Validating FTA card skin swabs as a source of DNA from wild amphibians

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Submitted in Partial Fulfilment of the Requirements of the Degree of MSc by Research, May 2016

Contents:

List of tables…….....3

List of figures……4

Acknowledgement……5

Abstract……6

Chapter One. Introduction

1.1 The use of DNA in conservation ……..…8

1.2 marking and tissue sampling in amphibians ……...…10

1.3. Swabbing as a source of DNA in non –amphibian vertebrates ……14

1.4. Non-invasive methods of DNA collection in amphibians ……...16

1.5. Whatman FTA cards as means to retrieve and store DNA ……..17

1.6. The great crested newt (*Triturus cristatus*)…….……..…20

1.7 Thesis aims…….…….…….24

Chapter Two. Methods

2.1. DNA sample collection…….…..……26

2.2. DNA extraction……...…28

2.3. PCR reactions……..……...30

2.4. Sequencing…….….32

Chapter Three. Results

3.1. Microsatellite locus *Tcri*13……....35

3.2. Microsatellite locus *Tcri*27……...…….36

3.3. Microsatellite locus *Tcri*29……..…….38

3.4. Microsatellite locus *Tcri*35……..……..39

3.5 Microsatellite locus *Tcri*36…….…..……..41

3.6. Microsatellite locus *Tcri*43…….……..……43

3.7. Microsatellite locus T*cri*46……...……44

3.8. MtDNA (ND4) amplification.……...…….46

Chapter Four. Discussion

4.1 General considerations……...49

4.2 Experimental design and protocol…….....50

4.3. Differences between genetic markers……...……51

4.4. Conclusions and recommendations...…….……..53

6. References……...55

7. Appendices…….….72

List of tables

Table 2.1……..……...…..31

Table 2.2…….……..…..32

Table 3.1.…….34

Table 3.2……..35

Table 3.3……..37

Table 3.4...……...39

Table 3.5..……40

Table 3.6……..42

Table 3.7…...……43

Table 3.8…...……...….45

List of figures

Figure 1.1……...…….21

Figure 1.2...……...……22

Figure 2.1…….……27

Figure 2.2...……...……27

Figure 3.1...……...……36

Figure 3.2……..38

Figure 3.3…..……41

Figure 3.4……...……..42

Figure 3.5...……...……44

Figure 3.6…….……45

Figure 3.7…...……...……47

Acknowledgements

I hereby would like to thank the University of Salford for accepting my application to conduct my Masters by Research and providing the facilities in which I was able to carry out my work. I am extremely grateful to David Orchard for collecting both of the sample sets for me, allowing this research to be undertaken by myself in the labs.

My greatest gratefulness goes to my supervisor, Dr. Robert Jehle, who allowed me and provided me this amazing opportunity in which I was able to study further. Through his guidance and knowledge I have been able to complete this thesis, and when I have been through tough times his patience and understanding has allowed me to do the best to continue and progress in my thesis.

I would also like to thank my family for supporting me through these last couple of years, with particular mention to Alexander Brown who has had the pleasure of listening to me moan when things have not gone to plan as well as the hours of listening to me read my thesis to him. Thank you all for your support and love, I am truly eternally grateful.

Abstract

Amphibians are the most endangered groups of vertebrates, and there is currently an urgent need to implement efficient monitoring schemes based on insights from DNA. However, largely due to their unprotected skin which lacks dead keratinised tissue, current standard techniques for DNA sampling of endangered amphibians are either destructive (sacrifice of entire animals) or can involve distinctly invasive techniques such as tissue sampling based on toe or tail tip clips. The present MSc by Research for the first time quantitatively investigates whether skin swabs with commercially available Whatman FTA cards, a relatively uninvasive technique, can yield DNA samples of sufficient quality and quantity for PCR amplification. In total, swabs from 22 individual great crested newts (*Triturus cristatus*, the most endangered newt in the UK and an important flagship species for conservation) were analysed, and partly directly compared with toe clip samples. For amplification tests, 7 nuclear microsatellite loci and one mitochondrial locus (ND4) were used; the success rates of PCRs were quantified by replicate PCRs based on DNA extractions using different protocols. While PCR success rates differed across genetic markers, the study showed that, with sufficient replicates, it is possible to efficiently retrieve amplifiable amphibian DNA based on FTA-card skin swabs. The most successful protocol was based on the merging of eight punches from different areas of the FTA card for DNA extraction using the commercially available Qiagen kit, leading to PCR amplification rates which were largely indiscernible from PCRs based on tissue samples. The established new protocol for DNA collection should aid in reducing the invasiveness of DNA collection in future amphibian studies.

Chapter One: Introduction

"Amphibians have been around for over 360 million years, enduring at least three mass extinction events, including the one that eliminated the dinosaurs. But amphibian species are becoming extinct at a pace faster than anything we have experienced." (Beeby 2008).

1.1. The use of information drawn from DNA in conservation

Due to its role in transmitting information between generations, DNA can be used as a key element to increase our understanding of individuals and populations (e.g. Woese and Fox 1977). Moreover, information derived from DNA can also help in biodiversity conservation, for example to reach the targets set by the Convention on Biological Diversity (e.g. Hartvig et al. 2015). There are numerous ways in which information from DNA can be valuable for conservation. One important area of research relates to the identification of cryptic species as well as life stages that are difficult to identify (Thomsen & Willerster 2015). In another area of research, measuring standing amounts of genetic variation can help to understand historical population processes in endangered species, and enables to detect for example the negative consequences of inbreeding (Baedell et al. 2009). At an individual level, the use of genetic markers can elucidate gene genealogies as well as patterns of kinship and sexual selection which result in specific mating systems (Hamilton & Sadowsky 2010; Beebee 2010). A particular advantage of DNA-based inferences is that samples can also be taken from animal remains and sources such as feathers, scales, faeces and fur (Bellermain et al. 2005; Ogden et al. 2009).

A currently particularly lively field of biodiversity research is DNA barcoding. Easy access to molecular techniques enables the accurate identification of species and other taxonomic entities through the sampling of tissues followed by the PCR amplification of mtDNA genes

using universal PCR primers. Sequences can be compared to online databases (example: BOLD, Barcoding Of Life Database, www.barcodeoflife.org) to assign individuals to species and sometimes their geographic origin, without any knowledge of morphology (Hartvig et al. 2015). DNA barcoding can aid in conservation by supporting field workers in identifying species, by helping taxonomists to determine whether (groups of) species need more detailed analyses, and by facilitating the appropriate units and scales of conservation planning. For example, Francis et al. (2010) presented DNA barcoding results from 1900 morphologically distinct bat species, providing a platform for their genetic identification in conservation-relevant research.

DNA is also increasingly used in applied conservation efforts such as spatial management and protection plans (e.g. Ibrahim et al. 2012). DNA profiling can further provide key evidence to wildlife crime, both to assign animal remains to protected species and populations, as well as to convict individuals involved in criminal activities related to wildlife (Alacs et al. 2010). For example, parts of brown bears and the Asian black bear (both species are listed as Endangered by the IUCN) traded by poachers could be identified with DNA techniques (Bai et al. 2003). Moreover, Lorenzi (2005) also used DNA to help identify the individual who was responsible for the illegal killing of a wild boar. Further applications of DNA-based methods to conservation are frozen and stored germplasm for future conservation efforts, as well as the use of information derived from ancient DNA to providing a better framework for understanding the dynamics of populations (Holt and Pickard 1999, Baedell et al. 2009).

1.2. Marking and tissue sampling in amphibians

Population ecological studies often require the identification of individuals through physical marks, or the collection of DNA samples. However, due to the sensitive skin, overall small size as well as their potential for tissue regeneration, amphibians are notoriously difficult to mark in the field (e.g. Donnelly et al. 1994). Infectious diseases are one of the main causes of declines in both captive and wild populations, and stress from handling can cause individuals to become more susceptible (Schumacher 2006). One method of identifying individual amphibians is photo identification. It is based upon taking pictures of individually variable natural markings, which are then compared visually by researchers or through computer-based image analysis (Caorsi et al. 2012). While photo identification requires individuals to be captured, it does not involve the application of physical marks and therefore is rather un-invasive (Plaiasu et al. 2005). In a recent study on crested newts (*Triturus cristatus*), it has been shown that photo identification matches well with information about the identity of individuals drawn from DNA fingerprinting (Drechsler et al. 2015). For other species, photo identification can however be error-prone, leading to a high rate of misidentification of individuals (Kenyons et al. 2009, Bendick et al. 2013).

Applying physical tags represents another suite of methods for the collection of information from individuals. For example, alphanumeric tags did not appear to influence frogs under laboratory conditions (Heard et al. 2008), although they can cause friction as well as snagging and tearing in the wild (Christy 2006). Clemas et al. (2008) evaluated handling and processing time as well as recapture rates for VIA (Visible Implant Alphanumeric) tags, photo identification and toe clipping. They found that, although there was no negative effect upon the health of the frogs in the species tested, the tags did move which made them harder to find. Visible Implant Elastomer (VIE) tags are based on colour codes, and have been

shown to influence the physiology of marked individuals (Antwis et al. 2014). A further way of marking individual amphibians is the implantation of PIT tags (Passive Integrated Transponder tags). While PIT tags have been applied for a range of studies over approximately the last two decades (e.g. Ott and Scott 1999), their implantation is a rather invasive procedure as they are relatively large in size (Winandy and Denoel 2011). Guimares et al. (2014) compared toe clipping to PIT tagging, documenting similar survival rates for individuals marked, however with a negative correlation between recapture rates and the number of toe pads removed. Winandy and Denoel (2011) also stated that PIT tagging could lead to behavioural changes that result in increased predation.

DNA-based techniques of identification in amphibians require the removal of some kind of tissue, but enable an unambiguous tracking of individuals across different life stages (e.g. Ringler et al. 2015). For taxonomic studies, it is a rather common practice to sacrifice wild-collected amphibians for analyses based on e.g. liver samples (e.g. Barber 199, Berger et al. 2006, Stuart et al. 2006). Skin tissue is regularly used to detect disease agents such as *Batrachochytrium dendrobatidis*, and is usually connected to lethal sampling (Berger et al. 1991, Annis et al. 2004, Une et al. 2008). For studies which do not involve the sacrifice of individuals, DNA is often collected through a blood sample or via the removal of a phalange using surgical scissors (Ott and Scott 1999). Toe clipping can be used as an alternative method to lethal sampling for the detection of diseases (St-Amour and Lesbarreres 2007; Gray et al. 2012).

The method of collecting DNA from amphibians via toe clipping is simple and cost-effective, and has been used for decades. However, recent controversy has arisen involving various researchers and organisations with regards to the practice of toe clipping. For example, the Brazilian federal agency, which enforces Brazil's environmental policies, has stated that this

method is a form of mutilation and should be criminalised under federal law (Correa 2013). However, such views are met with criticism due to the insights the method of toe clipping has enabled over time. Funk et al. (2005) have stated that "without this technique it will be harder to obtain crucial information that could prevent amphibian species from becoming extinct." Finding a method that can appease both viewpoints would be vital for future amphibian conservation and research.

