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Chapter 7: Summary and Discussion

Snakes are a fascinating and intriguing group of vertebrates that have gone to extremes in adaptation. Their venom system belongs to one of the most sophisticated natural weapons systems in the natural world. It is composed of a venom gland, duct and fang, and in some phylogenetic groups an accessory venom gland. In Chapter 2 we have elucidated the evolution of the different types of snake fangs. There are three distinct groups of snake fangs found. The first is a rear-fanged type, where the fangs are positioned in the rear of the upper jaw and this fang-type is found in most snake species. Then there are two types of front-fangs found. The first is found in vipers and pitvipers, where the fangs are often large and lie recumbent in the upper jaw. They are mobile and can move back and forth. The second type is the one found in cobras and mambas and relatives and is smaller and immobile. Because these two groups do not form a monophyletic group, the evolution of these different fang types has always been subject to debate. We have collected many different snake embryos of all three groups and looked at the development of the dentition using the Sonic hedgehog gene as a marker. Using in situ hybridization followed by careful 3D reconstruction we could follow the development of the fang. Our results showed a great amount of similarity in morphogenesis of the fang of the three different groups of snakes. The two front-fangs start right at the base of the rear of the upper jaw in development, just as the rear-fang. The only difference is that the two front-fangs move forward in development by ontogenetic allometry (pushing the fang forward due to rapid growth of the upper jaw behind the fang) to its adult front position. Also, we showed that the rear-fang develops from its own dental lamina instead of with the same dental lamina as the front 'normal' teeth. It was this posterior dental lamina that showed great similarity to the dental laminae in the two front-fang snake groups. Our results thus suggest a scenario in which all three fang-types are homologous and descendent from a rear-fanged ancestor (by means of uncoupling the posterior from the anterior teeth), after which the two front-fanged groups moved their rear-fang forward during evolution. This probably allowed them to feed on larger and more dangerous prey items as a quick
'snap-and-release' was made possible by the front-fangs compared to the 'bite-and-hold-on' that is only possible with rear-fangs.

Another extreme of adaptation in snakes is their body elongation. Snakes have elongated bodies with numerous vertebrae, some even up to 500 of them. They have lost most clear morphological boundaries in their adult bodies. This has been of major importance in the evolution of the venom-delivery system, as snakes use their elongated bodies as a coiled spring that can strike prey at a distance and with terrifying speed, injecting the immobilizing venom. Without elongated bodies able to strike and bite, their venom-delivery systems (especially the fangs) would probably have evolved in a completely different manner. In chapter 3 we have analyzed and examined the expression of Hox genes in different snake embryos from different stages to examine the evolution of a serpentine body form. Hox genes determine the basis structure of animals, and it was assumed that the species with a snake-like body evolved such a body by a homogenization of the Hox expression domains along the primary axis. However, in our extensive analysis using in situ hybridizations we have found - on the contrary - that snakes do retain a collinear expression of the Hox genes in the developing embryo. Some Hox genes boundaries correspond to expected anatomical boundaries (the most anterior and posterior Hox genes), but many do not. The dorsal (thoracic), homogenous rib-bearing region of trunk has also retained regionalized Hox expression that is not obviously reflected in the anatomy. Our results suggest that the evolution of a deregionalized, serpentine body involved not only alterations in Hox gene cis-regulation but also a different downstream interpretation of the Hox code.

The snake venom secretions themselves consist of a complex mixture of proteins and peptides that have evolved to target important physiological pathways and immobilize prey items. These toxins are of major evolutionary and biomedical interest. From an evolutionary perspective, they undergo an accelerated form of evolution. The genes undergo accelerated evolution due to a larger amount of mutation in coding regions than in non-coding regions, while in almost all other known genes in the natural world this is the other way around - except the genes in the immune system. This allows fast
evolution and adaptation towards ever changing prey and prey physiology (evolution of resistance). In an analogous manner, our immune systems must fight ever changing pathogens. This arms-race is the driving force behind diversity. From a biomedical perspective, this diversity is right at the base of why snake venoms form such a potential goldmine for new pharmaceuticals. In chapter 4 we have sequenced the king cobra (*Ophiophagus hannah*) genome to look at the evolution (and recruitment) of the venom genes. They are thought to have evolved from normal, physiological genes by means of gene duplication followed by selective expression in the venom gland. However, in the absence of genomic resources these hypotheses remain mainly speculative. Our results show that venom genes evolve through distinct mechanisms. L-amino acid oxidase, cysteine-rich secretory proteins and metalloproteinases, evolved by tandem-duplication of ancestral physiological genes, followed by recruitment through selective expression in the venom gland. By contrast, nerve growth factor toxins appear to have evolved by duplication and dual recruitment, while hyaluronidase and phospholipase B evolved by recruitment of existing physiological genes without further duplication. The massive toxin families the 3FTXs and the PLAs have undergone extensive duplications that have resulted in (at least) 21 copies in the genome. These results show a much more dynamic evolutionary history in venom genes as previously assumed. It is yet unclear what is causing the recruitment into the venom gland, and we are currently working on answering this question.

The enormous diversity in venom genes, caused by the different ways of evolution and recruitment as shown in chapter 5, makes it a challenge to identify bioactives for a particular receptor in order to either study the venom for evolutionary studies, or to find new pharmaceuticals. In addition to this, the extreme low venom yield that most snakes have make it a very precious sample. For most traditional methods of evaluating a sample with mass spec and bioactivity studies you need a relatively large amount of venom, which is a major challenge for many species. We have developed in chapter 6 a new method to study the make-up of the venom and - at the same time and in the same run - look for bioactives that bind to acetylcholine binding protein. One test used up less than 5 ug of venom and took
less than 2 hours. We found 21 bioactives of which 7 were never reported on before. This technique includes a microfluidic on-line screening approach utilizing nano-LC–MS with parallel bioaffinity detection using the acetylcholine binding protein (AChBP). The system comprises a post-column nano-flow split which directs the chromatographic effluent to mass spectrometric and on-line biochemical detection allowing for simultaneous correlation of separated bioactive venom toxins with their corresponding accurate mass.

In conclusion, in this thesis I have shown that snakes have undergone multiple changes in their genome and embryonic development that has provided them with the variation to which natural selection could act. This thesis provides evidence for the variable mechanisms of venom gene evolution, which presumably is much more flexible than previously thought. But it also underscores the potential use of the many different types of snake venom toxins that could be screened for use against human disorders. And most of all, I hope I have contributed towards the fact that snakes are just an incredibly interesting group of vertebrates from both the perspective of ecology and life-style, as well as from a genomic and molecular perspective. They are, and will always be, my first and true love.