Rudimentary Organs and Genes: Evolution of the Visual System in Caecilians

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Declaration

The work contained in this thesis is the author's own, except where stated or referenced accordingly. The views expressed in this thesis are those of the author and do not represent those of the University of London.

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Abstract

As Darwin (1859 pp143-146) noted, each major group of animals has evolved a visual system exhibiting unique adaptations to visual tasks. Caecilians are elongate, snake-like amphibians (the order Gymnophiona) that, except for the secondarily aquatic Typhlonectidae, are to some extent fossorial as adults. Caecilian eye morphology shows various degrees of rudimentation but the literature contains many contradictory reports, and phylogenetic characters based on eye morphology are controversial. New physiological, molecular and morphological data are presented here in order to better characterise the caecilian visual system and to investigate the utility of visual gene sequences in phylogenetics.

I provide the first description of the morphology of the eye of a member of the Rhinatrematidae, the sister group of all other caecilians and additional observations on a range of caecilian species. Morphological discoveries include the presence of a poorly developed iris in both the Rhinatrematidae and Ichthyophiidae. I elucidate the previously unknown complete sequence of the caecilian rod opsin gene and present new data on the physiology caecilian rhodopsin. The obvious reduction in visual system morphology in caecilians is associated with a change in the genes involved in visual function. The rod opsin is the only visual pigment detected in caecilians suggesting a loss of colour and diurnal vision, which are probably unnecessary in these predominately burrowing vertebrates. A change in the maximum absorbance of the caecilian rhodopsin compared to other amphibians is reported and experimental confirmation that this is caused by

amino-acid substitutions in the rod opsin gene is presented. The pattern of substitutions implies a previously unknown mechanism of spectral tuning. Phylogenetic analyses of caecilian rod opsin sequences give results that reflect current understanding of caecilian relationships and suggest both coding and non-coding regions are useful markers for inferring phylogenetic relationships within caecilians.

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Chapter 1

Introduction

1.1 Overview of thesis

This thesis presents a study of the molecular and morphological evolution of the visual system of a clade of burrowing vertebrates, the Gymnophiona, commonly known as the caecilians. The visual system of caecilians provides an interesting model of regressive evolution as it shows direction of change. Visual systems of vertebrates are of interest due to the observed morphological complexity, variation and the growing knowledge of genes involved in development and signal transduction in visual pathways.

The senses of subterranean animals such as caecilians display impressive structural and functional convergent adaptations to life underground. Caecilians are elongate, limbless amphibians that are highly specialized for burrowing. With the extreme confinement to the underground dark environment, caecilian vision is reduced and replaced by other sensory systems e.g., the tentacle which has chemosensory and tactile functions (Himstedt and Simon 1995, Fig 3.1). Lightless subterranean ecotopes lead commonly to regression or disappearance of the vertebrate and invertebrate eye (Walls 1942). The English name caecilian (Latin caecus = blind) and the German name 'Blindwuhlen' suggests that the visual system of the Gymnophiona is non functional (Himstedt 1995) and their visual system has long been considered rudimentary and non-functional (Norris and Hughes 1918; Walls 1942) due to the relatively small size of the eye which also may be covered by glandular skin and by bone, and a lack of some or all

of the extrinsic eye muscles and their innervation (Wake 1985). This rudimentation has been attributed to their fossorial and possibly nocturnal habits. Under closer examination however, the caecilians as a group show a great diversity in eye morphology with some taxa, such as *Ichthyophis,* having been shown to have a functioning visual system at least to the extent of being able to distinguish light from darkness (Himstedt 1995). One of the aims of this project is to re-evaluate and extend previous morphological studies on caecilians.

Visual pigment genes, the opsins, have been extensively studied from biochemical, molecular and evolutionary perspectives (e.g. Hunt et al. 2001; Crandall and Hillis 1997). Here it is intended to study the opsins to see whether there have been significant changes in the nucleic acid and/or amino acid sequences that have affected the function of the gene in any way, i.e., made the opsin non-functional or caused a spectral shift.

To study the evolution of the visual system of Gymnophiona it is helpful to understand relationships within the order. The relationships between the three different orders of living amphibians as well as between the 'higher' caecilians are not completely resolved. Opsin sequences have been used successfully (Bossuyt and Milinkovitch 2000; Venkatesh et al. (2001)) to study the phylogeny of vertebrate clades and might therefore be expected to provide useful information for caecilian phylogeny, a topic that is also investigated in this thesis.

This chapter provides introductory background information on caecilians, visual pigments and the morphology of the amphibian eye. In Chapter 2, I will describe the materials and methods used in the molecular work. Chapter 3 will be a description of the

eye morphology of a caecilian with a relatively well-developed eye that has not been described before. I will characterize the sequence and physiology of the rod opsin gene of caecilians in Chapter 4. In Chapter 5, I investigate the use of rhodopsin as a tool for the elucidation of phylogeny. Finally, in Chapter 6 I present an overview and broad discussion of the findings of this project.

1.2 What are Caecilians?

Figure 1.1 The caecilian *Schistometopum thomense* photo by R.A Nussbaum from (Nussbaum and Wilkinson 1989)

The Gymnophiona, commonly known as caecilians, are one of the three orders of recent Amphibia. They are readily distinguished from frogs and salamanders by their sensory tentacles and annulated, limbless bodies, and are distinct in many other characters (e.g. Taylor 1968; Wilkinson and Nussbaum 2006). The mostly fossorial (except for the aquatic or semi-aquatic South American Typhlonectidae) and tropical living caecilians are perhaps the least well-known tetrapod order (Gower et al. 2002). Although they are a relatively small group (about 170 species), it is clear that they have a

remarkable morphological, ecological and reproductive diversity (Kupfer et al. 2004, Gower et al. 2004, Wilkinson and Nussbaum 1997).

The name caecilian derives from the Latin word caecus = blind, referring to the small or imperceptible eyes. The name Gymnophiona, derives from the Greek words *gymnos*, naked and *ophis*, snake, as the Caecilians were originally thought to be related to snakes. It was Johannes Müller in 1835 that discovered that larval caecilians had gill slits and were in fact amphibians (Duellman and Treub 1994). They share characteristics with the frogs and toads, salamanders and newts such as smooth (externally scaleless) epidermis and the presence of gonadal fat bodies, pedicellate teeth and specialized papillae in the ear (Duellman and Treub 1994).

1.2.1 Caecilian Sensory Behaviour

Since caecilians are seldom observed in the field, relatively little is known about their natural history, ecology and behaviour compared to other amphibians (Gower et al 2002, Warbeck and Parzefall 2001). The caecilians are believed to depend on chemical or tactile cues. Laboratory experiments with *Ichthyophis* cf. *kohtoaensis* revealed its ability to localize prey by using only chemical cues. This species was shown to use its nostrils when above ground and use the tentacle (Fig 3.1 in chapter 3) to aid the location of prey when in burrows (Himstedt and Simon 1996). Further experiments suggested that *Ichthyophis* have a limited visual function, perhaps only detecting light from dark as the specimens show a negative phototaxis after the first day of hatching and having no visually mediated response to experimental prey (Himstedt 1995).

Typhlonectes natans has been observed in laboratory experiments to mark shelter sites with chemical signals and are attracted to chemicals, emanating from aggregates of conspecifics (Warbeck et al 1996). This is similar to the blind aquatic cave salamander *Salamandra luschani* (Guilliaume 2000 and 2002) and to *Proteus anguinus* (Gautier et al 2006). A male *Typhlonectes* was aggressive to another but not necessarily territorial (Warbeck and Parzefal 2001).

It is indicated by laboratory experiments in *Typhlonectes natans* that these caecilians produce waterborne chemical signals that communicate about gender, reproductive state and kin (Warbeck and Parzefall 2001). Because natural population densities and tendancies to aggregate in the field have not been studied, the nature of this animals mating system remains unknown.

1.3 Visual Pigments

Visual pigments consist of an opsin protein moiety linked to retinal, a derivative of vitamin A, which together are responsible for the initiation of the phototransduction pathway. The vitamin A derived chromophore can be in either 11-cis retinal or 11-cis 3,4-dehydroretinal producing a rhodopsin or porphyropsin visual pigment respectively (Crescitelli et al. 1972). Porphyropsins have been found in lampreys, teleost fish, amphibians, elasmobranches and some lizards. (Hart and Hunt 2007) Different classes of opsin exist distinguished by sequence identity and maximum absorbance λ_{max}. The opsin is a GPCR (G-Protein coupled receptor) that function through the activation of a heterotrimeric guanine nucleotide binding protein, the G protein. Visual pigments have a common basic structure of an opsin protein attached to a chromophore via a Schiff base

linkage and a conserved lysine residue. The opsin protein in vertebrates consists of a single polypeptide chain of 340-370 amino acids that forms seven α-helical transmembrane regions connected by cytoplasmic and luminal loops. In the tertiary structure the seven TM regions form a bundle within the membrane creating a cavity towards the luminal side for the chromophore (Hunt et al. 2007).

Rod opsin is responsible for scotopic, dim light vision. Up to four additional classes of opsins that are associated with colour and diurnal vision can exist in vertebrates (Hunt et al. 2001, Bowmaker and Hunt 1999). These cone classes have peak sensitivities at maximum absorbances in the regions of about 500-570nm ('red', long-wave-sensitive, LWS), 470-520nm ('green', middle-wave-sensitive, MWS), 440-470nm ('blue', shortwave-sensitive, SWS) and 360-440nm (violet/ultraviolet-sensitive, V/UVS). These opsins are found in the outer segments of photoreceptor cells in the retina. Often in nocturnal, cave dwelling or deep-sea living vertebrates opsin genes can be lost or spectral shifted.

1.3.1 Spectral Shift

The shift in wavelength in the same opsin class in different species depends on the interaction between the chromophore and opsin. The mechanism where pigments in the photoreceptors of the retina are tuned to different wavelengths has been investigated using pigments drawn from a large number of different species (Bowmaker et al. 2006; Carvalho et al. 2006; Hunt et al 1995; Hart and Hunt 2007). A shift could be due to the type of chromophore, which is either an 11-cis retinal or 11 cis 3,4, dehydroretinal to give

either a rhodopsin or a porphyropsin pigment respectively. The effect of 11-cis 3,4 dehydroretinal is to longwave shift the λmax of the pigment. Also the shift can be caused by a difference in the amino acid sequence.

Properties of the amino acids surrounding the Schiff base and polyene chain of retinal tend to significantly affect the amount of energy required to dissociate the opsin and chromophore to start the phototransduction cascade. Amino acid changes that are polar or charged can be responsible for relatively large changes. However, other changes further from the Schiff base and polyene chain of retinal can also cause spectral shifts. It is possible to determine which amino acid substitutions in a sequence cause the spectral shifts between pigments and to relate these changes to molecular interactions between the opsin and the chromophore.

An adaptive example of spectral shifts has been studied in cottoid fish of Lake Baikal in eastern Siberia (Hunt et al. 1996, Cowing et al. 2002). Shifts occur in fish adapted to different depths towards the shorter wavelengths deeper in the lake. The authors were able to identify the amino acid substitutions that account for these shifts. Site-directed mutagenesis was used to check that the shifts occurred because of the detected amino acid changes (Cowing et al 2002).

Figure 1.2. A schematic representation of the three-dimensional structure of a visual pigment, showing the opsin seven transmembrane domains (TMD I-VII), 3 extracellular domains (ECD I-III), 3 intracellular domains (ICD I-III), amino- and carboxy-termini (N or C), and the retinal chromophore. Circles represent amino acids. The grey circles represents sites of known structural or functional importance. The black circles represent residues shown to influence the spectral sensitivity of vertebrate pigments from Davies et al 2007b

1.4 The complexity and perfection of the vertebrate eye

Gordon Lynn Walls (1942) subtitled his comprehensive book on the vertebrate eye 'and its adaptive radiation'. He used this subtitle to emphasise, that the vertebrate eye 'has modified its pattern to fit itself for the many different kinds of performance demanded of it by its adaptively radiated owners.' (Walls 1942) The origins of eyes, and the ways in which they reached they present highly developed states have posed an

intriguing series of challenges for evolutionary scientists from Darwin onwards (Walls 1942, Dawkins 1986).