While toe clipping is favoured as a non-lethal method for disease screening compared to for example tail clipping (Greer and Collins 2007), it is widely debated as an invasive method for the purpose of marking and DNA sampling alone (Boitani and Fuller 2000). Nevertheless, Brannelly et al. (2013) state that toe clipping remains the most effective method of DNA collection method for amphibians, despite obvious drawbacks. Waddle et al. (2008) modelled the effect of toe clipping on the survival rate of tree frogs, and discovered that the clipping of toes did not account for the observed heterogeneity in the probability of capture; a similar finding was obtained by Hartel & Nemes (2006) on toads. Phillott et al. (2008) list six points defending the clipping of toes, suggesting that decreased return rates could be due to behavioural changes or alterations in the environment instead of marking. Paulissen and Meyer (2000) share similar views, stating that the effects of toe clipping appear to be minimal and argue that toe clipping is a viable and useful method. Phillott et al. (2010) showed that return rates of three frog species only had a short-term effect (24 hours) on marked individuals of a single species, suggesting temporary behavioural changes as a reason. Jones and Bell (2010) similarly support the method of toe clipping, finding no support for a marking effect on short-term recapture rates except in reptiles (skinks). Grafe et al. (2011) discovered that toe clipping decreased survival in two out of four studied species, however

only when a large number of toes were removed. The study recommends that the functionally most important toes should not be clipped.

This series of studies which argue in favour of toe clipping is juxtaposed against evidence for detrimental effects, which raise ethical questions and also concerns with regard to the scientific validity of the data collected. For example, Polich et al. (2013) demonstrate that toe clipping has serious detrimental effects on survivorship and growth in salamanders. McCarthy and Parris (2004) re-analysed previous data on anurans using Bayesian statistics, and discovered that return rates decreased by 4-11% if one toe was removed, and an additional 3.5% when a further toe was removed. This is confirmed for salamanders by McCarthy et al. (2009), who demonstrated that return rates were affected by toe clipping. In a seminal essay, May (2008) stated in the journal Nature that studies are regularly compromised by the effects of toe clipping, calling for an abandoning of the procedure. Parris and McCall (2010) assessed ethical trade-offs between toe/tail clipping and swabbing in tadpoles and adult frogs. They concluded that, while tail tip clipping is the best method for tadpoles, swabbing is the preferred option for adult frogs, reinforcing the view that toe clipping should become avoided as a method of DNA collection from adult anurans

Investigating the physiological effects of toe clipping, Narayen et al. (2011) measured urinary corticosterone levels of cane toads after removal of toes, and discovered that levels remained high up to six hours after the clipping had taken place, returning to values which were close to normal after around twenty four hours. Fisher et al. (2013) however noted that the effects of handling of toads without toe clipping on levels of corticosterone were indiscernible from handling for the purpose of toe removal. To this end, Kinkead et al. (2006) found no significant differences in stress hormone response and behaviour between salamanders which were toe clipped with and without anaesthesia. Amphibians show no emotions in the way that

other vertebrate species may, which makes the individual effects of toe clipping difficult to interpret (Alworth and Harvey 2007). Researchers in support of toe clipping generally argue that it will likely cause less distress and pain compared to mammals (Phillott et al. 2007, Perry et al. 2008). The conflicting effects of toe clipping as observed in specific studies are likely due to differential methodologies and sensitivities of study species (for a summary of evidences see Perry et al. 2011).

1.3. Swabbing as a source of DNA in non-amphibian vertebrates

Genetic information is important for the conservation of species, but usually requires invasive methods to obtain the required samples (Jones et al. 2008). As an alternative to invasive techniques such as the removal of body parts, skin and mouth swabbing for the purpose of DNA collection has been routinely used for example in mammals (Harlin and Wursig 1999, Pilcher, 2002). Swabbing can also be used on animal parts and remains such as the inside of eggshells immediately after hatching, allowing for parentage analyses of birds without handling of individuals (Galvez et al. 2010). Kashiwagi et al. (2015) carried out a pilot study that showed that skin mucus can be collected from manta rays even by members of the public, using a toothbrush on extendable poles. Le Vin et al. (2010) also collected DNA from fish by swabbing the skin mucus to avoid fin clips, however highlighting a significant risk of contamination between individuals. Liva et al. (2005) collected mucus and buccal cells from fish on swabs using Whatman FTA cards, showing that this method is non-destructive and cheap.

Swabs can also be taken from terrestrial vertebrates. Kovrik et al. (2008) swabbed the noses and mouths of cats for a DNA-based diagnostic test for sporotichosis. Similar to amphibians,

the collection of DNA from mice often took place by removing tail, ear or toe clips (Mitrecic et al. 2008). Alternatively, Meldgaard et al. (2004) swabbed the inner cheek of mice and argued that it was faster, simpler and more reliable than tissue sampling. Similarly, Almeida Ferreira et al. (2008) swabbed the eyes of 46 dogs using cotton swabs, and discovered that the swabs outperformed blood samples to detect leishmaniasis. Andersson and McMillan (2006) collected skin samples from 54 Australian mammals using a moist cotton tipped swab, resulting in a sufficient amount of DNA for PCR amplification.

Buccal DNA swabbing can also be conducted on reptiles. Beebee (2008) sampled two native reptile species and proved the method to be more reliable than DNA samples based on tail, toe and scale clips. Miller (2006) also collected cloacal and buccal swabs in reptiles and successfully amplified both mitochondrial and microsatellite loci, stating that the swabs were fast, inexpensive, and easy to implement. Klenk and Kamar (2003) obtained oral and cloacal samples from three reptilian species and isolated pathogenic virus DNA.

Despite the frequent notion of the advantages of swabbing, early studies also highlighted problems related to repeatability and the imperfect detection of disease agents (Calsamiglia et al. 1999, Newton et al. 1999). However, advances in technology since these studies were conducted suggest that these problems may now have been overcome. Waits and Paetkau (2005) raised the problem of contamination linked to swabbing techniques, promoting the application of standardised protocols. Jones et al. (2008) suggest other methods of non-invasive DNA collection which include shredded skin and faeces as well as road kill.

1.4. Methods of DNA collection in amphibians

Due to a rise in awareness of animal welfare and ethics, methods to collect DNA in amphibians increasingly require to be as little invasive as possible (Perry et al. 2011). However, all sampling methods still involve some level of handling of individuals, and for example oral swabbing requires the researcher to open the individuals' mouth, which is a difficult procedure particularly in small species (R. Jehle, personal communication). Another aspect of DNA collecting that has to be accounted for is the ability to store DNA samples once they have been collected in the field.

Mendoza et al. (2012) collected blood from punctures in an anuran, and found no effect on the survival of sampled individuals. Davy et al. (2014) also used blood that had been taken through a caudle venepuncture with a syringe and then blotted onto Whatman FTA cards. Forzan and Wood (2013) evaluated the use of toe clips and compared them to the use of blood transferred onto filter paper using hepatic needles to detect ranavirus in wild post metamorphic green frogs. However, the study yielded in only inconclusive results on the efficiency of DNA collecting procedures. Swabbing is another method which is increasingly used to collect DNA from amphibians. Skin swabbing has been proven useful for species which are too small for buccal swabs, and also requires less handling time (Prunier et al. 2012, Simpkins et al. (2015). Gallardo et al. (2012) show that skin swabs enable genetic studies without affecting the survival of swabbed individuals. Despite potential problems such as increased allelic dropouts, buccal swabs are increasingly used for sampling amphibians non-invasively in the wild (Broquet et al. 2007, Spear and Storfer 2008, Maddock et al. 2014). Poschadel and Moller (2004) used standard cotton buds when swabbing amphibians. These cotton buds can absorb sufficient amounts of oral mucus, and are able to fit it into the oral cavity of even the smallest species. Oral swabs have also been used to

collect DNA in tree frogs, including tail clips as well as toe clips from tadpoles (Gvozdic et al. 2010). Obendorf and Dalton (2006) used fine tip swabs on oral disks of tadpoles, before returning them to the wild. Despite its obvious advantages, contamination is a major problem for PCR-based DNA analyses based on swabs (Bus and Allen 2014, Kolby et al. 2015). Muller et al. (2013) carried out a survey analysing eight microsatellite loci, comparing buccal, skin and cloacal swabs, and discovered high degrees of contamination, which can however be prevented by careful measures when swabbing the individuals. Cashins et al. (2009) handled amphibians individually using a fresh pair of gloves in order to prevent contamination. Shin et al. (2014) also stated that swabbing is the least reliable DNA collection method as it often fails to detect infections in individuals and so underestimates disease prevalence. Bishop et al. (2009) argue that swabbing may not be the best collection method in disease studies such as surveying for chytrid fungus. Another problem with swabs is the storage of DNA. Sherratt et al. (2008) and Simpkins et al. (2010) stated that the storing of swabs does not decrease PCR success, however they must be frozen for longer storage.

1.5 Whatman FTA cards as means to retrieve and store DNA

FTA (Flinders Technology Associates) Whatman cards are manufactured to provide an easy, secure and safe method of collecting DNA from individuals in the field. FTA cards, unlike other methods of DNA collection, do not need a storage medium such as ethanol or a buffer. They are impregnated with a protein denaturant that allows for the lysis of cells, and nucleic acids can be stored directly from tissue (Ahmed et al. 2011). FTA cards can be stored at room temperature, and DNA bound on the cards can be prepared for PCR reactions by punching small pieces of the card and then washing the punches (Halsall et al. 2008).

Apart from applications in the wide area of biodiversity research, FTA cards can also be used to collect and transport DNA from pathogens from hazardous or dangerous environments, including crime scenes (Bus and Allen 2014, Smith and Burboyne 2004). In medical applications, FTA cards were for example used to collect samples from patients with leishmaniasis in remote areas (Peru, Kato et al. 2010). FTA cards have also been used for the detection of prokaryotes and viruses. Abdelwhab et al. (2007) described that FTA cards were able to preserve bird influenza viral RNA for up to five months. Polido-Landinez and Laviniki (2012) also confirmed that, due to the applied chemical impregnation, FTA cards are a safe and economic method of transporting harmful viruses. This was also backed by Perozo et al. (2006), at however a timescale of only 14 days. Awad et al. (2014) added that the treatment of the FTA card cellulose paper with anionic detergents and buffer provides a stable matrix for the fixation of avian metapneumovirus DNA. However, Arif et al. (2011) record difficulties during the processing of the cards for accurate diagnosis of diseases and viruses, although long-term storage is possible in contrast to the use of for example filter paper despite prevention of complete removal of blood proteins from cards by washing. FTA cards have also been used in testing plant pathogens. However, unlike in animals and humans, plant material cannot be swabbed is therefore either placed directly on the FTA card (Grund et al. 2010), crushed using a flat headed bolt to disrupt cell walls before application onto the FTA card (Bunting and Burnett et al. 2014), or plant tissue is grinded into a liquid before spotting it onto FTA cards (Grund et al. 2010).

FTA cards often serve for the collection and long-term storage of blood samples (for example Guitierrez-Corchero et al. 2002, for avian blood), and have been proven to be more successful in PCR amplification than buccal swabs (Milne et al. 2006; 83% - 100% and 62-89%, respectively). Moreover, FTA cards are often used when other DNA collection methods

may be challenging, combining them with the use os DNA collected with other means prior to fixation onto FTA cards (Merino et al. 2009, Keeler et al. 2012, Magzio and Mengani 2014 for studies with birds). FTA cards generally allow for the collection and analysis of samples from anywhere in the world, without considering safety regulations during shipping and storage (Fujito and Kubo 2005, Muthuhrishnan et al. 2008, Cox et al. 2010, Kraus et al. 2011). Once the samples have been collected, the cards are dried and can be added directly into the PCR reaction (Fujito and Kubo 2005).

To date there have been few studies looking into the use of FTA cards for the collection of DNA from amphibians. Mendoza et al. (2012) used FTA cards to fixate amphibian blood from punctures before extracting DNA using a DNAeasy kit, and Obendorf and Dalton (2006) used FTA papers to detect the presence of chytrid fungus on the oral disks of amphibian tadpoles; both studies are however unrelated to the use of FTA card swabs as a novel and rather un-invasive method for DNA retrieval. Maddock et al. (2014) carried out a study with caecilians, using four FTA cards for skin swabbing of two individuals. While the study showed that PCR amplification is possible, it also revealed low success rates and did not develop a standardised protocol for FTA card-based swabbing based on a larger sample. Pichlmuller et al. (2013) mentioned the possible transfer of epithelial cells from salamanders dorsal cells onto FTA cards, but did not follow this statement up in the research.

