016). The Illegal Wildlife Trade: Inside the World of Poachers, Smugglers and Traders. Springer.

UNODC. Criminalization of Wildlife Trafficking. Available at: https://www.unodc.org/e4j/en/wildlife-crime/module-3/key-issues/criminalization-of-wildlife-trafficking.html. Accessed on: 03.04.2024

UNODC (2020). World Wildlife Crime Report 2020. Available at: https://www.unodc.org/documents/data-and-analysis/wildlife/2020/World_Wildlife_Report_2020_9July.pdf. Accessed on: 03.04.2024

Ursenbacher, S., Schweiger, S., Tomović, L., Crnobrnja-Isailović, J., Fumagalli, L., & Mayer, W. (2008). Molecular phylogeography of the nose-horned viper (Vipera ammodytes, Linnaeus (1758)): Evidence for high genetic diversity and multiple refugia in the Balkan peninsula. Molecular Phylogenetics and Evolution, 46(3), 1116–1128. https://doi.org/10.1016/j.ympev.2007.11.002

Wallis, G.P., Arntzen, J.W., 1989. Mitochondrial-DNA variation in the crested newt subspecies: limited cytoplasmic gene flow among species. Evolution 43, 88–103.

Wallner, S. (2023). Monitoring of an allochthonous viper population (Vipera ammodytes, Viperidae) and the accompanying herpetofauna in Lower Austria. Wien.

Wasser, S. K., A. M. Shedlock, K. Comstock, E. A. Ostrander, B. Mutayoba, and M. Stephens. 2004. Assigning elephant DNA to geographic region of origin: applications to the ivory trade. Proceedings of the National Academy of Sciences of the United States of America 101: 14847–14852.

Wasser, S. K., Brown, L., Mailand, C., Mondol, S., Clark, W., Laurie, C., & Weir, B. S. (2015). Genetic assignment of large seizures of elephant ivory reveals Africa's major poaching hotspots. Science, 349(6243), 84-87.

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