The complex anatomical features of the eye have been used historically, for at least 300 years, to be an example against evolutionary theory and to support the notion of an intelligent designer (Ray 1691, p261 Paley 1802, Behe 1996).

Living organisms, Paley argued, are even more complicated than watches. Only an Intelligent Designer could have created them, just as only an intelligent watchmaker can make a watch. The vertebrate eye was used as the first example in his book 'Natural Theology; or evidences of the existence and attributes of The Deity' to apply his argument (Paley 1802, Chapter 3).

Darwin (1859 pp143-146) under a section labelled 'Organs of Extreme Perfection and Complication' in 'The Origin of Species', stated his anxieties concerning the evolution of such a complex organ as the eye that is extremely specific to the purposes of judging distances, in different light conditions taking into account the spherical and chromatic aberration seemed 'absurd to the highest degree'. 'The complicated nature of the eye consisting of its many tissues working in harmony to focus an image using the lens and cornea on the retina consisting of large numbers of photoreceptors, whilst being kept moist and accessory is difficult to explain if one of these components did not exist'. (Darwin 1859 pp 143)

Darwin supposed that the evolution of the eye from a simple to complex form would not be subversive to the theory of natural selection if an eye could be shown at each gradation to be functional at some point throughout its development (Darwin 1859 p144-146).

Subsequently, evolutionists reduced the criteria of 'Perfection' and 'Complication' to explain the evolution of the eye. This was achieved by theorizing the number of steps or generations involved (Nilsson and Pelger 1994) and suggested that the eye does not require 'perfection' to be functional and confer advantages to an organism (Dawkins 1986).

In 'The Blind Watchmaker' Dawkins (1986) examines this problem of evolution of such a perfect instrument consisting of so many intricate components and explains that the vertebrate eye could be the result of natural selection and an accumulation of advantageous random mutations. He also explains that perfection is not required to convey advantages to a species. Poor eyesight e.g. shortsightedness, colour blindness or astigmatism is still a superior state than total blindness in an organism's survival ability to detect say a large predator or a potential mate.

Nilsson and Pelger (1994) showed in a simulation that in as few as 2000 small steps, each with a small fitness advantage would transform a photosensitive patch into a complex eye. The steps from simple light detection to complete vision could therefore be easily envisaged especially when the observed variety of eye forms that exist in different species is considered (Land and Nilsson 2001, Oakley and Cunningham 2002, Fernald 2004).

Currently even staunch supporters of Intelligent Design accept this step-wise gradual evolution of gross morphology of the vertebrate eye as a logical possibility (Behe 1996). However, creationists and/or followers of Intelligent Design (Kitzmiller et al. 2005) continue to try to find examples from nature to support their beliefs.

Heated debate persists as Intelligent Design proponents such as Behe have tried to

use biochemistry to convince the public about the use of intelligent design as a scientific theory (Forrest and Gross 2007, Behe and Snoke 2004). According to Behe, (1996) the complexity of light sensitivity at the molecular level and the minute biochemical reactions required for those first "simple patches of photoreceptor[s]" still defies explanation. Other examples of biochemical systems to seemingly defy Darwinian mechanisms are the vertebrate immune system, the blood-clotting cascade, and bacterial flagellum (Behe 1996). This argument is based on Irreducible Complexity theory (Behe 1996), which ascertains that certain biological systems are too complex to have evolved from simpler or 'less complete' predecessors through natural selection acting upon a series of advantageous naturally occurring chance mutations. The removal of any part of an irreducible complex biological system renders it functionless. 'Since natural selection can only choose systems that are already working, then if a biological system cannot be produced gradually it would have to arise as an integrated unit, in one fell swoop' (Behe 1996).

However, there are logical, factual and scholarly flaws (Forrest and Gross 2007) in the Irreducible Complexity theory that have been highlighted by scientists (Yeates 2007). One of these flaws is Behe's assumption that there is a single function that is permanently and unalterably associated with a particular biochemical system, or with a chemical structure (Behe and Snoke 2004). However, the functions of biological and biochemical systems can change throughout its history. This is known as 'preadaptation' or, using the more modern term, ' Exaptation' which is a character previously shaped by selection for a particular role (an adaptation) later co-opted by selection for a new and still current fitness role (Vrba 2002). Examples of exaptation are pleitropy as seen in

systematic genomics and Proteomics, especially amongst regulatory genes (Forrest and Gross 2007, King et al 2003). Additionally, many key cell signaling protein families have been shown to predate animal origins (King et al 2003). An additional argument against Irreducible Complexity is how complex macromolecules and macromolecular machines can arise has been established (Yeates 2007). Bridges between different protein folds can be demonstrated, that provide an important illustration of the kinds of dramatic steps that can be taken during molecular evolution (Yeates 2007).

This thesis will examine the evolution of the morphology and molecules involved in caecilian vision. The opsin gene family, which has undergone duplication and functional changes to exploit a range of wavelengths (Yokoyama 2000), the caecilian tentacle, which has co-opted parts of the eye (Chapter 3) and the reduction of elements of the caecilian eye, to be described later, could be seen as obvious examples that could refute Intelligent Design arguments of Irreducible Complexity.

1.5 Vertebrate Eye Structure

The vertebrate eye has many components derived from ectodermal and mesodermal tissues that constitute the refractive and photosensory tissues. The dioptric apparatus, of cornea and an epithelial lens, comes from the surface ectoderm. The fibrous sclerotic coat, lids, lachrymal system and bony orbit is from mesodermal tissues. The eyes of vertebrates consist essentially of a retina derived from neural ectoderm, a lens derived from the surface ectoderm, a uveal layer with a nutritive function, a protective tunic the anterior segment of which is transparent, and a dark chamber filled

with the vitreous body, the entire organ being encased in the orbital cavity and moved by a group of extra-ocular muscles (Duke Elder 1958, Walls 1942). All the variations in structure seen in the major classes within the phylum are incidental in nature and have evolved essentially as adaptations to differences in habitat or function (Duke-Elder 1958, Walls 1942).

Occasionally in abyssal fishes, cave-dwelling fishes and amphibians, and subterranean reptiles, mammals and caecilians the eyes have relatively degenerate features compared to phylogenetic close relatives (Described in more detail in chapter 3).

Amphibians pioneered the vertebrate colonization of the terrestrial environment. The transition from aquatic to terrestrial life was one of the major critical periods in vertebrate evolution (Herrick 1948).

1.5.1 Morphology of Amphibian Eyes

The requirement of aerial vision when amphibians left the water for the dry land were met by an optical reorientation of the aquatic eye to suit the new medium and the provision of lids equipped with elaborate glandular structures as a protection against drying (Reyer 1977). Accommodation in amphibians differs somewhat from fish due to the transition of from water to air. Terrestrial features are apparent in the more flattened shape of the lens, the more spherical shape of the eye (which does not need to be streamlined as in fishes).

Before describing the unique features of the caecilian eye (Chapter 3) it is important to appreciate the structure of a typical amphibian eye. Salamanders and Frogs

are generally thought to have well developed eyes, vision being a predominant sense (Dawley 1997) whereas in caecilians chemosensory and tactile sensory systems are used.

Figure 1.3 Diagram comparing amphibian eyes. Eye of a A Frog; B Salamander; and two caecilians C *Ichthyophis* cf. *kohtoaensis* D *Boulengerula boulengeri*; C=Cornea I=Iris L=Lens LM=Lenticular muscle N=Nictitating membrane OL=Upper lid P=Pigment Epithelial R=Retina UL=Lower lid Z=Zonule fibres (From Himstedt 1996).

Figure 1.3 shows the marked differences between eyes of the three orders of living amphibians. Anura are typically visually oriented predators, with a prey capture mechanism (projectile tongues) that depends on visual cues. Although some salamanders have independently evolved projectile tongue, prey capture is more commonly through jaw movement, with both vision and olfaction important in locating prey. Some salamanders are cave dwelling (e.g. *Proteus*) and some are aquatic throughout their lives (e. g. *Cryptobranchus*), often as neotenic forms with relatively small eyes (Grüsser-Cornehls and Himstedt 1976).

Disregarding the size of the eye, the frog eye and the newt eye are similar in structure (Reyer 1977) with some differences mentioned below. Both the frog eye and the newt eye have been extensively described (Walls 1942, Rochon-Duvigneaud (1943), Duke-Elder (1958), Reyer 1977). In the following section figures of the eye of *Triturus viridescens* will be used as an example of an eye exhibiting many of the typical gross morphological features of an amphibian.

Fig. 1.4a

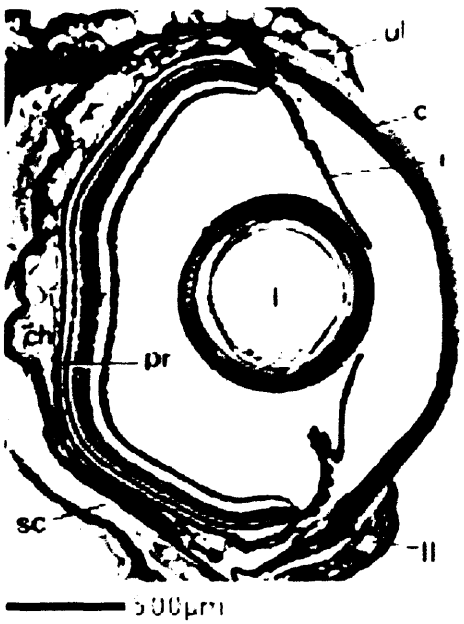


Fig. 1.4b

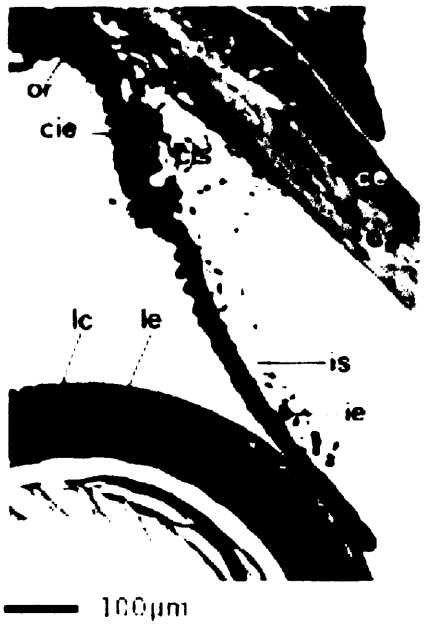


Figure 1.4a left Vertical meridional section of the eye of the newt, Notophthalmus viridiscens c=cornea, l=lens, I=iris, ul upper lid ll=lower lid, nr=neural retina, pr=pigmented retina, ch=choroid, sc =sclera.

Fig 1.4b right Detail of cornea, lens, dorsal iris, and ciliary body. (Masson's trichrome stain) lc=lens capsule, le=lens epithelium, is=iris stroma, cle =ciliary epithelium, cis=stroma of ciliary body, or=ora serrata, ce=corneal epithelium, cs=corneal stroma (substantia propria)

1.5.2 Fibrous tunic

The sclera and the cornea make up the fibrous tunic, the outer layer that completely surrounds the eye. The fibrous tunic provides essential support to the eye to resist the intraocular pressure and prevents any change in eyeball shape (Fig 1.4, Walls 1942, Smith 2000).

The cornea is the major refractive structure, especially in terrestrial animals, because of the high difference in density between the cornea and the surrounding air (Roth et al. 1998). The cornea is a transparent anterior continuation of the sclerotic coat, transparent due to the regular arrangement of collagen fibres (Smith et al. 1991). The bulk of the thickness of the cornea is the substantia propria. This layer is consists of lamellated fibrous connective tissue. There are no blood vessels but thread-like corpuscles that transport nutrients and remove waste to and from blood vessels surrounding the corneal margin (Walls 1942). Transparency is maximized by the lack of blood vessels in the cornea. The corneal epithelium, consisting of stratified epithelium cells, provides the outer surface of the cornea over the substantia propria. The corneal epithelium is continuous with the conjunctiva, which is in itself continuous with the skin and forms the linings of the eyelids. The innermost surface of the cornea is the corneal endothelium, which consists of non-dividing cells (Shimmura and Kawakita 2006). The endothelium is continuous with the iris and could therefore be considered the mesothelium of the anterior chamber (Walls 1942). The anterior chamber is the area anterior to the lens and posterior to the cornea. Between the endothelium and the substantia propria is the elastic membrane of Descemet (Walls 1942). Other cells that are

present are keratocytes and cells involved in the homeostasis of the cornea. Melanocytes, antigen presenting cells at the limbus area, nerve fibres and neural glial cells occur in the substantia propria (Shimmura and Kawakita 2006). Aquaporin 5 protein is also found in the cornea, the possible function of which is for water transport to prevent dehydration or to secrete watery products (Funaki et al 1998).