1.6. The great crested newt (*Triturus cristatus*)

Approximately 0.5% of all currently described animal species belong to the class Amphibia, and about 600 amphibian species belong to the order Urodela (Crump 2008). Amphibians are the most endangered class of vertebrates. According to recent IUCN lists, 32.4% (2,030 species) of the listed amphibians are globally threatened with extinction or already extinct (IUCN Red List 2015). The great crested newt (*Triturus cristatus*) is part of the family Salamandridae, which currently consists of 75 species and covers the largest range of all 10 urodelan families, spreading across northern America, Asia and Europe. All species in this family are aquatic as well as terrestrial, but differ in the time that they spend in both habitats (Halliday and Adler 2002).

There are currently seven species of crested newt recognised across Europe and Asia, with most species however only having become described within the last two decades (Jehle et al. 2011). *Triturus cristatus* is the species with the widest distribution area, ranging from Western Europe into Siberia, and was first described by Laurenti in 1768. Adult males usually measure between 115 mm 145 mm in total length. The great crested newt is a slender species with a flat broad head with a dark upper colouration, and a yellow-orange belly partially covered with dark large spots. Their throat can also be covered in these spots but is generally marbled yellow and black (Jehle et al. 2011, Figure 1.1).

Figure 1.1: A male great crested newt (*Triturus cristatus*). Taken from Jehle et al. (2011).

The natural history of great crested newts is relatively well studied. They reproduce in rather large, well vegetated, fish free ponds, where females can lay between 200 and 400 eggs during a prolonged breeding season. Males produce heterogametic sex chromosomes, whereas females are the homogametic sex. Depending on water temperature, larvae hatch from the eggs after 2-6 weeks (Froglife 2015). The larvae hatch without the ability to swim and thus remain attached to the egg. Forelimbs develop before hindlimbs, and larvae start swimming and foraging after about one week. After six weeks, the larvae are starting to resemble the adults, with black blotches appearing on the body and the tail. After about 16 weeks larvae undergo metamorphosis, reducing their gills and tail fins at a body length of about 45-90 mm long. After a largely terrestrial subadult stage, sexual maturity is reached at between two and four years of age, and documented maximal longevity in the wild is about 14 years (Jehle et al. 2011).

Figure 1.2 shows the distribution of the great crested newt throughout the United Kingdom. The majority of populations are found in England, with additional populations in both Scotland and Wales. *Triturus cristatus* is not found in Northern Ireland and the Republic of Ireland. It is estimated that there are currently around 75,000 populations of great crested newts in the UK (Jehle et al. 2011).

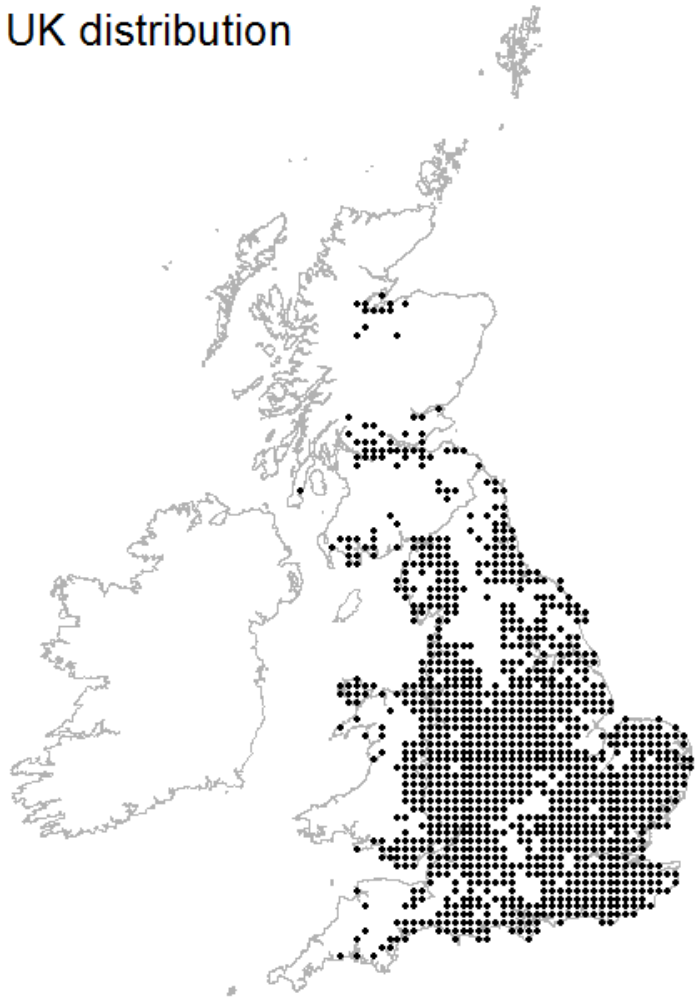

Figure 1.2: UK distribution of the great crested newt *(Triturus cristatus*). Taken from http://jncc.defra.gov.uk/publications/jncc312/species.asp?FeatureIntCode=S1166

Triturus cristatus is protected under a range of different statutes and laws. Under the Wildlife and Countryside Act 1981 of the United Kingdom, part 9 section 4, a person would be found guilty if they intentionally or recklessly disturb *T. cristatus* whilst it is occupying any structure or place which it uses as a shelter or for protection. This listing of *T. cristatus* also makes it illegal for any person to sell any wild-caught individuals, including transporting them for the purposes of sale. The great crested newt is further protected under the Conservation (Natural Habitats and co) 1994 Regulations, which state that it is an "offence to deliberately capture or kill a wild animal of European protected species, to deliberately disturb such animal, to deliberately take or destroy the eggs of such animal or to deliberately destroy a breeding site or resting site of such an animal". Internationally, great crested newts are protected under Appendix II of the Bern Convention, and under Annexes II and IV of Europe's Habitats Directive. Due to these legislations, an individual cannot handle or remove any great crested newt without having a licence.

For great crested newts, insights based on genetic markers have been important to improve our understanding of its population biology (featured in e.g. McCartney-Melstead and Shaffer 2015). For example, Jehle et al. (2001) used eight microsatellite markers to quantify the effective sizes of three populations, revealing that they were markedly below population census sizes. In a subsequent study, Jehle et al. (2005) measured migration rates between populations using assignment tests, and discovered asymmetric exchange of individuals between only a subset of the studied populations. In areas where populations of different species of crested newts overlap, the use of DNA markers have proven essential for quantifying the amount of gene flow between them (Dufresnes et al. 2016). Information from genetic markers can also identify whether a population has decreased in size due to reductions in population sizes in the past (Jehle et al. 2005), and help to assess whether

populations are native or not (O'Brien et al. 2015). As small, subdivided populations are vital for the long-term future (e.g. Griffiths and Williams 2000), such inferences contribute to the conservation management of great crested newts at the level of species as well as populations.

However, as in other amphibians, DNA-based studies on great crested newts (*Triturus cristatus*) are either based on the sacrifice of eggs (e.g. Jehle et al. 2005), gill clipping of larvae (e.g. Jehle et al. 2000), or on toe clipping and tail-tip clipping in adults. While tail tip clipping in adults has been shown to have no marked effect on growth and survival (Arntzen et al. 1999), however, no similar datasets exist on the effects of toe clipping for this species. Toe clipping is an invasive procedure, in which the researcher has to handle the individual before using a sterilised knife or blade to remove the toe. Discovering new less invasive ways to obtain DNA samples would currently be highly desired.

1.7 Thesis aims

The aims of this thesis are to:

(i) Quantitatively determine the suitability of DNA retrieved from amphibian skin swabs using FTA cards for PCR amplification,

(ii) Compare alternative DNA extraction protocols from FTA cards for their PCR success (direct amplification from processed FTA card punches, and card punches exposed to commercial Qiagen extraction kits), and

(iii) Compare PCR amplification success rates between FTA cards and tissue samples (toe clips) simultaneously taken from identical individuals.

Chapter Two: Methods and Materials

2.1 DNA sample collection

The presented research is based on samples collected during two successive breeding seasons. In 2014, twelve individual *T. cristatus* were swabbed by David Orchard (a PhD student from the University of Salford) at a pond near Preston on April 27. The samples were collected in one session, carefully avoiding any contamination between the different individuals whilst the swabbing was taking place. The DNA was collected by running the FTA cards along the whole of the body of individual *T. cristatus* several times, in order to ensure that much DNA as possible was gathered on them.

The second set of DNA collection took place on the 2nd of May 2015 at a pond on Gorse Hill Nature Reserve close to Ormskirk in Lancashire (Figure 2.1). Ten *T. cristatus* individuals were caught using mesh funnel traps as described in Madden & Jehle (2013, five males and five females). Each individual was wiped over an FTA card several times from the head to the tip of tail (Figure 2.2). While most of the DNA was captured with the central circle of the FTA card, more distal parts were also used for swabbing. For a direct comparison between FTA card swabs and tissue sampling, the distal half of the front right toe was removed using a knife sterilised with a flame (performed by David Orchard who was in possession of the required licenses). Toe tissues were stored in individual 1.5 ml tubes in absolute ethanol, and labelled with the same numbers (1-10) as the corresponding FTA card samples. The field collection took place under rainy conditions, which caused the FTA cards to become wet.

Figure 2.1. The pond from which the newts were collected at Gorse Hill Nature Reserve. (photograph by David Orchard).

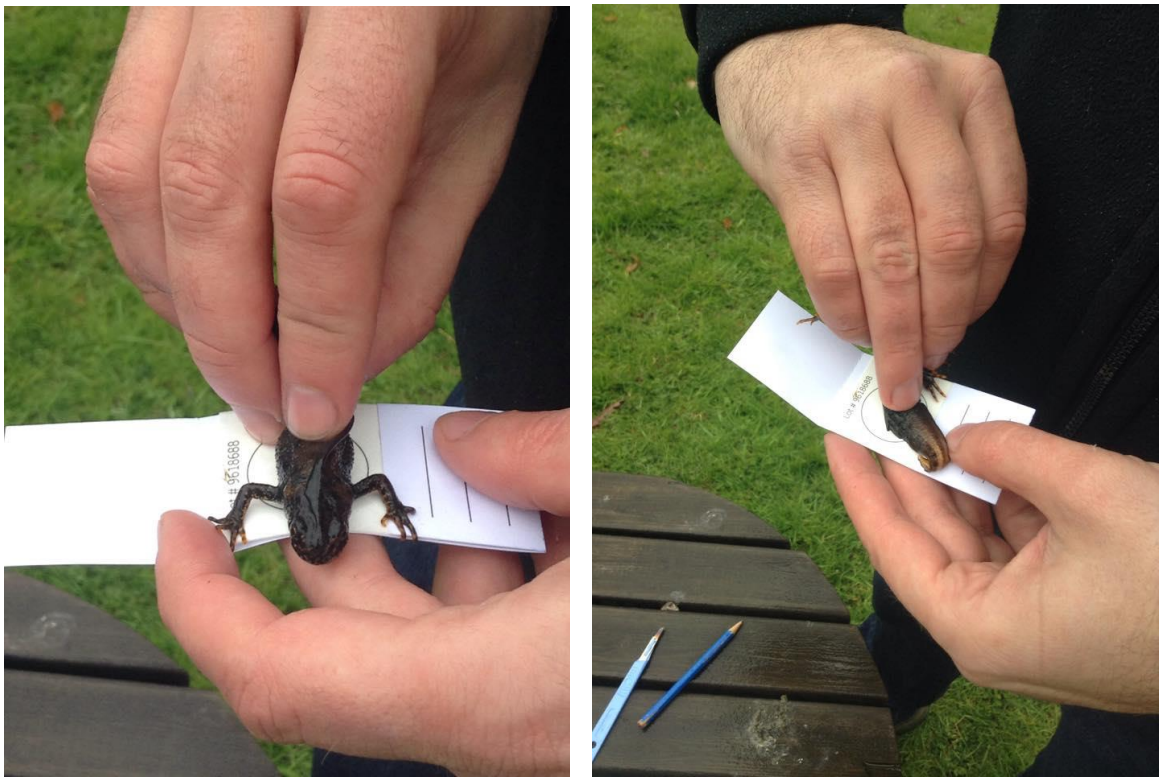

Figure 2.2. Swabbing of a male *Triturus cristatus* using an FTA card.

Once the FTA cards have dried, they can then be kept at room temperature for up to nine months, a time span linked to the chemical components that are combined within the cards, inhibiting the breakdown of DNA (Polido-Landinez & Laviniki 2012). In both 2014 and 2015, DNA extraction took place after approximately 6 months.

2.2. DNA extraction

Before the retrieved DNA was used for amplification with Polymerase Chain Reaction (PCR), FTA cards were processed broadly following the procedure described in Ahmed et al. (2011). Between one and five punches per FTA card were taken using a Harris wheeler punch to remove a circle with approximately 2 mm diameter of the FTA card. In order to avoid contamination, the Harris wheeler punch was cleaned by punching three holes on a filter paper between the use of FTA cards. The punches that were used were taken from multiple areas of the cards, both inside and outside of the circle on the paper, to reveal whether DNA was present on the entire surface area of the card (see Appendix 1). All 22 sampled individuals (12 FTA card swabs only in 2014, 10 FTA card swabs and tissue samples in 2015) were tested for a range of genetic markers (see below) as well as PCR protocols.

For DNA obtained with FTA cards, two alternative DNA extraction methods were used:

(I) For 2014 and 2015 samples (22 individuals in total), PCRs were conducted with washed FTA card punches directly added to the PCR reaction. Between 1 and 5 punches for each PCR reaction were trialled for each genetic marker, using an equal number (minimum of two) of PCR attempts per category. Punches were placed into individual 1.5 ml Eppendorf tubes, adding 200µl of FTA purification reagent (commercially supplied). The buffer was agitated in the Eppendorf tubes using a pipette to ensure that the punches are completely covered.

Punches were left in the buffer for 3-5 minutes, occasionally shaking the tube to ensure the purification reagent is removing any debris before removing the purification reagent with a pipette. This process was repeated three times. The punches were then washed in the same way with 200µl 1M TE buffer (10 mM Tris-HCL pH 8.0; 1 mM EDTA pH 8.0, Awad et al. 2014). This process was repeated twice to ensure that the punches in the tube were washed. Once the punches have been washed with both reagents, they were transferred to the top of the Eppendorf tube using a pipette in order to dry out (about 60 minutes). Dried punches were subsequently moved to Polymerase Chain Reaction (PCR) tubes for use in PCR reactions, or stored at -20°C for up to a week.

(II) As an alternative extraction protocol (2015 samples only, 10 individuals), eight FTA punches were pooled for Quiagen extraction, followed by 4 or 5 PCRs attempts for each individual and marker used. In a 1.5 ml tube containing the tissue sample or the FTA cards punches, 20 µl of Qiagen Proteinase K and 200 µl of Qiagen buffer was added before vortexing for fifteen seconds. This mixture was then incubated at 56°C for three hours or for 36°C overnight. After incubation, 200 µl of buffer AL was added before vortexing, adding 200 µl of 100% ethanol and vortexing again. The mixture was placed into mini spin columns and centrifuged at 6000 x for one minute. The filtrate was then discarded, and the spin column was moved to a new microcentrifuge tube. After adding 500 µl buffer AI and centrifugation at 6000x for one minute, the through flow was discarded and the spin column was placed into a new tube. After adding 200 µl of solution AW2, it was centrifuged at 20,000 x for three minutes. Once again the collection tube and filtrate was discarded, and the spin column placed into a new tube. The DNA was then eluted by adding 200 µl of buffer AE to the centre of the membrane in the spin column, before incubating at room temperature for

one minute and centrifugation for one minute at 6000 xg. In order to increase the DNA yield, this step was repeated another time.

In addition to PCRs based on FTA card extracts, 4 or 5 PCR attempts for each marker and individual were conducted based on DNA directly derived from tissue (toe clips, ten 2015 individuals only), using the same Quiagen-based procedure as described above to allow for a direct comparison with the respective FTA card PCRs. About half of the toe was used for extraction.

2.3: PCR Reactions

In order to set up a PCR reaction, a master mix was created with quantities multiplied according to the number of PCR reactions (plus 10% to allow for human error; see Table 2.2 for details). For each FTA card in the PCR reaction an extra 1 µl of water was added to increase the volume up to that needed, and the master mix was set up and kept on ice.

Table 2.1. The reagents used for a 25 µl single PCR reaction including FTA cards.

Reagents used in Master Mix	Quantities used per sample (ul)
Bioline NH_4 buffer	2.5
50 mM $MgCl_2$	1
DNTP mix	0.25
Forward primer	0.5
Reverse Primer	0.5
Water	19.75
Taq	0.5
Total	25ul

Two different types of genetic markers were amplified (Table 2.3): seven nuclear microsatellites (using primers as described by Krupa et al. 2002) and a mitochrondrial gene (ND4, using primers as described in Wielstra et al. (2010) to produce a 658 base pair PCR product). The microsatellite loci were chosen based on their availability and an ongoing study (Krupa et al. 2002; Orchard et al. in preparation). For the amplification of microsatellites, primer-specific temperature profiles as outlined in Krupa et al. (2002) were used. The success of a PCR reaction was determined by the presence of a clearly visible band on an agarose gel visualised under ultraviolet light (see the respective figures in the Results chapter). While the microsatellite banding profiles were not consistently scored e.g. using Applied Biosystems (ABI) technology, agarose gels have a significantly lower power to detect weak PCR products than ABI capillary sequencers (R. Jehle, personal communication).

Table 2.2. Characterisation of microsatellite loci and the mtDNA (ND4) marker tested for *T.cristatus* samples. Expected PCR product sizes were taken from Krupa et al. (2002) and Wielstra et al. (2010).

Locus Name	Primer Sequence	Expected PCR product size (bp)
*Tcri*13	F:GTGATGATTGCCAAGC	114-130
	R:GATCCAAGACACAATATTTAG	
*Tcri*27	F: GATCCACTATAGTGAAAATAAATAATAAG	246-295
	R: CAAGTTAGTATATGATATGCCTTTG	
*Tcri*29	F:CGAGTTGCCCAGACAAG	298-330
	R:GATCACATGCCCATGGA	
*Tcri*35	F:CCAACTGGTTGGCATTC	185-229
	R:GATCACAGAAACTCTCAATATAAGC	
*Tcri*36	F:GATCATCTGAATCCCTCTA	266-282
	R:ATACTTCATGACGTTTGG	
*Tcri*43	F:GAAGTAACTGAAAGATAACATGTAG	262-298
	R:GTTTCTATTCATTTTTGTTACCCAC	
*Tcri*46	F:CAAGTTTCCTCTGAAGCCAG	260-296
	R:GTTTCTTGCCTGACAAAGTAATCCTTC	
ND4	F:AGCGCCTGTCGCCGGGTCAATA	658
	R:GTGTTTCATACTCTTCTTGGT	

2.4: Sequencing

To verify that the PCR products on agarose stem from *T. cristatus* amplification products, three random ND4 mtDNA PCR products were commercially sequenced. Amplified products were cleaned using a standard ethanol/salt precipitation method and sequenced in both directions. Sequences were examined using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2013), and sequences were blasted against published nucleotide sequences available on Genbank (http://www.ncbi.nlm.nih.gov/nuccore/).

Chapter Three: Results

A summary of PCR attempts and success rates across samples collected in 2014 and 2015 is shown in Table 3.1 (see also Appendix 1 for a more detailed list of samples and PCR reactions). A total of 1284 PCR reactions were carried out. Out of these, 624 (48.6%) were completed using the FTA cards based on the 2014 swabbing of 12 individuals, with a total of 341 (54.6%) successful PCRs. A total of 660 (51.4%) PCR reactions were carried out using the samples collected in 2015 from 10 individuals, with 428 (64.8%) working successfully. More reactions failed using the FTA cards collected in 2014 (45.4%), compared to the FTA card set collected in 2015 (35.2%). To compare FTA card-based methods with tissue sampling, toe clips were also taken alongside FTA card swabs in 2015. A total of 350 PCR reactions were completed, 77% of which worked successfully.

Table 3.1. Overview of PCR results for eight genetic markers (mtDNA ND4 and seven microsatellite loci), based on two sampling years and different DNA retrieval and extraction protocols (FTA cards and tissue samples, for more details see the Methods chapter). For FTA PCRs, equal numbers of PCR attempts using between 1 and 5 FTA punches per PCR reaction were pooled.

Locus	2014 (FTA only)		2015 (FTA)		2015 (FTA Quiagen)		2015 (tissue)	
	PCR attempts	Success rate	PCR attempts	Success rate	PCR attempts	Success rate	PCR attempts	Success rate
*Tcri*13	48	83.3%	80	75%	50	62%	50	60%
*Tcri*27	72	37.5%	60	60%	50	64%	50	66%
*Tcri*29	84	40.5%	80	58.8%	40	91%	40	90%
*Tcri*35	72	58.3%	70	57.1%	40	95%	40	90%
*Tcri*36	96	53.1%	90	67.8%	40	85%	40	77%
*Tcri*43	48	83.3%	70	72.9%	50	80%	50	74%
*Tcri*46	96	51%	80	68.8%	40	87%	40	82%
ND4	108	53.7%	130	60%	40	85%	40	80%
Total	**624**	**54.6%**	**660**	**64.8%**	**350**	**81.25%**	**350**	**77%**

3.1 Microsatellite locus *Tcri*13

Table 3.2 shows an overview of PCR success rates for microsatellite locus*Tcri*13. Overall, 85% of FTA- based PCRs in 2014 were successful although rates varied across individuals (for example individual 11 had a low success rate of 5/10, i.e. 50%). PCRs based on FTA cards collected in 2015 had approximately 10% lower success rate than PCRs based on FTA cards collected in 2014, with however one individual (13) having a high success rate of 90%. Alongside FTA punches used from the 2015 cards, the DNA of eight punches from each card was extracted using the Qiagen protocol, resulting in overall higher PCR success rates (Table 3.2, Figure 3.1).

Table 3.2: PCR success rates for microsatellite locus *Tcri*13.