Collagen fibres in the sclera are organized into ribbon-like bundles, which provides strength and inelasticity.

The limbus (rim) area between the sclera and the cornea is the weakest part of the fibrous tunic due to the arrangement of the fibres. With relatively few cells in the sclera and low metabolism there is no need for a direct blood supply (Walls 1942). The extrinsic muscles that move the eye are attached to the sclera (Smith 2000ß).

In anuran tadpoles, an outer cornea of epithelium and connective tissue is present which is continuous with the skin of the head. Beneath this is a very thin, inner cornea continuous with the outer surface of the ciliary body. At metamorphosis, the outer and inner corneas fuse to complete the substantia propria of the typical cornea.

1.5.3 Uveal tract

The uveal tract consists of three major parts: the choroid, the triangular ciliary body, and the iris which extends in front of the lens (Fib 1.4 b Walls 1942; Duke Elder 1958; Smith 2000). The choroid is a heavily vascularized pigmented layer closely adherent to the sclera. The pigmentation prevents internal reflections and penetration of light through the eyeball into surrounding tissue and the high density of blood supplies the highly metabolic retinal layer. The retina lines the innermost layer of the uveal tract.

The photosensitive part of the retina (pars optica) covers the choroid and sclera. The retina continues anteriorly at the ora serrata, also known as the ora terminalis, with a much thinner epithelial layer, the pars caeca, which terminates at the rim of the pupillary aperture.

The ciliary body appears at the ora terminalis of the retina. It is not as highly vascularized or pigmented as the choroid, but is muscular on its inner surface anteriorly, and has a large number (70-80) of radially arranged, fin-like ciliary processes (Walls 1942). The retinal epithelial covering of the ciliary processes continuously produces aqueous humour in the anterior chamber, the anterior chamber is the area anterior to the lens and posterior to the cornea. The aqueous humour provides much of the intra-ocular pressure, rather than the more static, gelatinous vitreous humour made in the posterior chamber by the retina during development. It is thought that the low viscosity of the aqueous humour is advantageous, especially in fishes, in the facilitating of movement of the lens during accommodation. At the base of the ciliary body, zonule fibres extend to the lens capsule. They arise from the surface of the ciliary epithelium and their chief function is to support the lens (Walls 1942). Most of the fibres extend out to the anterior face of the lens at the equator, less extend to the posterior face at the equator and some run from one ciliary process to the other without joining the lens (Walls 1942).

The iris is the rest of the uveal tract with the non-photosensitive continuation of the retina that bends sharply away from the sclera at the corneal limbus (Walls 1942; Smith 2000). The filtration angle is the area of connective tissue at the periphery of the iris at the point of the corneal limbus. The canal of Schlemm occurs in the filtration angle in the fibrous tunic and drains excess aqueous fluid. The iris is an opaque disk of tissue

with a hole, the pupil in its centre (Walls 1942). Contractile elements in the iris regulate the size of the pupil to vary the amount of light that is focused onto the retina. Involuntary muscles make up some of the contractile elements in the iris and are organized into a ring-shaped 'sphincter pupillae' closely surrounding the pupil. The sphincter pupillae contracts to decrease the size of the pupil. The opponent of the sphincter is a complex consisting of the elasticity of the iris tissue, the radial blood vessels, and the myoid lamina 'dilatator pupillae'.

There are numerous ciliary processes in the frog eye but only a single, midventral ciliary fold in the newt eye. In the frog, both dorsal and ventral protractor lentis muscles pass from the corneoscleral junction through the ciliary body to insert on hypertrophied ciliary processes. When these muscles contract in accommodation, the lens is pulled forward by means of the zonule fibres. Only a ventral protractor lentis muscle, having a similar function, is present in the newt eye. The muscle originates from the corneoscleral junction, passes through the surface layers of the ciliary body and perforates the pigmented iris epithelium near its junction with the ciliary epithelium. The iris epithelium projects inward as a horizontal fold on either side of this cleft. The fold ventral to the muscle is continuous with the single ciliary process where the muscle terminates. From the ciliary body, zonule fibres pass to the lens capsule. Dorsally and ventrally, in both frogs and newts, smaller bundles of smooth muscle, the tensor choroideae, extend from the sclera in a meridional direction to insert on the choroid close to the ora serrata. In the stroma of the iris of the iris are located the iridial vessels and the sphincter and dilator pupillae muscles. There are also scattered melanophores and iridophores, giving the characteristic pattern of pigmentation.

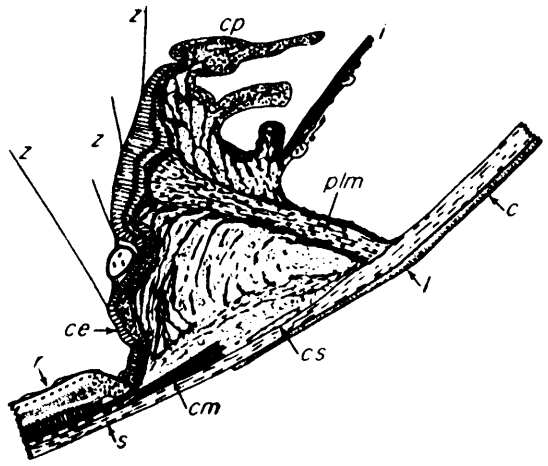


Figure 1.5 The ventral ciliary process and associated structures in a frog, *Rana pipiens* x50 (from Walls 1942) c=cornea; ce- ciliary epithelium, cm-ciliary muscle, cp-ciliary process, cs-canal of Schlemm; I-iris, l-limbus cornea, plm-protractor lentis muscle; r- sensory retina, s-sclera, z-zonule fibres.

Ciliary processes extend to the back of the iris to the papillary margin and form pupillary nodules. Their function may be to keep the iris away from the lens and thus allow the aqueous humour to flow backwards when the lens is drawn forward in accommodation. The mass of the triangular ciliary body is occupied by a meshwork of vascularized pigmented pigment tissue. Smooth fibres of the ciliary muscle run backwards to be inserted into the choroid. The protractor lentis muscle arises from the corneal margin, enters the ciliary triangle and is inserted into the hypertrophied ciliary processes (Walls 1942). Figure 3.2 shows the form of the ciliary region. In frogs there is typically a dorsal and ventral protractor lentis muscle. In salamanders there is only a ventral protractor lentis muscle and a dorsal suspensory ligament Walls, 1942).

1.5.4 Lens

The crystalline lens is present posterior to the iris and anterior to the membranous surface of the vitreous humour in the posterior chamber of the eye. The vitreous and the iris as well as the zonule fibres provide some support to the lens. The main body of the lens is made of long thin fibres arranged into layers like the coats of an onion. A membrane, the lens capsule surrounds the main body in a continuous elastic covering (Smith 2000).

The lens in amphibian larvae is spherical and approximates the cornea. In terrestrial adult amphibians the lens is displaced posteriorly leaving a deep anterior chamber and becoming somewhat flattened in the antero-posterior direction (Walls 1942). However there are exceptions. For example, adult *Triturus* exhibits considerable aquatic behaviour and have a spherical lens (Himstedt 1995).
Accommodation occurs by moving the lens as a whole rather than the shape of the lens being changed by ciliary muscles

1.5.5 Retina

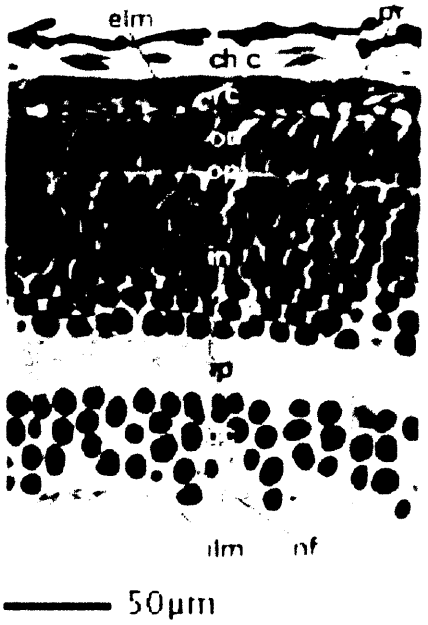


Fig 1.6 Neural and pigmented retina of *Triturus viridescens*. Ilm=internal limiting membrane, nf=nerve fibre layer, gc =ganglion cell loayer, ip =inner plexiform layer, in=inner nuclear layer, op=outer plexiform layer, on=outer nuclear layer, elm =external limiting membrane, rc =layer of rods and cones, pr=pigmented retinal epithelium, ch.c = chorio capillaries.

The innermost coat within the eyeball is the retina (Fig 1.4). The main layers that form the retina of a vertebrate eye are two plexiform layers, the inner and the outer nuclear layers, the ganglion cell layers, the outer segments and the pigmented epithelial. The outer segments of rod and cone photoreceptors contain the photosensitive pigments. The outer nuclear layer contains the inner segments of the photoreceptors and their nuclei. The two plexiform layers are the main sites of synaptic contacts between the retinal cells. Synapses form between the photoreceptors with the bipolar neurons in the outer plexiform layer. This layer is also where the synapses are formed between horizontal cells and the neighbouring rods and cones as well as with other horizontal cells. In the

inner plexiform layer the bipolar neuron terminal forms synapses with one or more ganglion cells and additional horizontal cells known as amacrine cells. The optic nerve is formed from axons coming from the ganglionic cells converge at one point which then pierces the choroid and sclera and makes its way to the brain.

The photoreceptor cells are of two general types namely the rods and cones (Fig 1.5 a and b,). The rods are associated with scotopic or dim light vision whereas the cones are used for daylight and coloured vision. This is due to specific visual pigments, namely the opsin proteins, that are associated with the rod and cone photoreceptors as well as the morphological properties of the photoreceptor cells themselves. However, these photoreceptor cells have essentially the same principal parts, the outer and inner segments, nucleus, and foot piece (Walls 1942). The outer segment is a long membrane-enclosed pile of discs. About 80% of the disk membrane protein is the visual pigment. The outer segment is connected to the inner segment by a connecting cilium. The inner segment has with an ellipsoid region that is mitochondrion rich and an inner zone, the myoid, of well-developed endoplasmic reticulum and golgi apparatus. These organelles are involved in the synthesis of the discs in the outer segments. The discs are constantly being renewed and then eventually digested by the pigment epithelium. Beneath this is an axon or fibre that expands to form the foot or spherule. The spherule has many synaptic vesicles sometimes organised into ribbons and has depressions to form a synaptic surface (Smith 2000).

Figure 1.7. Photoreceptor cells A Rod photoreceptor and B Cone photoreceptor cc = connecting cilium; e = ellipsoid; f = foot or spherule; m = myoid; n = nuclear region; is = inner segment; os = outer segment (from Smith 2000).

Cone photoreceptors (Fig 1.7 B) differ from rods in that the discs are continuous with the boundary membrane, the outer segment is relatively small and the discs are smaller towards the distal end, the inner segments and pedicle are large (Smith 2000).

Figure 1.8 on the next page illustrates the diversity of photoreceptor cells in frogs and salamanders.

Figure 1.8. Frog and salamander cone and rod photoreceptors. A single cone, double cone, red rod and green rod of leopard frog, *Rana pipiens*. b. A single cone, double cone red rod, and green rod of tiger salamander, *Ambystoma tigrinum*. (From Walls 1942)

There are commonly four types of amphibian photoreceptor cells, a red or green rod, and a single or double cone. The outer segment of the red rod is unusually large and the nucleus in contact with the external limiting membrane, a level generally occupied by the nuclei of cones (Walls 1942). The proportions of the rods, cones and double cones are correlated with relative degree of diurnal and nocturnal activity (Grüsser-Cornehls and Himstedt 1976). The green rod differs from the red rod in having a shorter outer segment and an elongate inner segment compared to red rods. The single cone has a much smaller, tapering outer segment compared to the red rod and a plump inner segment containing both an ellipsoid of mitochondria, less densely packed than in the rod, and a darkly staining oil or lipid droplet. An oil droplet is present in the former and lacking in

the latter (Mariani 1986). Differences have been noted between newt retinas and frog retinas is the presence of oil droplets, cytoplasmic extensions from the accessory member which clasp the principal member of the double cone to that of a rod, and more extensive areas of cell-to-cell contact among the different photoreceptors (Keefe 1971).

In amphibians, the pigmented retinal epithelium is characterized by long cytoplasmic processes which extend between the parts of the photoreceptors protruding beyond the external limiting membrane. In response to bright light, the pigment granules move into these processes, thus shielding the outer segments of the rods and cones. The reverse occurs in dim light. An extensive smooth endoplasmic reticulum is present in the pigmented retinal cells of the newt. Fragments of rod outer segments are also observed embedded in the cytoplasm of these cells.

1.5.6 Adnexa

The adnexa consist of the extrinsic eye muscle, eyelids and lachrymal system.

There are six extrinsic muscles that facilitate movement of the eyeball attached to the sclera. There are four rectus (superior, inferior, lateral and medial) and two oblique (superior and inferior) muscles. The muscles can be identified by the position and innervation. The oculomoter supplies the superior, inferior, medial rectus and inferior oblique extrinsic muscles of the eye, the trochlear nerve controls the superior oblique muscles and abducens supplies the lateral rectus muscle of the eye. In addition to the six extrinsic muscles that occur in a vertebrate eye, most amphibians possess a retractor bulbi and a levator bulbi that move the eye as a whole. The retractor bulbi muscle is probably derived from the external rectus, and the levator bulbi from jaw musculature (Duke-Elder

1958). If the eye is touched, the retractor muscle retracts the eyeball while simultaneously pulling the nictitating membrane over the cornea. The levator bulbi pulls the eyeball forward again and the lower lid falls back to its normal position. Eye retraction accompanies feeding and is likely an aid to swallowing food (Levine et al. 2004).

The eyelids are folds of skin that function in cleaning and moistening of the cornea. The lachrymal gland produces tear fluid. The gland lies against the superior temporal quadrant of the eye in the anterior part of the orbit. The tears are mixed with mucus secreted by scattered cells in the conjunctiva.

Eyelids and glands are adapted to protect the eye that is exposed to air rather than water. In the majority of amphibians that live their adult life on land, a short upper and lower lid develop during metamorphosis (Walls 1942). Amphibian larvae and aquatic adult frogs lack lids like fishes. Glands lubricate the eye. Primitive lachrymal glands exist on the temporal side of the eye. The Harderian glands are hypertrophied and occupy the nasal half of the orbit (Walls 1942).

1.5 Why study caecilian visual systems?

Caecilians visual evolution is of particular interest due to their fossorial and nocturnal habits and their long divergence times with the other amphibian orders. The establishment of the amphibian orders dates from the Triassic/early Jurassic, 200 to 300 million years ago (Roelants et al. 2007). This is in contrast with other rudimentary vertebrates with visual systems vertebrates which have had much less time since their divergence from their more visually orientated relatives such as the families of burrowing

mammals are thought to have diverged from other mammals in the Eocene between 10 and 40 million years ago (Nevo 1999). The evolution of the troglobitic *Astyanyx mexicanus* from the epigeal form is thought to have occurred between 2.2 –5 million years (Porter et al. 2007).

Relatively little of the research on amphibian visual systems has concentrated on caecilians (Roth et al. 1998) and yet the caecilian system has become of interest to phylogeneticists (Wake 1993a, 1994a; Wilkinson1997, Forey and Kitching 2000; O'Keefe and Wagner 2001). The complexity of the visual system provides many features for study of caecilian adaptation to the environment and phylogeny. The evolution of the visual system can be studied morphologically by examining the features of the eye of caecilians. Genetic adaptations of visual system proteins, specifically opsin, can be studied by inspection of the molecular sequence and physiology.

Chapter 2

Materials and Methods

This chapter details the molecular protocols and materials used to obtain results for Chapters 4 and 5. Other materials and methods required for morphological and phylogenetic analysis are detailed in Chapters 3, 4, and 5.

2.1 Materials

2.1.1 Specimens

- Tissues for DNA extraction kept in 70-100% Ethanol at -20°C
- *Ichthyophis cf. kohtoaensis* kindly supplied by Werner Himstedt Institute of Zoology, Darmstadt University of Technology for mRNA extraction.
- *Typhlonectes natans* specimens acquired through the pet trade used for in situ hybridization sections, and for mRNA extraction

2.1.2 Kits

- pGem®-T Easy vector system (Promega)
- High Pure PCR Product Purification Kit (Roche)
- Nucleic Acid Detection kit (Roche)
- DIG RNA labelling Kit (SP6/T7) (Roche)

- 5'3'RACE Kit (Roche)
- DIG High Prime DNA labelling and Detection Starter Kit II (Roche)
- Illustra Quickprep micromRNA purification kit (GE Healthcare)
- Qiaquick Gel extraction kit (Qiagen)
- Genelute HP Plasmid Maxiprep kit (Sigma)
- Genelute HP Plasmid Miniprep kit (Sigma)
- KOD XL DNA Polymerase (Novagen)
- SMART™ cDNA Library construction kit
- Gigapack III XL packaging extract

2.1.3 General Reagents and solutions

- 10 x NH_4 Reaction Buffer: 160mM $(NH_4)_2SO_4$, 670mM Tris-HCL (pH 8.8), 0.1% (w/v) Tween 20 (Bioline)
- 50 mM $MgCl_2$ (Bioline)
- dNTPs 100mM (Promega)
- Ethidium bromide (Fluka)
- Phenol (BDH)
- Chloroform (BDH)
- Isoamyl alcohol (BDH)
- Ethanol (100%) (BDH)
- SDS: Sodium Dodecyl Sulfate (BDH)
- 20x SSC: 3M NaCl, 25mM EDTA pH 8.0
- NE Buffer: 100mM NaCl, 25mM EDTA pH 8.0

- PBS (phosphate buffered saline)(Oxoid)
- Ammonium acetate 8M (BDH)
- Biotaq™ DNA Polymerase 5U/µl (Bioline)
- EcoR1 12U/µl fungal) (BDH Laboratory supplies)
- Ampicillin 50mg/ml (Calbiochem)
- X-gal 20mg/ml (Calbiochem)
- 1PTG: Isopropyl-Beta-d-Thiogalactopyranoside 100mM (Calbiochem)
- Hyperladder II (Bioline)
- Hyperladder I (Bioline)
- EDTA: Ethylenediaminetetraacetic acid- disodium salt (BDH)

2.1.4 Primers

Primers were synthesised by Sigma –Genosys unless supplied in kits

2.1.5 Electrophoresis Reagents

- Agarose (Bioline)
- 50x TAE buffer(National Diagnostics)
- Loading dye (Bioline)

2.1.6 Culture Media

- S.O.C (Super Optimal Catobolite repression): 0.5% Yeast extract, 2.0% tryptone, 10mM NaCl, 2.5mM KCl, 10mM MgCl2, 20mM MgSO4, 20mM glucose.

- LB (Luria Bertani) broth 1%(w/v)tryptone (Oxoid), 0.5%(w/v) yeast extract(Oxoid) ,1% (w/v) NaCl in ddH_2O
- LB agar: LB broth, 1.5% (w/v)bacteriological agar

2.1.7 Bacterial strains

- JM109 competent cells (Promega)
- XL1-Blue MRF'(Stratagene)
- SOLR™ (Stratagene)

2.1.8 Tissue Culture Cells, Media and Reagents

- Human embryonic kidney (HEK) 293 T human fibroblast cell line (European collection of cell cultures, Salisbury, Wiltshire)
- Dulbecco's Modified Eagle Medium with GlutaMAX I ™ (GibcoBRL, Life Technologies)
- Foetal calf serum (First Link (UK) Ltd).
- Penicillin (1000 u/ml)/Streptomycin (100 mg/ml) (GibcoBRL, Life Technologies)
- Trypsin-EDTA: 0.5g Trypsin, 2g EDTA per litre Modified Puck's Saline.

2.1.9 Additional reagents for Reconstitution of Opsin with 11-cis retinal.

- DDM: Dodecyl-maltoside: 2,1 and 0.1% (w/v) in 1x PBS.

- PMSF: Phenylmethylsuphonylfluoride (50mg/ml) in propan-2-ol
- 11-cis retinal (donated by Dr. Rosalie Crouch)
- Peptide 1: Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala (Sigma Genosys)

- Cyanogen bromide (CnBr)-activated Sepharose 4B (Pharmacia)
- Anti-1D4 mouse monoclonal antibody (NIH cell culture centre, USA)

2.1.10 Additional Southern blot reagents

- Neutralisation Solution:1.5 M NaCl, 1M Tris pH 7.5
- Denaturing Solution 1.5M NaCl, 0.5M NaOH

2.1.11 Additional in-situ hybridization reagents

- Prehybridisation and Hybridisation solution: 50% Formamide (BDH), 5M SSC, 50µg/ml tRNA (Invitrogen), 1% SDS, 50µg/ ml Heparin (Invitrogen), ddH_2O
- Solution 1: 50% Formamide (BDH), 5M SSC, 1% SDS, ddH_2O
- Solution 3: 50% Formamide (BDH), 1M SSC, ddH_2O
- 10x TBS: 1.4M NaCl, 27mM KCl, 250mM Tris-HCL pH 7.5, ddH_2O
- TBST: 1xTBS, 1% Tween-20
- NTMT: 100mM NaCl, 100mM Tris-HCl, pH 9.5, 50mM $MgCl_2$, 1% Tween 20 ddH_2O
- NBT stock solution: 75mg/ml in 70% dimethylformamide
- BCIP stock solution: 50mg/ml in 100% dimethylformamide

- Staining solution: NTMT, 4.5µl NBT stock solution, 3.5µl/ml BCIP stock solution

2.1.12 Additional cDNA library reagents

- 1M Mg SO_4(BDH)
- LB/ Mg SO_4 agar plates :10mM Mg SO_4, 15g Agar, 1 litre LB broth
- LB/ Mg SO_4 Broth: 10mM Mg SO_4, 1 litre LB broth
- Top agar: 10mM Mg SO_4, 7.5g Agar, 1 litre LB broth
- λ dilution buffer: 1M NaCl, 0.1 M Mg $SO_4.7H_20$, 0.35M Tris-HCL (pH 7.5)

2.2 Methods

2.2.1 Microspectrophotometry (MSP)

A modified Liebman dual beam microspectrophotometer under computer control was used to make measurements from photoreceptors. With the help of an infrared converter, the measuring beam (normally 2µm square cross-section) was aligned to pass transversely through the photoreceptor outer-segments or piece of skin while the reference beam passed through a clear space adjacent to the object examined. Spectra were scanned from 750 to 350nm in 2nm steps and back from 251 to 749 nm at the interleaved wavelengths. Only one absorption spectrum was usually obtained from a given outer segment to minimize the effects of bleaching. To verify the presence of a photolabile pigment, putative outer segments were bleached by exposure to white light

from the monochromator passing through measuring beams of the microspectrophotometer.

A standardized computer program was used to estimate the λmax for each outer segment. The spectra obtained from the outer segments were averaged to obtain a mean curve. The curve was then referred to a standard template curve in order to obtain an estimate of λmax. The standard template curve used was the Dartnall standard curve for rhodopsin placed with its λmax at 502nm and expressed on an abscissal scale of log frequency (Govardovskii et al. 2000).

2.2.2 Nucleic Acid Extraction

2.2.2.1. mRNA isolation

The QuickPrep™ micro mRNA Purification kit (Amersham Biosciences) was used to directly synthesise polyadenylated RNA (mRNA) from small amounts of tissue.