2014 (FTA)		**2015**			
Individual number	PCR success rate	Individual number	FTA PCR success rate	Qiagen FTA PCR success rate	Tissue PCR success rate
1	8/10	13	9/10	3/3	4/4
2	7/10	14	8/10	3/3	4/4
3	8/10	15	8/10	2/3	4/4
4	7/10	16	7/10	3/3	3/4
5	7/10	17	8/10	3/3	4/4
6	7/10	18	8/10	2/3	4/4
7	8/10	19	7/10	2/3	3/4
8	6/10	20	7/10	3/3	4/4
9	8/10	21	8/10	3/3	4/4
10	6/10	22	7/10	3/3	4/4
11	5/10				
12	8/10				
Total	**85/120**		**77/100**	**27/30**	**38/40**

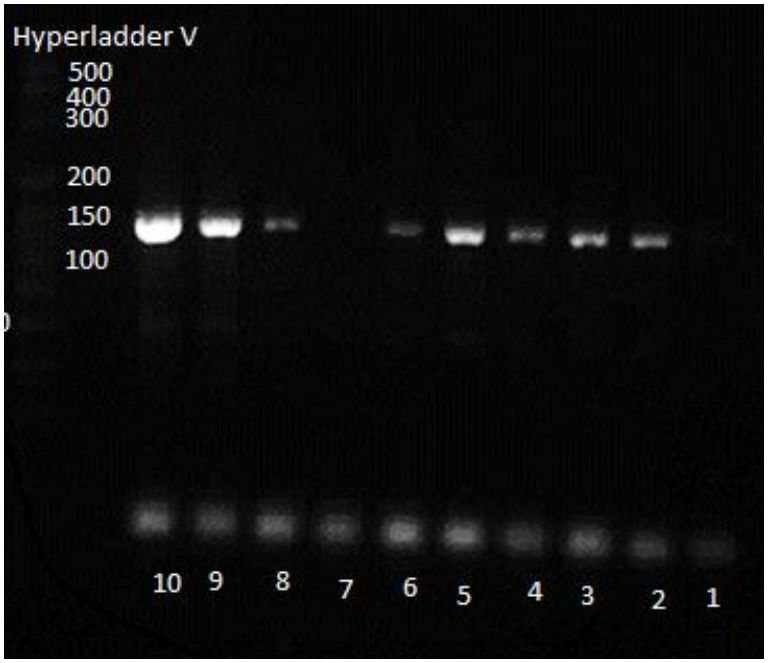

Figure 3.1. Agarose gel image of PCRs conducted with microsatellite *Tcri*13 using punches from cards 1 -10 from the 2015 sample set.

3.2 Microsatellite locus *Tcri*27

Table 3.3 shows an overview of PCR success rates for microsatellite locus *Tcri*27. Overall success rates for FTA- based PCRs in 2014 were in the order of 40%, although success rates varied across individuals (for example individual five had a low success rate of 2/10, i.e. 20%). PCRs based on FTA cards collected in 2015 had approximately 20% higher success rate than PCRs based on FTA cards collected in 2014, with however one individual (18) having a significantly lower success rate of 40%. FTA punches used for Qiagen DNA extraction resulted in overall high PCR success. The band of the successful PCR matches with the expected product size of the locus (Figure 3.2).

Table 3.3: PCR results for individual punches from all the FTA cards from both sample sets using microsatellite locus *Tcri27*.

2014 (FTA)		2015			
Individual number	PCR success rate	Individual number	FTA PCR success rate	Qiagen FTA PCR success rate	Tissue PCR success rate
1	5/10	13	4/10	3/3	4/4
2	6/10	14	7/10	3/3	4/4
3	5/10	15	5/10	3/3	3/4
4	5/10	16	7/10	2/3	3/4
5	2/10	17	5/10	2/3	4/4
6	6/10	18	4/10	3/3	3/4
7	3/10	19	8/10	2/3	3/4
8	5/10	20	8/10	2/3	3/4
9	3/10	21	4/10	2/3	4/4
10	6/10	22	7/10	3/3	4/4
11	3/10				
12	6/10				
Total	**55/120**		**59/100**	**25/30**	**35/40**

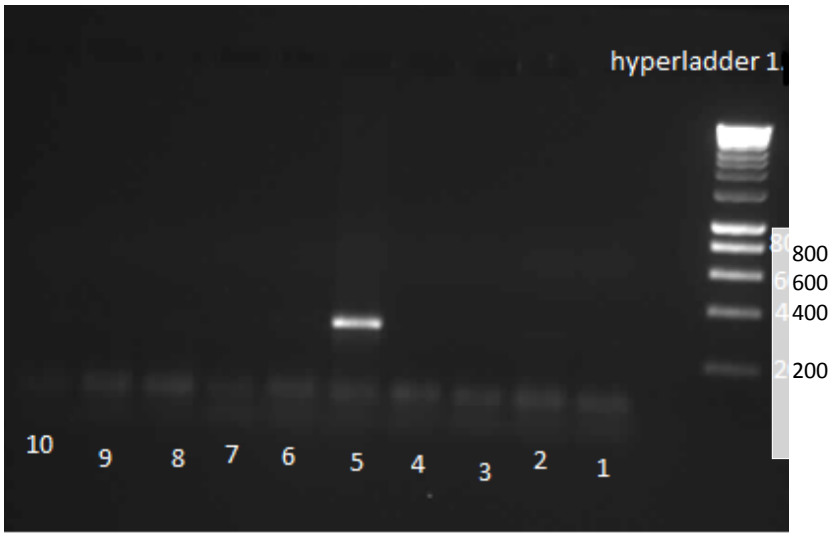

Figure 3.2. Agarose gel images of PCRs conducted with microsatellite *Tcri* 27 using punches from cards 1 -10 from the 2015 sample set.

3.3 Microsatellite locus *Tcri*29

Table 3.4 shows an overview of PCR success rates for microsatellite locus *Tcri*29. Overall success rates for FTA- based PCRs in 2014 were in the order of 40%, although success rates again varied across individuals (for example individual 12 had a low success rate of 3/10, i.e. 30%). PCRs based on FTA cards collected in 2015 had approximately 20% higher success rate than PCRs based on FTA cards collected in 2014. PCRs based on Qiagen extractions overall produced the best results (Table 3.4).

Table 3.4: PCR results in fractions for the individual punches from all FTA cards from both sample sets using microsatellite loci *Tcri*29.

2014 (FTA)		**2015**			
Individual number	PCR success rate	Individual number	FTA PCR success rate	Qiagen FTA PCR success rate	Tissue PCR success rate
1	5/10	13	7/10	2/2	4/4
2	4/10	14	6/10	2/2	4/4
3	6/10	15	7/10	1/2	4/4
4	5/10	16	5/10	1/2	4/4
5	5/10	17	9/10	2/2	3/4
6	4/10	18	7/10	2/2	3/4
7	4/10	19	6/10	1/2	3/4
8	4/10	20	7/10	1/2	4/4
9	5/10	21	7/10	2/2	4/4
10	4/10	22	5/10	2/2	4/4
11	5/10				
12	3/10				
Total	**59/120**		**66/100**	**16/20**	**37/40**

3.4 Microsatellite locus *Tcri*35

Table 3.5 shows an overview of PCR success rates for microsatellite locus *Tcri*35. Overall success rates for FTA- based PCRs in 2014 were in the order of 58%, although they varied across individuals (for example individuals three and ten had low success rates of 50%). PCRs based on FTA cards collected in 2015 had approximately 2% lower success rate than PCRs based on FTA cards collected in 2014, with however one individual (22) having a low success rate of 40%. Despite significant stutter bands in several agarose gels (e.g. Figure 3.4), the PCRs produced a band at the expected product size of about 190bp.

Table 3.5: PCR results for the individual punches from all FTA cards from both sample sets using microsatellite loci *Tcri*35.

2014 (FTA)		**2015**			
Individual number	PCR success rate	Individual number	FTA PCR success rate	Qiagen PCR success rate	Tissue PCR success rate
1	6/10	13	8/10	1/2	3/4
2	8/10	14	6/10	1/2	4/4
3	5/10	15	5/10	1/2	4/4
4	8/10	16	8/10	1/2	4/4
5	6/10	17	4/10	2/2	4/4
6	6/10	18	8/10	2/2	3/4
7	6/10	19	6/10	2/2	3/4
8	5/10	20	6/10	1/2	3/4
9	5/10	21	5/10	1/2	4/4
10	5/10	22	4/10	2/2	4/4
11	8/10				
12	5/10				
Total	**73/120**		**60/100**	**14/20**	**36/40**

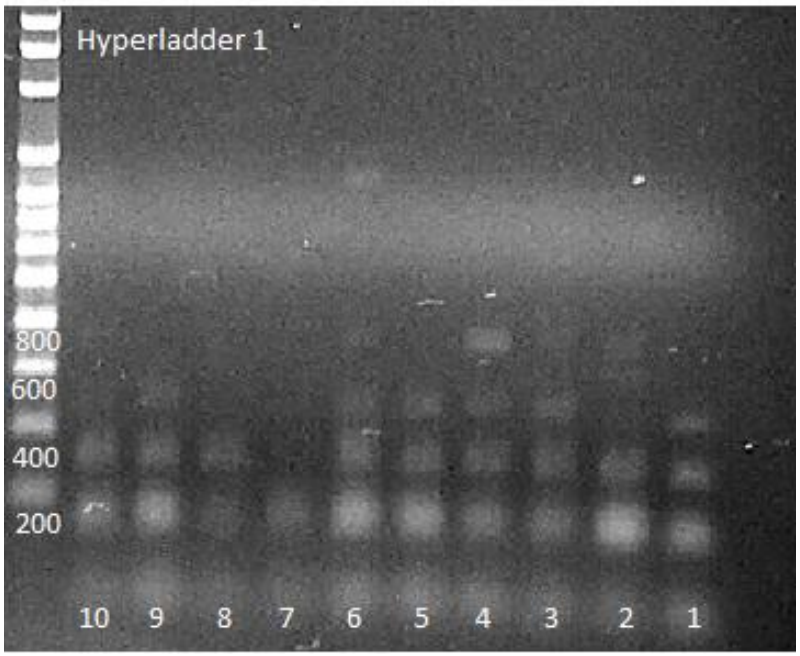

Figure 3.3. Agarose gel images of PCRs conducted with microsatellite *Tcri*35 using punches from cards 1 -10 from the 2015 sample set.

3.5 Microsatellite locus *Tcri*36

Table 3.6 shows an overview of PCR success rates for microsatellite locus *Tcri*36, which, based on FTA cards, were in the order of 50%. PCRs based on FTA cards collected in 2015 had approximately 14% higher success rate than PCRs based on FTA cards collected in 2014, with can however be largely attributed to one individual (19) with a high success rate of 80%. Using Quiagen extraction based on eight punches increased the proportion of successful PCRs (Fig. 3.5).

Table 3.6: PCR results for the individual punches from both sample sets using microsatellite locus *Tcri*36.

2014 (FTA)		2015			
Individual number	PCR success rate	Individual number	FTA PCR success rate	Qiagen PCR success rate	Tissue PCR success rate
1	8/10	13	5/10	1/3	3/4
2	4/10	14	6/10	2/3	4/4
3	5/10	15	4/10	1/3	4/4
4	8/10	16	7/10	1/3	3/4
5	9/10	17	7/10	1/3	4/4
6	8/10	18	5/10	2/3	3/4
7	8/10	19	6/10	2/3	3/4
8	8/10	20	6/10	2/3	3/4
9	7/10	21	5/10	1/3	4/4
10	7/10	22	5/10	1/3	4/4
11	8/10				
12	8/10				
Total	**92/120**		**56/100**	**16/30**	**35/40**

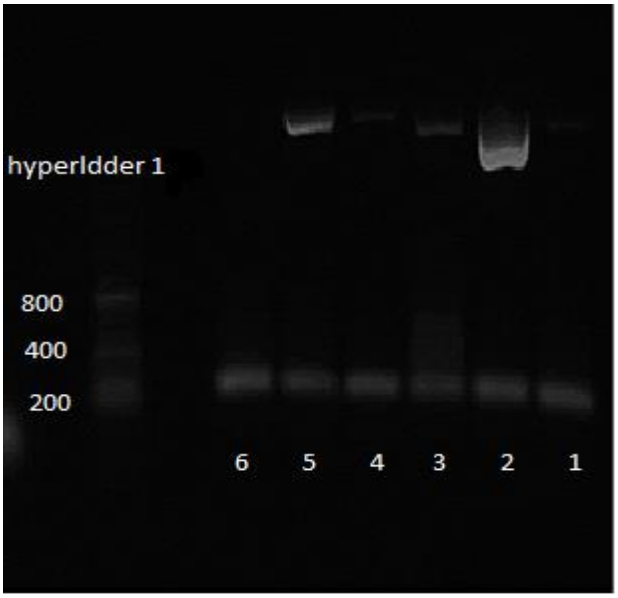

Figure 3.4. Agarose gel images of PCRs conducted with microsatellite *Tcri*36 using punches from cards 1 -6 from the 2014 sample set.