The protocol provided by the manufacturers of the kit was followed. Three eyes from *Ichthyophis* cf. *kohtoaensis* and 10 eyes from *Typhlonectes natans* were used in separate reactions. The eye tissue was homogenized using a small sterile pestle, cleaned in 100% ethanol, which fitted in an eppendorf tube containing the tissue and extraction buffer. The extraction buffer contained guinidium thiocyanate which quickly inactivates endogenous RNases. The extract was then diluted in elution buffer (10mM Tris-HCl, pH 7.5 and 1mM EDTA) to reduce the concentration of guinidium thiocyanate so binding of RNA to oligo(dT) attached to the cellulose can occur in the next step. The extract were initially purified by brief centrifugation which pelleted contaminating proteins that are

precipitated during the three-fold dilution. The supernatant was then transferred to a microcentrifuge tube containing oligo(dT) –cellulose, mixed for 3 minutes by inverting the tube then centrifuged for 10 seconds at top speed. The supernatant was then removed. The pellet of oligo dT cellulose with mRNA was washed several times in high (10mM Tris HCl, pH 7.5, 0.5 M NaCl and 1 mM EDTA) and low salt buffers (10mM Tris HCl, pH 7.5, 0.1 NaCl and 1mM EDTA)before the mRNA was eluted in 0.2 ml of elution buffer prewarmed to 65°C.

2.2.2.2. Quantification of mRNA and DNA

The quantity of DNA was determined by spectrophotometry by using the formula

[mRNA]=A_{260} x 40μg/ml

[DNA]= A_{260} x 50μg/ml

2.2.2.3 Reverse transcription- Polymerase Chain Reaction (RT-PCR) to create cDNA

Complementary DNA (cDNA) is synthesized from a mature mRNA template in a reaction catalysed by the enzyme reverse transcriptase.

The First strand cDNA was synthesized in a reaction using 5 μl Oligo dT or Random 6-mer / 9-mer (500 ng) or for 3' RACE the primer (5'GCGAGCACAGAATTAATACGACTCACTATAGGT_{12}VN-3'), 10 μl (1 – 2 mg) mRNA, 15.5 μl water. The final volume was 30.5 μl. The tube was incubated at 85°C for 15 minutes and chilled on ice for 2 minutes to denature and remove secondary structures from the mRNA.

10 µl of 5x First strand buffer (Invitrogen), 5 µl 0.1M DTT, 2.5 µl dNTPs mixture (10 mM of each nucleotide) and 1 µl of RNaseOUT inhibitor (Invitrogen 40 U/ ml) were added to the tube and incubated at 25°C for 2 minutes. Then 1 µl Superscript II Reverse Transcriptase (Invitrogen) was added, incubated at 25°C for 5 minutes, followed by 42°C for 2 hours.

1 µl RNase H (NEB) was then added to remove template DNA. The solution was then incubated at 37°C for 10 minutes, then 55°C for 10 minutes.

2.2.2.4. Extraction of gDNA from Liver

This procedure extracted high concentrations of intact genomic DNA for Southern blotting and PCR with a modified phenol-chloroform extraction method.

Liver tissue that had been stored at –80°C was weighed and ground up in liquid nitrogen using a pestle and mortar. 5ml of ice cold NE buffer (100mM NaCl, 25mM EDTA, pH 8.0) was added, and spun at 4000 g for 7 minutes in a Beckman J2-21 centrifuge at 7°C. The supernatant was removed and the pellet resuspended in another 5ml NE buffer before being spun again. This was repeated 3 or 4 times until the supernatant was clear. The pellet was resuspended in 5ml NE buffer, with the addition of proteinase K, to digest proteins, and SDS, to lyse the cells, at concentrations of 400µg/ml and 10% respectively and incubated overnight at 50°C. The DNA was extracted from this solution by mixing gently with an equal volume of buffered phenol/chloroform (BDH) for 20 minutes then spinning at 5000g/ 10 minutes. The aqueous layer was aspirated and then mixed and spun again with the buffered phenol/chloroform. The aqueous layer was removed and mixed twice with an equal volume chloroform isoamyl (24:1 chloroform: isoamyl

alcohol) for 10 minutes and spun for 10 minutes at 5000g. The gDNA was then precipitated by addition of 5M NaCl to a final concentration of 0.4M plus 2 volumes ethanol, mixed gently for 2 minutes and spooled into a fresh tube. The DNA was then dissolved overnight in 1ml ddH_20 at 4°C, then precipitated using 500 µl 8M ammonium acetate plus 6 volumes ethanol. This tube was mixed gently and the DNA removed to a fresh tube, washed in 70% ethanol, dried and dissolved in 1ml ddH_20.

2.2.3 DNA amplification

2.2.3.1 Polymerase Chain reaction (PCR)

Polymerase Chain reactions were performed to selectively amplify regions of DNA from gDNA or cDNA templates

The reaction mixture typically contained 10mM dNTPs, 2µM $MgCl_2$, 0.1µM each of the forward and reverse primers, 2.5 U Biotaq DNA polymerase and between 20ng to 100ng cDNA or gDNA in a total volume of 50µl. Cycling conditions were an initial denaturing step at 94°C for 5 minutes, then 35 cycles of denaturing at 94°C for 30 seconds, annealing for 1 minute and extension at 72°C for 1minute 30 seconds with a final extension at 72°C for 10minutes. Negative controls were run in parallel containing the same reagents but without the addition of DNA. Temperature gradients, where the annealing temperature was varied across the PCR block, were applied to duplicate samples to obtain optimal conditions for amplification of target sequences.

2.2.3.2 Gel electrophoresis of PCR fragments

On completion 25µl of PCR reaction with 5µl of loading dye was run on a 1-2% (w/v) agarose gel in 1x TAE buffer containing ethidium bromide at 1µg/ml. The agarose gel was prepared by melting agarose powder in buffer in a microwave. The molten agarose was poured into a mould and cooled with a comb to make wells for the PCR samples. 5µl of DNA ladder, Hyperladder I or Hyperladder II was run for size determination of DNA fragments. The gel was then viewed on a UV transilluminator and a digital image taken using a digital camera.

2.2.3.2 DNA Extraction from electrophoresis gel.

If a band was visualized of the expected size using gel electrophoresis, it was cut out using a sterile blade. The Qiaquick gel extraction kit (Qiagen) was used to extract the DNA. The gel fragment was melted in a low pH solution (3 x volume of agarose block in solution QG) then applied to a silica spin filter column which removes the agarose using centrifugation (17900 g for 1 minute). The bound DNA was then washed of impurities by centrifugation at 17900 g for1 minute with 750µl solution PE. The column was spun again before addition of 25 µl of ddH_20 for elution.

2.2.3.4 Rapid Amplification of cDNA Ends (RACE).

RACE is a PCR based technique that facilitates the cloning of full length cDNA sequences when only a partial cDNA sequence is available.

2.2.3.4.1 3' RACE.

Takes advantage of natural poly (A)-tail of mRNAs as a priming site for PCR amplification. The primer used in first strand synthesis is an anchored poly (T) adapter. This cDNA produced in the above protocol for first strand synthesis can be used for 3'RACE. 3' RACE uses a nested P.C.R protocol to isolate the sequence. A PCR reaction mixture was made as described in section 2.2.3.1 but containing 1μl of first strand 3' RACE cDNA, 1μl 10mM Outer primer designed to adaptor (5'-GCGAGCACAGAATTAATACGACT-3'), 1μl 10mM 3' Outer gene specific primer. The PCR reactions were thermocycled as before. A second round of PCR was set up with a 1/10 dilution of the PCR product, Inner primer (5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3') and Inner gene specific primer.

2.2.3.4.2 5'RACE.

The 5' end of the visual pigment gene was amplified using the 5/3 RACE kit (Roche). First strand synthesis is was with a gene specific primer, tailed with a adaptor of known sequence at the 3'end of the cDNA before nested PCR with gene specific primers and primers designed to the adaptor.

5μl of mRNA, 4 μl cDNA synthesis buffer, 2μl dNTPs, 1μl cDNA gene specific primer (AAG TTG CTC ATG GGT TTA CAG ACC ACT ACG)(12.5μM), 1ul of reverse transcriptase and 5μl of ddH_20 was added to a sterile PCR tube. The tube was incubated for 60 minutes at 55°C and for 5 minutes at 85°C then spun briefly to collect tube

contents at the bottom of the tube. The cDNA was then purified using the High Pure PCR product Purification kit (Roche). All centrifuge spins were at 8000g. The cDNA was mixed gently with 100µl of Binding buffer and transferred onto a filter column in a 2ml collection tube. The tube was spun in a centrifuge for 30seconds and the flowthrough discarded. 500µl of wash buffer was added to the column and spun for 30 seconds. The eluate was discarded and 200µl of wash buffer was added. The tube was then spun for 2 minutes at 17900 g to ensure the filter was dry before elution. The filter column was placed in a fresh tube. The cDNA was eluted by spinning the tube at 8000g for 30seconds with 50µl of Elution buffer.

A known sequence is added (tailed) on to the 3'end of the first strand cDNA. A homopolymeric tail is appended using Terminal Transferase and ATP. A reaction mixture of 19µl of cDNA sample, to 2.5 µl of reaction buffer and 2.5 µl of 2mM dATP was made. The tube was incubated at 94°C for 3 minutes and chilled on ice. 1µl of Terminal Transferase (80Units/µl) was added and incubated at 37°C for 20 minutes. The tube was incubated at 70°C for 10 minutes to heat inactivate the enzyme. The tube was spun and placed on ice ready for PCR amplification.

A PCR reaction for 5' RACE (5µl of the tailed cDNA, 1µl of Oligo T anchor primer (12.5mM) , 1µl of gene specific primer (12.5 mM), 1µl of dNTP, 1µl of 1/10 dilution Biotaq, 5µl of reaction buffer and 36µl of ddH_20) was cycled in a thermal cycler (94°C for 2minutes 35x(94°C for 15 seconds, 60°C for 30 seconds, 72°C for 40 seconds) 72°C for 7 minutes). A second round of nested PCR was set up with a 1/10 dilution of PCR

product and inner gene specific and anchor primers. The product was visualized by gel electrophoresis, gel extracted and cloned into pGEM T-easy vector before sequencing.

2.2.4 Cloning into vector and Transformation of bacterial cells.

Individual PCR products were ligated into a vectors, transformed into bacterial cells. Screening of positive colonies used the blue-white detection system.

2.2.4.1 Ligation of pGEM T-Easy (Promega) vector and PCR product.

The ligation reaction consisted of 4μl of PCR product, 5μl of ligation buffer, 0.5μl of pGEM vector and 0.5μl of T4 ligase. The reagents were mixed gently using a pipette and either left for 1hour at room temperature or left overnight at 4°C to ligate.

2.2.4.2 Ligation into pMT4 vector.

The pMT4 vector ligation used a higher ratio of PCR product to vector (7μl of digested PCR product, 1μl 10x ligase buffer (Promega), 1μl T4 DNA ligase, 1μl pMT4 vector).

2.2.4.3 Transformation of cells.

The ligation mixture was briefly pulsed in a centrifuge to mix. 25μl of JM109 High Efficiency competent cells (Promega) were removed from -80°C storage and placed on ice to thaw for 5 minutes. 1μl of ligation mix was placed into 1.5ml tube. 25μl of competent cells, flicked to mix, was placed into the tube with ligation. The tube was then flicked to mix the ligation and cells. The tubes were left on ice for 20 minutes. The tube was then placed in a water bath at 42°C for 45-50 seconds, then placed on ice for 2

minutes. This 'heat-shocks' the cells so that the plasmid is taken up by the bacterial cells. 450ul of room temperature S.O.C is added to the tube. The tube was then placed in a shaking incubator at 37°C for 90 minutes.

2.2.4.4. Plating and screening of colonies by blue-white detection.

The transformed cells were plated on LB agar plates containing 50μg/ml ampicillin, 20μg/ml IPTG and 20μg/ml X-gal. The plates were incubated overnight at 37°C. The following morning the plates were placed at 4°C for at least 30 minutes. The plasmid is present cells that will be able to grow in the presence of ampicillin. The plasmid infers ampicillin resistance so if present, cells are able to grow in the presence of antibiotic.The IPTG present in the agar induces β-galactosidase production, which when present breaks down X-gal producing a stable dark blue product. The cloning site within the plasmid is in the coding sequence for β-galactosidase. A cloned fragment interrupts this gene and prevents the production of β-galactosidase. Colonies that contain an insert do not produce β-galactosidase enzyme and are white.

2.2.4.5 Isolation of plasmid DNA from 5ml overnight culture (Miniprep)

The GenElute plasmid miniprep kit (Sigma) was used for the isolation of plasmid with the insert from the JM109 bacterial cells grown in 5ml of overnight cultures. The kit uses a modified alkaline-SDS lysis procedure followed by absorption on to silica in the presence of high salts. Contaminants are removed by washing with a solution containing ethanol. The DNA is eluted from the silica column with ddH_20. Bacterial cells were pelleted from the overnight culture in a centrifuge 5000 g, 4°C for 10 minutes. The

supernatant was removed. The pellet was resuspended in 200µl Resuspension solution containing RNase A to remove contaminating RNA and transferred to a 1.5ml tube. The cells were lysed by addition of 200µl of Lysis solution. The tubes were inverted 6 times before the reaction neutralized by addition of 350µl of Neutralization buffer. The tube was then spun at 17900 g for 10 minutes. Cell debris, proteins, lipids, SDS and chromosomal DNA form a pellet. The supernatant contains the plasmid with insert. The column in a collection tube was prepared by addition of 500µl of Column Preparation Solution and spinning at 17900 g for 1 minute. The cleared lysate was added to the column and the spun for 1 minute at 17900 g. The flow-through was again discarded. DNA was now bound to the column at this stage. 500µl of Optional Wash was added to remove nuclease contamination and spun for 1 minute at 17900 g. The flow-through was discarded and 750µl of the Wash solution was added to the column. The column was spun at 17900 g for 1 minute. The flow-through was then discarded. The column was respun at 17900 g for 1 minute to remove excess ethanol. A new sterile collection tube was used for the final elution spin at 17900 g for 1 minute using 100µl of ddH_20.

2.2.4.6.Maxiprep of high quantities of plasmid.

To achieve a higher yield of plasmid for the regeneration of visual pigments maxipreps were used 500µl of overnight was used to inoculate sterile 250ml of LB in a conical flask. The new culture was incubated overnight in a shaking incubator at 37°C. Isolation of the plasmid is again using a kit, GenElute Plasmid Maxiprep Kit that uses a modified alkaline-SDS lysis procedure followed by adsorption of the DNA on to silica in the presence of high salts. The cells were pelleted at 5000 g for 10 minutes in 50 ml conical

tubes, supernatant removed and pellets resuspended in 6ml of Resuspension solution. 6ml of Lysis buffer was added and the tube inverted 6 times. 6ml of Neutralization solution was added to the tube and inverted to form a precipitate of cell debris, proteins, lipids, SDS and chromosomal DNA. The tube was spun at 17900 g for 10minutes to pellet the precipitate. The GenElute Maxiprep binding column, in a 50 ml collection tube, was prepared by applying 12ml of Column preparation solution and spinning at 3000g for 1 minute. The eluate was discarded. The supernatant from the cleared lysate was transferred to the binding column and spun at 3000g for 1 minute to bind the DNA to the silica column. The flow through liquid was discarded. 8ml of Optional wash solution was added to the column and spun at 3000g for 1 minute. The eluate was discarded. The column was washed with 15ml of Wash solution spun at 3000g for 1 minute. The flow through discarded and the tube spun again to remove ethanol from the column. The DNA was eluted in a sterile 50ml tube with 5ml of ddH_20 spun at 17900 g for 1 minute.

2.2.5 Southern blot

Southern blotting is a method for identifying the presence of known DNA sequences (genes) in DNA. The DNA to be probed is digested with restriction enzymes, denatured and bound to a membrane. The membrane is probed with a piece of the gene attached to an antigen that reacts with a chemiluminescent antibody, which can be viewed on exposure to photographic film.

2.2.5.1. Preparation of Gel for Southern Blotting

Genomic DNA was restriction digested (reaction mixture 5U EcoR1, 5μl of buffer, 0.5 μl BSA and genomic DNA) overnight at 37°C. A low percentage agarose gel (1%) was prepared. The gel was loaded with Hyperladder 1 and digested DNA and run overnight at low voltage to ensure separation of fragments. The ladder section of the gel was then cut off, soaked in Ethidium bromide solution for 5minutes and placed next to a ruler and photographed using a transilluminator to visualize the bands. This was then used to determine the size of the band containing the gene of interest. The rest of the gel was depurinated with 0.2N HCL to nick any fragments larger than 25kb so as to improve membrane transfer. The gel was then denatured for 45 minutes then rinsed again with fresh Denaturing solution. The denaturing solution was changed every 15 minutes. The gel was then neutralised for 45 minutes with Neutralization solution. After 30 minutes fresh solution was added. The gel was then ready for transfer of the DNA to the membrane.

2.2.5.2. Southern Blotting Transfer of DNA to membrane.

A thick piece of Whatman 3MM blotting paper was placed on a sheet of glass to form a support that was longer and wider than the gel. The ends of the blotting paper draped over the edges of the plate. The support was placed inside a large baking dish. The dish was filled with 20xSSC until the level of the liquid reached almost to the top of the support. When the blotting paper on the top of the support became wet, air bubbles were smoothed out with a glass rod or pipette. The gel was placed on the support so that it centred on the wet blotting paper, making sure that air bubbles were not trapped in

between. The gel was surrounded but not covered by Saran wrap to prevent 'short circuiting' of buffer flow. 20xSSC solution was placed on top of the gel to wet it. A piece of positively charged nylon membrane (Hybond N+, Amersham) was cut to the shape of the gel. This membrane was then placed on top of the gel so the corners aligned whilst avoiding making air bubbles. Four pieces of blotting paper (Whatman 3MM) cut to the size of the gel were placed on top of the wet membrane. A stack of paper towels the size of the gel were placed on top of the blotting papers. A glass plate was placed on top of the stack with a suitable weight. The transfer of DNA to membrane was left to proceed overnight. The paper towels and blotting paper were then removed and the membrane placed on a piece of dry blotting paper. The orientation points and gel slots were marked on the membrane. The gel was then stained with Ethidium bromide in ddH20 to determine the efficiency of transfer. The DNA was then fixed to the membrane by baking at 120°C for 30 minutes.

2.2.5.3. Construction of probes.

1 μg of 200-300bp gene fragments were labelled using DIG High Prime DNA labelling and detection kit(Roche). This kit randomly labels when a dUTP is incorporated in the probe sequence. The fragment was first denatured by boiling for 10 minutes and then placed on ice. 4μl of DIG High Prime (contains random primers, nucleotides, DIG-dUTP, Klenow enzyme and buffer) was added and incubated overnight at 37°C. 0.2M EDTA at pH8 was added and heated to 65°C for 10 minutes to stop the reaction.

The efficiency of the labelling reaction was determined by preparation of a dilution series of the probe and control supplied with kit. 8 dilutions were prepared with decreasing concentration from 10pg/µl to 0.01 pg/µl. 1µl dots of each dilution was placed on a positively charged nylon membrane (Hybond N+ by Amersham) with 1µl of ddH_20. The membrane was placed on blotting paper, wrapped in foil and baked at 120°C for 30 minutes to fix DNA to membrane. The DIG High Prime DNA labelling and detection kit (Roche) was used to detect efficiency of the labelled probed. The membrane was placed in a box with 20ml Maleic acid buffer and placed on a shaking platform for 15minutes. The buffer was poured away then 10ml of blocking solution added and incubated for 30 minutes. The blocking solution was poured away then 10ml of antibody solution was added. This was washed twice for 15minutes in 10ml washing solution to remove excess antibody. 10ml of detection buffer was added to adjust the pH and incubated for 5 minutes. The membrane was placed in a plastic hybridization folder. 0.1ml of 'CSPD ready-to-use' was distributed over the membrane and immediately covered with the second sheet of the folder. Air bubbles were squeezed out before sealing. The membrane was incubated in the chemical solution for 5 minutes before exposing to x-ray film for up to 20 minutes.

2.2.5.4 Hybridization of probe to Southern blot membrane.

10ml per100cm^2 of the Southern blot membrane of 'DIG Easy Hyb' was heated to the hybridization temperature 37°C. The membrane was prehybridized by addition of this pre-heated solution and incubating for at least 30 minutes at 37°C in a hybridization oven. 25ng of probe per ml of prehybridization solution was denatured in a boiling water

bath for 10 minutes, then rapidly cooled in ice. The probe was added to the preheated hybridization solution (3.5ml/100cm^2 of membrane). The prehybridization solution was poured off the membrane and the probe mixture added. The membrane was incubated overnight at 37°C.

2.2.5.5. Stringency Washing.

The membrane was placed in a box and washed twice at room temperature in wash solution 1 (2xSSC, 0.1% SDS) and placed on a rotary platform shaker. Wash solution 2(0.5 x SSC, 0.1 %SDS) was prewarmed to 65°C. The membrane was washed twice in prewarmed wash solution 2 and placed in a shaking incubator at 65°C.

2.2.5.6. Immunological detection

Immunological detection occurs at room temperature using the Nucleic acid detection kit (Roche). The Southern blot membranes were processed using the same protocol that was used to detect the efficiency of the probes but with different volumes. After stringency washing, the membrane was rinsed in Washing buffer for 2 minutes. The buffer was poured away then 100ml of blocking solution was added and incubated for 30 minutes. The blocking solution was poured away then 20ml of antibody solution was added. Then washed twice for 15minutes in 100ml washing solution to remove excess antibody. 20ml of detection buffer was added to adjust the pH and incubated for 5 minutes. The membrane was placed in a plastic hybridization folder. 1ml of 'CSPD ready-to-use' was added to the membrane and immediately covered with the second sheet of the folder. Air

bubbles were squeezed out before sealing. The membrane was incubated at 37°C for 10 minutes in the chemical solution before exposing to x-ray film for up to 20 minutes.

2.2.6 In situ hybridization of *Typhlonectes* eye sections.

Localization and detection of genes to photoreceptors was attempted using fragments firstly of rod opsin isolated from *T. natans* and cone visual pigments from *Xenopus laevis* to sections of eye using in-situ hybridization techniques.

2.2.6.1. Crysectioning

The head of *Typhlonectes natans* was skinned so the eyes were exposed. 1-2 days after fixing in 4% PFA solution, the head was decalcified in a saturated solution of EDTA for 4 days. The heads was cryoprotected in sterile PBS buffer and 4% sucrose at 4°C overnight

The head was placed in a mould with O.C.T. embedding resin (Raymond Lamb laboratory supplies). The resin was frozen by immersion of the mould in acetone cooled with dry ice. The head was sectioned to a 14 μm thickness in a Cryostat (Leica CM1850) at -20°C. The sections were stored at -80°C until ready to probe.

2.2.6.2. Creation of riboprobes

The riboprobes were created using the DIG RNA labeling kit (SP6/T7)(Roche). The kit incorporates digoxigenin-UTP by in vitro transcription with SP6 and T7 RNA promotion to generate sense and anti-sense probes. The PCR product was cloned into pGEM vector. Linearization of the vector and insert was accomplished through cutting at a restriction

enzyme site downstream from the cloned insert. The restriction enzyme used was EcoR1 to produce the sense strand and Sal1 to produce the antisense strand. The antisense strand is complementary to the gene and should hybridize with mRNA in the section. The sense strand is the same as the gene and should not hybridize to the transcipt and acts as the negative control. The products from the restriction digest were cleaned and precipitated using a phenol-chloroform extraction.

1μg of digested DNA was added to a sterile 0.2ml tube and made up to 13μl using ddH_20. The tube was placed on ice. Then 2μl of10x NTP labeling mixture, 2μl of 10x transcription buffer, 1μl of Protector RNase inhibitor, and RNA polymerase SP6 or RNA polymerase. The reagents were mixed gently using a pipette, and a centrifuge. The tubes were incubated for 2h at 37°C. 2μl of DNase I, was then added and incubated for 15 minutes to remove template DNA. 2μl of 0.2M EDTA (ph8) was added. The RNA transcripts were then analyzed by gel electrophoresis. A dilution series and spot test was performed similar to the probes produced for the Southern blot to measure the labeling efficiency.