3.6 Microsatellite locus *Tcri*43

Table 3.7 shows an overview of PCR success rates for microsatellite locus *Tcri*43. Overall success rates for FTA- based PCRs in 2014 were in the order of 80%, which was approximately 10% higher than in 2015. Again, the Quiagen-based protocol showed overall better results than PCRs conducted directly with punches. The PCR product sizes were as expected from the known sequence of the locus (Fig. 3.6).

Table 3.7: PCR results for the individual punches from all the FTA cards from both sample sets using microsatellite loci Tcri43.

2014 (FTA)		**2015**			
Individual number	PCR success rate	Individual number	FTA PCR success rate	Qiagen success rate	Tissue PCR success rate
1	9/10	13	8/10	3/3	4/4
2	9/10	14	7/10	3/3	3/4
3	8/10	15	6/10	2/3	3/4
4	9/10	16	8/10	2/3	3/4
5	9/10	17	7/10	2/3	4/4
6	8/10	18	7/10	3/3	4/4
7	9/10	19	8/10	3/3	4/4
8	9/10	20	8/10	3/3	4/4
9	9/10	21	8/10	3/3	4/4
10	9/10	22	6/10	2/3	3/4
11	8/10				
12	8/10				
Total	**104/120**		**73/100**	**26/30**	**36/40**

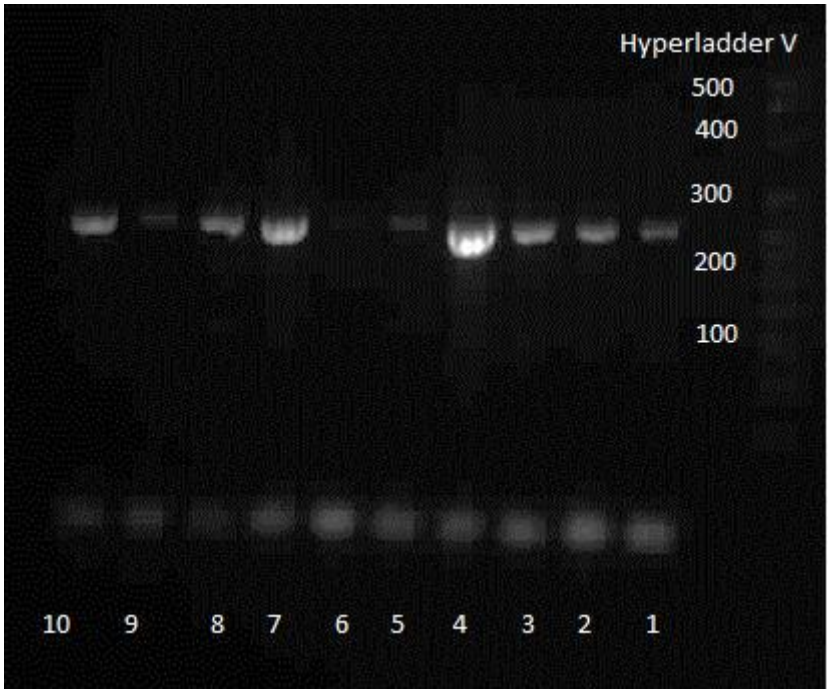

Figure 3.5. Agarose gel images of PCRs conducted with microsatellite *Tcri*43 using punches from cards 1 -10 from the 2015 sample set.

3.7 Microsatellite locus *Tcri*46

Table 3.8 shows an overview of PCR success rates for microsatellite locus *Tcri*46. Success rates for FTA- based PCRs in 2014 were on average 51%. PCRs based on FTA cards collected in 2015 had approximately 17% higher success rate than PCRs based on FTA cards collected in 2014. Alongside PCRs conducted with FTA punches from the 2015 cards, eight punches from each card were also pooled for Qiagen extraction, yielding in >90% PCR success rates. The PCR product size matched the expectation based on the known sequence (Figure 3.7).

Table 3.8: PCR results for the individual punches from all the FTA cards from both sample sets using microsatellite loci *Tcri*46.

2014 (FTA)		2015			
Individual number	PCR success rate	Individual number	FTA PCR success rate	Qiagen success rate	Tissue PCR success rate
1	7/10	13	6/10	2/2	4/4
2	6/10	14	7/10	2/2	4/4
3	7/10	15	8/10	2/2	3/4
4	4/10	16	7/10	2/2	4/4
5	7/10	17	7/10	1/2	4/4
6	6/10	18	6/10	1/2	4/4
7	7/10	19	8/10	2/2	3/4
8	6/10	20	8/10	2/2	4/4
9	6/10	21	6/10	2/2	4/4
10	4/10	22	6/10	2/2	3/4
11	7/10				
12	4/10				
Total	**71/120**		**73/100**	**18/20**	**37/40**

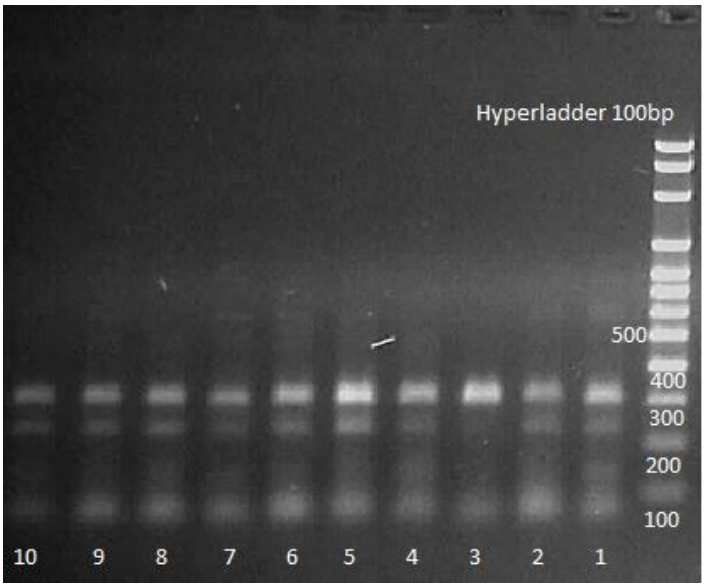

Figure 3.6. Agarose gel images of PCRs conducted with microsatellite *Tcri*46 using punches from cards 1 -10 from the 2015 sample set.

3.8 MtDNA (ND4)

Table 3.9 shows an overview of PCR success rates for the mitochondrial ND4 locus. Overall success rates for FTA- based PCRs in 2014 were in the order of 54%, although success rates varied across individuals (for example individual 9 had a low success rate of 3/10, ie 30%). PCRs based on FTA cards collected in 2015 had a slightly (4%) higher success rate than PCRs based on FTA cards collected in 2014 (Figure 3.8). As was the case for microsatellite loci, PCRs based on Qiagen extracts from 8 pooled punches yielded in a high success rate than PCR conducted directly with punches.

Table 3.9: PCR results for individual punches from all of the FTA cards from both sample sets using mtDNA ND4.

2014 (FTA)		**2015**			
Individual number	PCR success rate	Individual number	FTA PCR success rate	Qiagen success rate	Tissue PCR success rate
1	4/10	13	7/10	3/3	4/4
2	5/10	14	7/10	3/3	3/4
3	8/10	15	7/10	2/3	4/4
4	3/10	16	6/10	3/3	4/4
5	6/10	17	8/10	2/3	4/4
6	6/10	18	8/10	2/3	3/4
7	7/10	19	6/10	2/3	3/4
8	7/10	20	7/10	2/3	4/4
9	3/10	21	6/10	3/3	4/4
10	6/10	22	6/10	3/3	3/4
11	5/10				
12	9/10				
Total	**69/120**		**68/100**	**25/30**	**35/40**

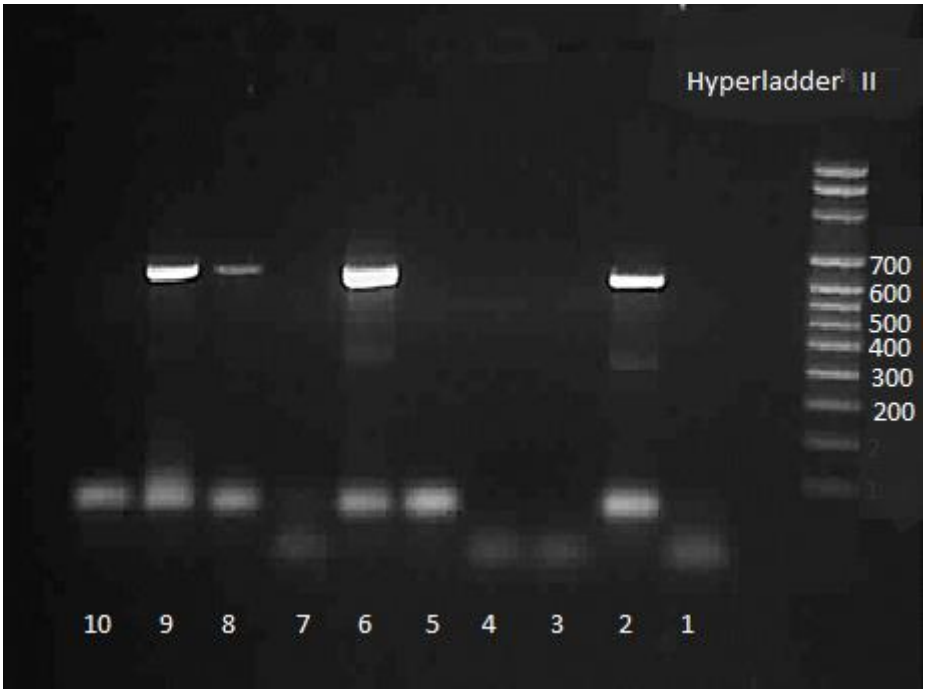

Figure 3.7. Agarose gel images of PCRs conducted with mtDNA (ND4) using punches from cards 1 -10 from the 2015 sample set. Four out of 10 punches yielded in a successful PCR.

All three sequenced individuals shared one common ND4 halpotype, having identical sequences (Table 3.10). Blasting the sequence revealed a 100% match with two sampled deposited on Genbank, including an individual for which the complete mtDNA sequence is available (Wielstra & Arntzen 2011). The obtained sequences clearly confirm that the PCRs amplified *T. cristatus* mtDNA.

Table 3.10: Sequence obtained from the *T. cristatus* mtDNA ND4 PCR from FTA cards.

ATCCTGGCAGCAGTATTGCTCAAGCTTGGGGGATATGGCATTATTCGAATTACCA
TAATCCTTACCCCACTAACAAAAGAAATGGCATATCCATTTATAATTCTAGCCTT
ATGAGGGGTCGTGATAACAAGCCTAATCTGCATACGACAAACAGACCTTAAGTC
GCTAATTGCATATTCGTCTGTAAGCCACATAGGCCTGGTCATTGCCGCTATTATA
ATTCAAACACCGTGAAGCCTAACGGGGGCTGTTATTTTAATGATCTCACACGGGC
TTATTTCATCCGCCCTATTTTGCCTAGCAAACCTAAATTATGAACGAACGCACAG
TCGAACTCTACTATTAGTTCGAGGGGCCCAGGCAGTCATACCATTAATGGCAACT
TGGTGACTCATTGCAAATTTATCAAATATAGCACTTCCTCCAACAATAAATCTAT
GAGGAGAGCTGACAATTATAGTCTCGCTGTTTAACTGATCCCCCTGGACAATCCT
AATCACGGGCTTGGGGGCCTTAATCACTGCCTCTTACACCTTATACATATTCCTA
ATAACACAACGTGGCCCAACCCCCCCACATCTTGGCCAAATTAACCCATCTTACA
CTCGAGAACACTTCTTACTTATAATACACTTAGCCCCTATATTACTGCTAATTC

Chapter Four: Discussion

4.1. General Considerations

This thesis was undertaken to verify whether the method of skin swabbing with FTA cards was a viable and non-invasive option for the collection of DNA from great crested newts (*T. cristatus*), providing an alternative to historically more commonly used invasive methods such as toe clipping. Using FTA cards for tissue collecting is a common practice in species such as livestock (Muthuhrishnan et al. 2008). Given the vulnerable skin of amphibians combined with their high conservation concerns, FTA cards have a clear potential for a wider use, although detailed quantitative studies were so far lacking. After a general quantification of the applicability of FTA cards to retrieve PCR-amplifyable DNA (Aim 1), alternative DNA extraction methods were compared with regard to resulting PCR success rates (Aim 2). Moreover, PCR success based on FTA cards was compared against tissue samples (toe clips) which were simultaneously collected from a subset of study individuals (Aim 3). The results of the present study suggest that skin swabs performed with FTA cards are a viable method of DNA collecting for *T. cristatus*, and the method is therefore likely applicable for amphibians as a whole.

Only a small number of studies exist which have previously tested the use of FTA cards for the collection of DNA from amphibians. Maddock et al. (2014) summarised a range of different approaches for non-lethal sampling of caecilian amphibians, also including swabbing of the body using a FTA card. While a success rate of 25% (2/8 PCR tests) combined with a low DNA yield resulted in a rather low performance compared to 15 other methods of DNA collection, the study was only conducted on two individuals and not in a rigorous quantitative way. Forzan et al. (2013) stored blood collected from the eye of an anuran species using a capillary on FTA cards, and documented that this method was overall performing better than toe clipping. This cemented the idea for this study that FTA cards

could be used efficiently and with success in swabbing of amphibians. Various alternative materials including cotton swabs and filter paper have been used to swab amphibians, with however variable success rates and the additional requirement for storage of samples for example in ethanol in the field (Poschadel and Moller 2004, Obendof and Dalton 2006). The present study convincingly shows that the trialled method is a useful alternative to other tissue collecting methods, although several FTA card punches per FTA cards are needed for PCR success rates similar to those conducted with tissue samples such as based on toe clips (see also Smith and Burgoyne 2004 for a general overview on other taxa).

4.2. Experimental design and protocol

Initial PCR tests revealed that the number of punches used per PCR reaction (1-5 punches were trialled, Appendix 1) had an influence on PCR success rates. Using 4 or 5 punches usually led to PCR failures, likely due to their too high volume in PCR reactions, and success rates were higher for 1-3 punches per PCR reaction (detailed data not shown). Although the probability to capture a part of the FTA card with tissue fixed increases with the number of punches, a large number of punches in PCR reaction apparently decreased its efficiency. A direct comparison of different extraction methods using identical punches was unfortunately not possible given that the punches were consumed in the PCR reaction. Perozo et al. (2006) stated that DNA on FTA cards only lasted for 14 days, while in the present study PCR products were still obtained after several months. The comparatively lower PCR success rate based on FTA card punches directly used for PCR reactions is likely a result of spatial heterogeneity in the amount of DNA bound to the card, as individual punches only cover a very restricted area. All areas of the FTA cards collected in both years were used to retrieve punches (see Appendix 1), and apparent PCR failures could have resulted from distal punches

which were taken from regions of the FTA card which were not swabbed over the individual newt. Based on the findings presented in the Results section, the pooling of DNA from eight punches through Quiagen extraction is however generally recommended as the protocol for future studies.

The present study revealed significantly higher FTA card-based PCR success rates than Maddock et al. (2014), whose results are however based on only two FTA cards and four PCR tests each. The differences between the studies could be related to different sizes of study organisms (caecilians are more slender than urodeles), insufficient swabbing and skin cell retrieval, and storage of FTA cards.

While the present study was restricted to *T. cristatus*, the general findings are likely transferrable to other amphibians (urodeles, anurans and caecilians) for which a comparable amount of skin cells can be captured with FTA cards. While replicate PCRs will be needed when card punches are not merged for Qiagen kit-based DNA extraction, it should aid in reducing the impact of DNA collection on threatened amphibians in general.

4.3. Difference between genetic markers

Eight different genetic markers (1 microsatellite and the mitochondrial DN4 region) were amplified to gather detailed knowledge across a range of marker types and PCR conditions. As expected, due to its smaller size and availability of larger numbers of copies per cell compared to nuclear genes, the amplification of the mtDNA gene had an overall high success rate. In 2015, a direct comparison in PCR amplification success was available between DNA derived from FTA cards with DNA derived from tissue (toe clips). PCR success rates suggest that amplification rates are similar between the two methods whenever Harris wheeler punches are taken from areas of the FTA card that contain sufficient amounts of DNA.

Each card from both sampled sets (collected in 2014 and 2015) was tested with every primer used, as well as using a representative number of punches, revealing different amplification success rates depending on the genetic marker. Using two punches for cards 1-10 each, for example, both microsatellites *Tcri*29 and *Tcri*36 had a 100% success rate (Table 3.1). However, when using five punches from each of the ten cards in the reactions, only two and four PCRs for *Tcri*29 and *Tcri*36 were successful. Individual cards often worked better with specific primers. For example, individual 12 showed an 80% (8/10) PCR success for locus *Tcri*13, but only a 30% (3/10) success for *Tcri*29; a similar pattern can be observed for e.g. individual 17. The two sets of FTA cards also had varying overall success rates when using the PCR reactions. Reactions using *Tcri*43 based on 2014 sampled proved more successful (83%) compared to PCRs based on the 2015 sample set (72%). A similar pattern was also revealed by *Tcri*13 (83% and 75% PCR success rates in 2014 and 2015, respectively. Amplification rates based on Qiagen extractions were significantly higher than those of direct FTA card PCRs, which could be due to the cumulative amounts of DNA that was able to be extracted from the eight punches. Nevertheless microsatellites *Tcri*35 and *Tcri*36 had lower overall success rates of 70% (14/20) and 53% (16/30), respectively. These results are generally linked to low amplification success of these loci (see Appendix 1). Microsatellite *Tcri*36 produced higher PCR success rates for samples collected in 2014 compared to 2015, which is in disagreement with the overall findings across all loci tested. Unscorable smears (detailed data not shown) observed for this marker in 2015 coincided with the ordering of a new batch of PCR primers, which might be responsible for the observed discrepancy due to the uncertainty of the product quality. Another difficult locus is represented by the microsatellite *Tcri*27, which had low and inconsistent success rates in both 2014 and 2015. While PCR failures were observed in all genetic markers tested, all other loci consistently amplified at least half of the test samples in both years of investigation. As expected due to

previous works, the ND4 (mtDNA) PCR reaction had among the overall highest success rates. The strengths of PCR products appear to not be affected by the DNA retrieval method, as strong bands were observed for PCRs both based on tissue samples as well as FTA card extracts (detailed data not shown). For example, the cards 13, 14 and 15 respectively had *Tcri*13 PCR success rates of 9/10, 8/10 and 8/10 for FTA cards, which is comparable to amplification success rates produced by the corresponding tissue samples (3/3, 3/3 and 2/3, respectively). This is similar to results from ND4 amplifications, where success rates for individuals 17 and 18 were 8/10 each, corresponding to 2/3 success rates based on tissue samples.

4.4. Conclusions and recommendations

In an age where species are becoming extinct and endangered due to deforestation, climate change and global development, it is vital that we continue to explore methods of retrieving DNA for conservation-relevant research in the least invasive way. This thesis set out to discover whether a non-invasive method of testing for DNA in great crested newts demonstrated that the PCR success rate of FTA card-based swabbing can be comparable to PCRs based on toe clips. While there is scope for further optimisation of the trialled protocol (optimisation of the number of punches, ensuring that cards used contain sufficient amounts of swabbed DNA), the results from this thesis demonstrate that the developed method can fully replace more invasive methods such as toe clipping. Based on the comparison of different methods of DNA extraction from FTA cards in this thesis, we generally recommend the Qiagen protocol combined with the pooling of several punches (8 in our case) taken from single cards as this proved consistently most effective.

Triturus cristatus is currently decreasing in numbers (for a summary see e.g. Jehle et al. 2011), and a genetic monitoring program based on minimal invasive techniques could enable

improved future protocols to insure the species' survival. Amphibians in general are an indicator species and help with playing an important role in our ecosystem. Currently 32.4% of global amphibians are classed as Extinct or Threated with Extinction, with an additional 25% of species having insufficient data to determine their status (IUCN Red List 2016). What would happen if the 32.4% were to become extinct? Imagine what fascinating and interesting creatures future generations would miss out on.

6. References

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7. Appendices

Appendix 1: List and details of samples used.

Date	Number of cards used	Number of punches used	Where punches were taken from	Primer used	Punches that worked
09/12/2014	6	1	inside circle	mtDNA	4
10/12/2014	6	1	outside circle	mtDNA	4
11/12/2014	12	2	inside circle	mtDNA	10
12/12/2014	4	2	outside circle	mtDNA	1
13/12/2014	12	4	inside circle	mtDNA	4
14/12/2014	12	4	outside circle	mtDNA	6
15/12/2014	12	3	inside circle	mtDNA	11
16/12/2014	12	3	outside circle	mtDNA	9
17/12/2014	6	2	1 in, 1 out of circle	mtDNA	4
18/12/2014	6	2	1 in, 1 out of circle	mtDNA	5
19/12/2014	12	2	1 in, 1 out of circle	mtDNA	12
20/12/2014	12	3	inside circle	mtDNA	12
04/01/2015	12	4	outside circle	mtDNA	5
05/01/2015	12	5	outside circle	mtDNA	3
06/01/2015	12	4	inside circle	mtDNA	4
07/01/2015	12	5	inside circle	mtDNA	4
08/01/2015	12	3	inside circle	mtDNA	8
09/01/2015	12	1	inside circle	mtDNA	9

10/01/2015	12	2	outside circle	mtDNA	10
11/01/2015	6	1	outside circle	35	4
12/01/2015	6	3	inside circle	35	5
13/01/2015	6	2	inside circle	35	5
14/01/2015	12	2	outside circle	mtDNA, tcri 36	7
15/01/2015	12	2	outside circle	mtDNA, tcri 36	10
16/01/2015	12	1	inside circle	35 and 36	9
17/01/2015	12	1	outside circle	35 and 36	8
18/01/2015	12	3	inside circle	35 and 36	10
19/01/2015	12	3	outside circle	35 and 36	9
20/01/2015	12	4	inside circle	35 and 36	6
21/01/2015	12	2	outside circle	mtDNA, tcri 36	9
22/01/2015	12	2	inside circle	mtDNA, tcri 36	11
23/01/2015	12	4	inside circle	35 and 36	6
24/01/2015	6	5	inside circle	35 and 36	3
25/01/2015	6	5	inside circle	35 and 36	2
26/01/2015	6	3	inside circle	35 and 36	2
27/01/2015	6	3	both in and out of circle	mtDNA, tcri 46	2
28/01/2015	6	1	inside circle	tcri 36, tcri 46	4
29/01/2015	6	1	inside circle	46	5
30/01/2015	12	1	outside circle	46	9
31/01/2015	12	2	inside circle	46	10
01/02/2015	12	2	outside circle	46	11