2.2.6.3 Probing Cryosections with Labeled Gene Fragment.

The slides were thawed for 30 minutes. All pretreatment before hybridization occurred at room temperature. The slides were transferred to a slide holder, which could contain 5 slides, and 15ml of PBS was added and incubated for 5 minutes. 5 slides were used for the antisense reaction and 5 slides for the sense reaction. Each slide contained between 8-10 sections of eye. The PBS was poured off from each slidemailer and 4% PFA solution was added to prevent RNA degradation and left for 15 minutes. The PFA was

poured off, PBS added to the slide mailers and incubated for 5 minutes. The PBS was poured off and a proteinase K solution added (proteinase K 5μg /ml in PBT) to inactivate any enzymatic activity that may degrade the mRNA. The proteinase K enzymatic activity was stopped by pouring off the solution from the slide holders then incubating in 2mg/ml glycine solution in PBT. The glycine was then removed, washed twice with PBT for 5 minutes. The slides were then postfixed in 4% PFA for 20 minutes.

2.2.6.4. Hybridizing of probe to sections

The hybridizing solution was heated to 65°C in a water bath, added to the slidemailers and incubated for 30 minutes. 10μl/ml antisense and sense probe suspended in hybridization solution were denatured at 85°C. The slides were transferred to a plastic sandwich box containing filter paper soaked in 50% formamide solution in ddH_20. 85μl of probe solution was added to each slide and covered with a plastic coverslip. The slides were incubated overnight at 65°C.

Post hybridization washes and antibody hybridization were carried out as follows. Solution 1 and Solution 3 were heated in water bath set to 65°C and 60°C respectively. The coverslips were removed and the slides placed inside the slide mailers. The slides were washed 3 times in solution 1 for 20 minutes each time at 65°C. Solution 1 was removed and then washed 3 times for 20 minutes in solution 3. Solution 3 was removed and the slides washed at room temperature in TBST 3 times for 5 minutes at a time. The slides were removed from the slide mailers and dried around the sections. The sections were circled with a PAP pen, which prevents liquid from going over the edges of the slide. The slides were transferred to a large square dish covered with foil. 1ml block

solution was placed on each slide for 30 minutes to prevent unspecific binding of antibody. 1 in 2000 dilution of anti-DIG-Ab (Roche) was prepared in 1% sheep serum in TBST. 85µl of antibody dilution was added to each slide and covered with a plastic coverslip. The slides were incubated for 2 hours at room temperature then placed at 4°C overnight.

2.2.6.5. Post Antibody wash and Chemiluminescent reaction.

The coverslips were removed and the slides transferred to slide holders and washed 4 times for 15 minutes in TBST. NTMT solution was added and incubated for 10 minutes. This wash is repeated twice. The slide mailers were covered in foil to optimize the chemical reaction.

2.2.7 Reconstitution of Opsin with 11-cis retinal

When the full coding sequence of an opsin gene had been identified, an expression construct was made and recombinant proteins were expressed using a mammalian cell line. The opsin protein was then isolated and regenerated in the dark with 11-cis retinal. Finally absorbance spectra were obtained by the use of a spectrophotometer. Dark and bleached spectra were obtained.

2.2.7.1. Amplification of full length opsin with modified primers.

The full length opsin sequence was amplified using KOD XL DNA polymerase kit (NOVAGEN). This kit incorporates a proof-reading polymerase which results in a low rate of mutation caused by the PCR reaction. Modified primers were designed to include

restriction enzyme sites to enable cloning into the expression vector pMT4. The forward primer was designed using an EcoR1 site before the 5' end of the caecilian opsin gene. The reverse primer contained a Sal I site, the 3'end of the gene at the position of the stop codon.

The KOD PCR reaction (1μl first strand cDNA, 5μl 2mM dNTPs, 2μl 25mM $MgSO_4$, 1μl 12.5mM forward primer, 12.5mM reverse primer, 5μl 10x KOD buffer, 1μl 2.5U/μl KOD Polymerase) was thermocycled (94°C 2minutes, 35x(94°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute. 72°C for 5minutes). The product was then ligated into pMT4, transformed, cultured and maxipreped to produce a high yield of plasmid.

2.2.7.2. Transfection of HEK 293T Cells with pMT4/Opsin

All tissue culture work was carried out in a microflow safety cabinet. HEK 293T cells were grown for 3 days in DMEM medium with 10% foetal calf serum and penicillin/streptomycin (DMEM/FCS/Pen/Strep). The medium was removed and the cells gently washed with 5ml of PBS. PBS was removed and 1ml of trypsin in EDTA was added. The flask was incubated for 5 minutes at 37°C and 5% carbon dioxide for 5 minutes. The trypsin dissociated the cells from the flask. The flask was tapped to dislodge the cells and the cells were pippetted up and down approximately 30 times to mix. The cell suspension was divided and transferred to 11 tissue culture flasks containing 13 ml DMEM/FCS/Pen/Strep solution prewarmed to 37°C. The cells were incubated for 2 days. The cells were dislodged with trypsin and transferred to 25ml of DMEM/FCS/Pen/Strep prewarmed to 37°C. The cell solution was mixed and placed in

12 140mm Petri dishes containing 17ml DMEM/FCS/Pen/Strep. The cells were incubated for 2 days.

A precipitate containing calcium phosphate and DNA was formed by slowly mixing a HEPES-buffered saline solution with a solution containing calcium chloride and DNA. This precipitate adheres to the surface of cells. In a 15ml falcon tube 300ug of DNA (plasmid and insert) and 1.5ml of 2.5M $CaCl_2$ was added. Then sterile water was added to make up a 15ml solution. 15ml of 2X HeBs was put in a sterile 50ml conical tube and placed in a rack. A mechanical pipette was attached to a plugged 1ml or 5ml pipette to bubble the 2X HeBs. A Pasteur or Gilson pipette was used to slowly add the DNA/$CaCl_2$ mixture drop wise to the conical tube of bubbling HeBs. After the last drop of DNA/$CaCl_2$ was added, the solution was vortexed for 30 seconds immediately.

The precipitate was incubated at room temperature for 20 min. 2.5ml of the precipitate was distributed over each of the 12 plates and gently agitated to mix precipitate and medium. The plates were incubated for 15min at room temperature and 12.5ml of medium was added and incubated overnight at 37°C/5% CO_2. The medium was aspirated, washed with 12.5ml of PBS and fed by adding 20ml of fresh medium. The cells were incubated for 48 hours at 37°C/5% CO_2 before being harvested.

2.2.7.3. Harvesting

The medium was removed from 4 plates at a time and the plates washed gently with 15ml PBS so as not to dislodge the cells. The PBS was removed and 5ml of fresh PBS was added to each plate. Using a cell scraper, the cells were scraped and transfer to a 50ml conical tube. A further 5ml of PBS was used to wash the plate so as to remove any

remaining cells, transferring this volume from plate to plate before finally transferring to the tube containing the harvested cells. The cells were centrifuged at 1000g for 5 minutes at 4°C and the supernatant removed. The cells were resuspended in 20ml PBS and respun. Resuspension in PBS was repeated twice more before finally removing the supernatant. The pellet was stored at -80°C until ready for regeneration of the pigment.

2.2.7.4 Regeneration of pigment

The visual pigments were regenerated with 40 μM 11-cis retinal. Tubes containing retinal in this experiment were wrapped in foil to keep dark. Once the retinal was added to the foil covered tubes of harvested cells, the tube was spun for 1 hour at 4°C in a rotary mixer.

2.2.7.5. Isolation of the pigments.

The pigments were then isolated by incubation with 1% (w/v) dodecyl-maltoside(DDM) and 20 mg/ml PMSF before passage over a CnBr Sepharose binding column coupled to an anti-1D4 monoclonal antibody. After the tube of cells and retinal had been spun for 1hour, the supernatant was removed and the pellet resuspended in 5 ml of 2% DDM and 5ml of PMSF. The tube was then spun for another hour at 4°C. The CnBr Sepharose binding column was prepared by washing with 1% DDM at 3000g for 30 seconds and the supernatant removed. This washing step was repeated twice. This washing was repeated twice more. After the last wash a small amount of DDM was left so the column did not dry out. After 1 hour the supernatant from the cells was added to the column in the dark and rotary mixed at 4°C for 2 hours. Whilst the column is spun, 0.2ml of glass wool was placed in a 1ml syringe and compacted down with a plunger to compress the fibres. 400μl of 0.1% DDM was added to peptide 1 and kept on ice. After 2 hours the column was spun at 5000 g for 5 minutes in the dark. The supernatant was removed except for 1ml. The column was resuspended and added to the syringe and placed in a 15ml conical tube. The tube was spun for 10 seconds. 1ml of 0.1% DDM was added carefully so as to

avoid air bubbles. Then centrifuged for 15 seconds. This was repeated 8 more times, emptying the falcon after every 2 spins. Parafilm was added to the end of the syringe. 200 μl of peptide I was added to the column and flicked to remove air bubbles. It was then wrapped in foil and left on ice for 30 minutes. The film was removed from the syringe and the syringe placed in a new 15ml tube. The tube was then spun for 30 seconds. 200μl more of Peptide I was added and spun for 30 seconds at 5000 g.

2.2.7.6. Measurement of absorption spectra and rhodopsin template fitting.

Absorption spectra were recorded in the dark using a dual-path spectrophotometer (Spectronic Unicam, Cambridge, UK). The spectrophotometer was blanked with 0.01 DDM. Scans were taken between 200-800nm to find the λmax. Pigments were bleached by exposure to light for 15 min. The λmax value for each pigment as determined by subtracting the bleached spectrum from the dark spectrum to generate a difference spectrum. This was then fitted to a standard Govardovskii rhodopsin A1 template (Govardovskii et al 1996) to determine the λmax.

2.2.8 cDNA Library construction

A cDNA library of brain and eyes of *Ichthyophis cf. kohtoaensis* was constructed. mRNA was isolated and double stranded cDNA synthesised using PCR methods. The cDNA PCR products were then digested, ligated into a vector and packaged into λ phage to form the library. The library was screened for the gene of interest and excised into plasmid for sequencing. The library was constructed using the kit Super smart Clontech which uses

PCR methods to generate relatively large yields of cDNA from a small amount of mRNA.

2.2.8.1. First strand synthesis.

3µl of *Ichthyophis* cf. *kohtoaensis* mRNA, obtained by methods detailed in section 2.2.2.1, was added to 1µl of SMART IV Oligonucleotide and 1µl of 3DS III/3'Primer. This was mixed briefly in a centrifuge for 30 seconds at 5000 g. The reaction was incubated in a water bath at 72°C for 2 minutes and placed on ice for 2 minutes to denature the mRNA. The tube was spun briefly in a centrifuge before adding 2µl of First Strand buffer, 1.0 µl DTT (20 mM), 1µl of dNTP Mix (10 mM) and 1.0µl of Powerscript Reverse Transcriptase(Clontech). After mixing gently the tube was incubated at 42°C for 2hours in a thermal cycler with a heated lid.

2.2.8.2. cDNA Amplification by PCR

A PCR was set up with 2µl of First Strand DNA 10µl of Advantage PCR buffer, 2µl of 50x dNTPs, 2µl of 5' PCR primer, 2µl CDS III/3' PCR Primer, 2µl of 50x Advantage 2 Polymerase Mix and made up to 100µl with ddH_20. The reaction was mixed by flicking and centrifuged briefly. The tube was placed in a thermal cycle set at 95°C for 20 seconds and cycle between 95°C for 5 seconds and 68°C for 6 minutes 26 times.
The next step was to clean and precipitate the double stranded cDNA. 50µl of the double stranded cDNA and 2µl of Proteinase K was placed in a tube and incubated at 45°C for 20 minutes. 50 µl of ddH20 and 100µl of phenol: chloroform: isoamyl alcohol was added to the tube and mixed by gentle inversion for 2 minutes. The tube was centrifuged

at 17900 g for 5 minutes to separate the phases. The top aqueous layer was moved to a clean 1.5 ml tube. 10µl of 3M Sodium Acetate, 1.3µl of Glycogen (20µg/µl) and 260µl of 95% ethanol were added to the tube and centrifuged at 14,000 for 20 minutes at room temperature. The supernatant was removed from the pellet by aspiration with a pipette. The pellet was then washed with 100µl of 70% ethanol and air dried. 79µl of ddH_20 was added to resuspend the pellet.