02/02/2015	12	2	inside circle	35	11
03/02/2015	12	3	outside circle	35	10
04/02/2015	12	2	inside circle	35 and 29	11
05/02/2015	12	1	inside circle	35 and 29	10
06/02/2015	12	1	outside circle	29 and 13	9
07/02/2015	12	2	inside circle	35	9
08/02/2015	12	4	inside circle	35	9
09/02/2015	12	4	outside circle	35	5
10/02/2015	12	1	outside circle	13	8
11/02/2015	12	2	1 in, 1 out of circle	29 and 35	10
12/02/2015	12	2	1 in, 1 out of circle	29 and 35	10
13/02/2015	12	1	inside circle	29 and 13	7
14/02/2015	12	2	outside circle	29 and 13	6
15/02/2015	6	2	inside circle	29 and 13	3
16/02/2015	6	3	outside circle	29 and 13	3
17/02/2015	6	3	inside circle	29 and 13	4
18/02/2015	6	2	inside circle	29 and 36	4
19/02/2015	6	3	inside circle	29 and 36	4
20/02/2015	6	3	2 inside, 2 outside circle	13 and 27	3
21/02/2015	6	1	inside circle	29	4
22/02/2015	6	4	inside circle	29	3
23/02/2015	6	4	outside circle	29	3
24/02/2015	6	1	outside circle	36 and 46	5
25/02/2015	6	1	inside circle	36 and 46	5
26/02/2015	6	2	inside circle	29	5

27/02/2015	6	2	outside circle	29	5
28/02/2015	6	2	outside circle	46	6
01/03/2015	6	4	inside circle	29	5
02/03/2015	6	4	outside circle	29	6
03/03/2015	6	2	inside circle	27 and 46	5
04/03/2015	6	2	outside circle	27 and 46	5
05/03/2015	6	1	outside circle	27 and 46	5
06/03/2015	6	1	inside circle	27 and 46	6
07/03/2015	6	2	outside circle	27 and 46	6
08/03/2015	6	3	inside circle	27 and 46	5
09/03/2015	6	3	outside circle	27 and 46	5
10/03/2015	6	2	outside circle	mtDNA	6
11/03/2015	6	1	outside circle	mtDNA	6
12/03/2015	6	2	outside circle	mtDNA	4
13/03/2015	6	1	outside circle	mtDNA	4
14/03/2015	6	2	inside circle	mtDNA	5
15/03/2015	6	3	inside circle	mtDNA	6
16/03/2015	12	3	inside circle	35 and43	8
17/03/2015	12	3	outside circle	35 and43	9
18/03/2015	12	1	outside circle	35 and43	7
19/03/2015	12	1	inside circle	35 and43	10
20/03/2015	12	2	inside circle	35 and43	11
21/03/2015	12	2	outside circle	35 and43	11
22/03/2015	12	3	inside circle	35 and43	11
23/03/2015	12	3	outside circle	35 and43	11
24/03/2015	12	4	inside circle	35 and43	7

25/03/2015	12	4	outside circle	35 and43	6
26/03/2015	12	1	outside circle	13	7
27/03/2015	12	1	inside circle	13	9
28/03/2015	12	2	1 in, 1 out of circle	29 and 46	10
29/03/2015	12	2	1 in, 1 out of circle	29 and 46	10
30/03/2015	12	2	1 in, 1 out of circle	29 and 46	11
31/03/2015	6	3	inside circle	29 and 46	5
01/04/2015	6	3	inside circle	29 and 46	5
02/04/2015	6	5	outside circle	29 and 46	3
03/04/2015	6	5	inside circle	29 and 46	2
04/04/2015	6	5	inside circle	29	2
05/04/2015	6	5	inside circle	13	3
06/04/2015	6	4	in and out of circle	27	3
07/04/2015	6	4	in and out of circle	27	3
08/04/2015	6	4	in and out of circle	35	3
09/04/2015	6	4	in and out of circle	36	3
10/04/2015	6	3	in and out of circle	27	4
11/04/2015	6	3	inside circle	27	4
12/04/2015	6	3	in and out of circle	13	5
13/04/2015	6	2	inside circle	mtDNA	5
14/04/2015	6	2	outside circle	mtDNA	6
15/04/2015	6	2	inside circle	mtDNA and 46	6
16/04/2015	6	1	outside circle	mtDNA and 46	6
17/04/2015	6	1	inside circle	35 and 13	5
18/04/2015	12	1	inside circle	46 and 27	10
19/04/2015	12	2	inside circle	35 and 13	11

20/04/2015	12	2	outside circle	46 and 27	11
21/04/2015	12	3	outside circle	35 and 13	11
22/04/2015	12	3	inside circle	46 and 27	9
23/04/2015	12	4	inside circle	35 and 13	6
24/04/2015	12	4	inside circle	46 and 27	7
25/04/2015	12	5	inside circle	35 and 13	3
26/04/2015	12	5	inside circle	46 and 27	5
27/04/2015	10	1	inside circle	35 and 13	9
28/04/2015	10	2	inside circle	35 and 13	10
29/04/2015	10	3	inside circle	35 and 13	10
30/04/2015	10	4	inside circle	35 and 13	9
01/05/2015	10	5	inside circle	35 and 13	6
02/05/2015	10	1	inside circle	13 and 43	8
03/05/2015	10	2	inside circle	13 and 43	9
04/05/2015	10	3	inside circle	13 and 43	9
05/05/2015	10	4	inside circle	13 and 43	9
06/05/2015	10	5	inside circle	13 and 43	4
07/05/2015	10	1	inside circle	29 and 36	9
08/05/2015	10	2	inside circle	29 and 36	10
09/05/2015	10	3	inside circle	29 and 36	9
10/05/2015	10	4	inside circle	29 and 36	4
11/05/2015	10	5	inside circle	29 and 36	2
12/05/2015	10	1	inside circle	mtDNA	6
13/05/2015	10	2	inside circle	mtDNA	10
14/05/2015	10	3	outside circle	mtDNA	10
15/05/2015	10	2	inside circle	mtDNA and 46	9

16/05/2015	10	4	inside circle	mtDNA	7
17/05/2015	10	2	outside circle	mtDNA	9
18/05/2015	10	5	inside circle	27 and 46	6
19/05/2015	10	2	inside circle	27 and 46	7
20/05/2015	10	1	inside circle	27 and 46	9
21/05/2015	10	2	inside circle	27 and 46	10
22/05/2015	10	3	inside circle	27 and 46	10
23/05/2015	10	4	inside circle	27 and 46	7
24/05/2015	10	5	inside circle	27 and 46	5
25/05/2015	10	1	inside circle	mtDNA and 29	10
26/05/2015	10	2	inside circle	mtDNA and 29	10
27/05/2015	10	3	inside circle	mtDNA and 29	10
28/05/2015	10	4	inside circle	mtDNA and 29	9
29/05/2015	10	5	inside circle	mtDNA and 29	4
30/05/2015	10	1	outside circle	13	6
31/05/2015	10	1	inside circle	27	7
01/06/2015	10	2	inside circle	13	9
02/06/2015	10	2	outside circle	27	9
03/06/2015	10	3	in and out of circle	13	10
04/06/2015	10	3	in and out of circle	27	10
05/06/2015	10	1	outside circle	13	9
06/06/2015	10	1	inside circle	13	9
07/06/2015	10	1	outside circle	29	9
08/06/2015	10	2	inside circle	29	9
09/06/2015	10	2	inside circle	27	9
10/06/2015	10	2	outside circle	27	9

11/06/2015	10	2	inside circle	35	10
12/06/2015	10	3	outside circle	35	8
13/06/2015	10	3	inside circle	36	10
14/06/2015	10	4	outside circle	36	7
15/06/2015	10	4	inside circle	mtDNA	6
16/06/2015	10	4	2 inside, 2 outside circle	mtDNA	5
17/06/2015	10	4	inside circle	36	8
18/06/2015	10	5	outside circle	36	4
19/06/2015	10	1	inside circle	mtDNA	7
20/06/2015	10	1	inside circle	mtDNA	8
21/06/2015	10	1	outside circle	mtDNA	9
22/06/2015	10	4	2 inside, 2 outside circle	36	9
23/06/2015	10	4	inside circle	46	7
24/06/2015	10	4	2 inside, 2 outside circle	46	8
25/06/2015	10	4	inside circle	29	9
26/06/2015	10	3	inside circle	27	8
27/06/2015	10	3	inside circle	46	8
28/06/2015	10	3	inside circle	29	9
29/06/2015	10	2	outside circle	13	9
30/06/2015	10	2	in and out of circle	29	9
01/07/2015	10	2	inside circle	27	9
02/07/2015	10	1	outside circle	27	9
03/07/2015	10	1	inside circle	36	10
04/07/2015	10	2	inside circle	35	10

05/07/2015	10	2	outside circle	mtDNA	10
06/07/2015	10	2	inside circle	mtDNA	10
07/07/2015	10	2	inside circle	27	10
08/07/2015	10	2	inside circle	mtDNA	10
09/07/2015	10	2	outside circle	mtDNA	9
10/07/2015	10	3	inside circle	13	8
11/07/2015	10	3	outside circle	13	8
12/07/2015	10	2	outside circle	43	9
13/07/2015	10	1	inside circle	43	9
14/07/2015	10	1	inside circle	27	10
15/07/2015	10	1	outside circle	27	7
16/07/2015	10	3	inside circle	29	7
17/07/2015	10	3	inside circle	29	6
18/07/2015	10	3	outside circle	29	6
24/08/2015	10	3	inside circle	mtDNA	6
25/08/2015	10	3	outside circle	mtDNA	6
26/08/2015	10	2	inside circle	13	8
27/08/2015	10	2	outside circle	13	7
28/08/2015	10	1	inside circle	mtDNA	8
29/08/2015	10	1	outside circle	mtDNA	9
30/08/2015	10	3	inside circle	mtDNA	10
31/08/2015	10	3	outside circle	mtDNA	7
01/09/2015	10	2	inside circle	36	6
02/09/2015	10	1	outside circle	36	6
03/09/2015	10	2	1 in, 1 out of circle	mtDNA	7
04/09/2015	10	2	inside circle	35	7

05/09/2015	10	1	outside circle	35	6
06/09/2015	10	4	inside circle	29	8
07/09/2015	10	2	inside circle	mtDNA	9
08/09/2015	10	2	inside circle	36	6
09/09/2015	10	3	inside and outside	46 and 29	7
10/09/2015	10	1	inside circle	29	10
11/09/2015	10	1	outside circle	29	10
12/09/2015	10	2	in and out of circle	27	9
13/09/2015	10	3	inside circle	13	7
14/09/2015	10	2	in and out of circle	46	8
15/09/2015	10	2	in and out of circle	mtDNA	10
16/09/2015	10	3	in and out of circle	mtDNA	10
17/09/2015	10	3	inside circle	29	8
04/09/2015	10	2	inside circle	35	9
05/09/2015	10	1	outside circle	35	7
06/09/2015	10	4	inside circle	29	9
07/09/2015	10	2	inside circle	mtDNA	9
08/09/2015	10	2	inside circle	36	10
09/09/2015	10	3	inside and outside	46 and 29	9
10/09/2015	10	1	inside circle	29	8
11/09/2015	10	1	outside circle	29	6
12/09/2015	10	2	in and out of circle	27	5
13/09/2015	10	3	inside circle	13	7
14/09/2015	10	2	in and out of circle	46	9
15/09/2015	10	2	in and out of circle	mDNA	10

16/09/2015	10	3	in and out of circle	mtDNA	9
17/09/2015	10	3	inside circle	mtDNA	10