2.2.8.3. Sfi I restriction enzyme digestion

79µl of the cleaned cDNA was added to a tube with 10µl 10x Sfi I Buffer, 10µl of Sfi I enzyme and 1µl of BSA. The reagents were mixed and the tube placed in a water bath at 50°C for 2 hours. 2µl of 1% xylene cyanol dye was added to the tube and mixed well.

2.2.8.4. cDNA size fractionation by Chroma spin -400

Sixteen 1.5ml tubes were labelled and arranged in order on a rack. The Chroma spin column was prepared by warming to room temperature and resuspending the gel bead matrix using a pipette. The column, attached to the stand, was drained of storage buffer until the top of the gel beads could be seen. When the storage buffer had been fully eluted, fresh 700µl of column buffer was added. Once the column had dripped out then the Sfi I digested cDNA was added. After the sample had been absorbed into the column then 100µl column buffer was added. Following elution, 600µl of column buffer was added. Single drop (40µl) fractions were collected in the labelled tubes. The profile of the fractions was determined by gel electrophoresis at a high voltage (150v) for 10 minutes. The first 3 fractions that had cDNA were pooled together and used for the next

steps. The fractions were cleaned by phenol-chloroform extraction and the resulting pellet resuspended in 7µl of ddH_20. The Sfi I digested cDNA is ready for ligation into the λTriplEx2.

2.2.8.5. Ligation into vector

1µl of cDNA, 1µl of λTriplEx2, 0.5µl of ligation buffer, 0.5µl ATP(10mM), 0.5µl T4 DNA ligase and 1.5µl of ddH20 was added together in a tube and placed in an incubator set at 16°C overnight.

2.2.8.6. Packaging into λ vector

The library was packaged using the Gigapack III XL packaging extract (Stratagene). 4µl of ligated DNA was added to the packaging extract in a 1.5ml tube. The tube was incubated at room temperature at 22°C for 2 hours. 500µl of λ buffer was added to the tube. 20µl of chloroform was added and mixed into the packaging. The supernatant contains the packaged ligated cDNA ready for amplification into a library.

2.2.8.7. Titering of packaging.

Before amplification can take place the packaging efficiency must be measured.
A single colony of Xli-blue MRF^- bacterial cells was used to inoculate 15ml of $LB/MgSO_4$. This was placed in a shaking incubator set at 37°C and incubated overnight until the OD_{600} reached 2.0. The cells were centrifuged for 5 minutes at 5000 g, the supernatant removed, and the pellet resuspended in 7.5ml of 10mM $MgSO_4$. The cells were diluted to an OD_{600} of 0.5. 1µl of the packaging material was added to 200µl of

bacterial cells in a culture tube. In another tube, 1µl of 1 in 10 dilution of packaging and 200µl bacterial cells was placed. The tubes were incubated at 37°C for 15minutes to allow the phage to attach to the cells. 5ml of top agar, melted and cooled to 48°C, was added to each tube and poured on to LB agar plates and allowed to set for 10 minutes. The plates were incubated overnight in a 37°C incubator. The number of plaques of lysed cells as counted and the pfu per ml calculated as the number of plaques x the dilution factor x the total packaging volume/µl of phage plated. A high concentration pfu, 6-7 x 10^4 pfu per 150mm plate of LB/$MgSO_4$ plate is needed for amplification of the library.

2.2.8.8. Amplification

6 culture tubes with 500µl XL1-Blue MRF' cells at OD_{600}, 200µl of lysate, with 12ml of top agar was placed on prewarmed 150mm agar plates after incubation at 37°C for 15minutes. The plates were incubated until the plaques became confluent (6-18) hours. 8ml of λ dilution buffer was added to each plate and the plates placed on a rotary shaker at 4°C overnight followed by incubation at room temperature. The λ-phage lysate was poured from each plate and pooled together into a sterile beaker. The amplified library was then cleared of debris by centrifugation in a Beckman J2-21 centrifuge at 7000 rpm (15000 g) for 10 minutes. The supernatant was pooled together in a sterile 50ml tube. Dilutions of amplified library was then titred to find the amount of library required for screening.

2.2.8.9. Screening of clones.

The bacteriophage library was plated out to produce confluent plates with the number of plaques expected to represent the library. DNA was then hybridized to nylon membranes and probed with non-radioactive probes.

2.2.8.9.1 Hybridization of DNA to membrane.

The plates were precooled for 30 minutes to prevent the smearing of plaques and separation of the top agar when lifting the membrane. Hybond N membranes were cut to the size of the plates. The membranes were placed on the surface of the plates taking care not to trap any bubbles or move the membrane once it had touched the surface. Asymmetric orientation marks were stabbed through the membrane and whole agar layer and marking the same position on the base of the plate with a marker pen. After 1 minute, the membrane was removed from the Petri dish in one continuous movement using blunt ended forceps. The membrane was placed with the plaque side uppermost on a sheet of 3MM Whatman filter paper. The DNA must be liberated from the bacteriophage, denatured and then fixed to the membrane following a neutralisation step. This is achieved by placing the membrane plaque uppermost on a series of 3MM Whatman paper soaked in solution. The membrane was left for 5 minutes with plaque side uppermost on a pad of absorbent filter paper (2 pieces of 3mm Whatman paper) soaked in Denaturing solution taking care not to trap air bubbles under the membrane. The membranes were removed from the Denaturing pad and placed on a pad soaked in neutralization solution for 3 minutes and this was repeated with a fresh pad soaked in the

same solution. The membrane was washed briefly in 2xSSC to remove proteinous debris. The membrane was transferred to a dry filter paper and air dried ready for alkali fixation. A pad of paper was soaked in 0.4N NaOH. The membrane was left on the alkaline pad for 3 minutes and then rinsed in 5x SCC with gentle agitation for 1 minute. The membranes were placed on dry filter paper and air dried until damp. The membranes were wrapped in Saran wrap and placed at 4°C until ready to be screened.

A control blot was prepared to determine the efficiency of the probe. 100ng of the purified probe DNA was denatured by heating to 95°C for 5 minutes and chilling on ice. Samples of dilutions of the DNA was spotted on to a piece of membrane pre- wetted in 10x SSC in 2µl aliquots. The membrane was allowed to dry before placing on a pad of denaturing solution for 5 minutes. The membrane was transferred to a filter pad with neutralising solution for 1 minute before alkali fixation on a pad of alkali for 3 minutes.

2.2.8.9.2 Labelling of probe using ECL detection kit.

The DNA to be labelled was diluted to a concentration of 10ng/µl. 100ng of the DNA sample was denatured by heating for 5 minutes in a boiling water bath for 5 minutes and cooled on ice. 10µl of the labelling reagent was added to the DNA and mixed. 10µl of gluteraldehyde solution was added to the reagents and mixed. The tube of reagents was incubated for 20 minutes at 37°C. The probe had to be used within 15 minutes.

2.2.8.9.3 Pre-hybridisation and Hybridisation

The membranes were sandwiched between mesh prewetted with 2xSSC. Air bubbles were removed using a glass tube as a roller. The mesh with the membranes were rolled

up and placed in a hybridization bottle. 20ml of hybridization buffer was added to the bottle and placed in a hybridization oven for 1 hour for 42°C. Following pre-hybridisation, the labelled probe was added to the hybridization bottle and the incubation continued overnight.

2.2.8.9.4 Blot washing

In a plastic box an excess of primary wash buffer was added to the membranes and agitated twice for 10 minutes at 55°C. The membranes were placed in a clean container at room temperature and an excess of secondary wash buffer was added. The membranes were washed in fresh secondary wash buffer for a further 5 minutes.

2.2.8.9.5. Detection

An equal volume of detection reagent 1 and detection reagent 2 was mixed to give sufficient solution to cover the blot. Excess secondary wash buffer was removed from the blots and placed in fresh containers on a sheet of Saran wrap with the DNA side up. The developing reagent was added directly to the membrane and incubated for 1 minute at room temperature. Excess detection reagent was drained off and wrapped in Saran wrap. Air bubbles were smoothed out. The blots were placed DNA side up in a film cassette. A piece of X-ray film (Hyperfilm ECL) was placed on top of the membrane. The cassette was closed and exposed for 5 minutes. The film was removed and developed. Positive plaques were cored out from the agar plate, placed in a tube of 500µl lambda buffer and stored overnight at 4°C.

2.2.8.9.6. Excision into plasmid

The cDNA library can be converted to a plasmid library by an excision procedure. The insert was originally cloned into the *Eco*RI and *Xho*I sites of the 41kb Uni-ZAP XR λ insertion vector. The DNA flanking the *Eco*RI and *Xho*I sites in the λ vector consists of the 3kb plasmid vector pBluescript SK-. On coinfection of the XL1-Blue MRF' strain with the λphage and the ExAssist helper phage, excision of the pBluescript phagemid from the Uni-ZAP XR λvector occurs. The ExAssist helper phage also provides the phage proteins that encapsulate the single stranded DNA phagemid DNA. The packaged filamentous phage particles are secreted into the medium and can be purified by centrifugation of the cells and collection of the supernatant. Plasmids are more convenient for sequencing than directly from lambda libraries. 200μl of XL1-blue MRF (OD_{600} = 1), 250μl of phage stock of the cored plaque and 1μl of ExAssist helper phage($1x10^6$ pfu/ml) were incubated at 37°C for 15 minutes to allow infection of the *E.coli* cells (phage will absorb into cells at room temperature but λ cannot inject DNA until incubated at 37°C. 3ml of LB was added and the tube placed in a shaking incubator at 37°C for 3 hours. The tube was then heated to 70°C for 20 minutes to kill the XL1-Blue cells. The tube was spun at 1000g for 15 minutes. The supernatant was transferred to another sterile tube which now contains the excised phagemid, a plasmid with an M13 phage origin of replication encapsulated as single stranded DNA into a phage protein particle capable of infecting any E.coli cell that has an F pilus. 200μl of SOLR bacterial cells (OD_{600} 1.0) 100μl of the excised pBluescript phagemid were incubated for 15 minutes to allow infection of the E. coli cells. Both the phagemid and the ExAssist helper phage are infectious on the SOLR cells, although the ExAssist helper phage cannot

replicate in this strain because it contains an amber mutation (UAG) that requires a suppressor tRNA to grow. 200µl of the cell mixture from each tube was plated onto LB agar plates with 100µg/ml Ampicillin. The plates were incubated overnight at 37°C. Single colonies were picked and cultured in 5ml LB with 100µg/ml and incubated overnight at 37°C in a shaking incubator. The cultures were then minipreped and sequenced.

2.2.9 Cycle Sequencing

PCR products and ligated plasmid were sequenced on an ABI 3730. The samples were prepared for sequencing by performing a sequencing PCR using the reagents from the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit, (PE Applied Biosystems). The kit provided the reagents to perform a fluorescence-based cycle sequencing reaction using either gene specific or vector primers. Sequencing reactions contained 4µl BigDye, 2µl Big Dye Sequencing buffer, 6µl of gene or plasmid, 1µl of sequencing primer (3.2 mM) and 7µl of ddH_20. The reactions were cycled in a thermal cycler (25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 30 seconds). The samples were cleaned by precipitation. 0.5µl of 0.5M EDTA, 2µl 3M sodium acetate and 50µl of 96% ethanol was added to the sequencing reactions and mixed. The samples were covered in foil to keep dark for 15minutes. The tubes were then spun at 17900 g for 30 minutes. The supernatant was aspirated with a pipette and 70µl of 70% ethanol was added. The tubes were then spun at 17900 g for 15 minutes. The ethanol was removed and the pellet dried by incubation at 65°C for 5 minutes. The pellet was resuspended in 10µl HiDi™ Formamide (Applied Biosystems) and then transferred to a sequencing 96 well plate. The samples were then run on the ABI 3730.

Chapter 3

Morphology of Caecilian Eyes

3.1 Introduction

3.1.1 Overview

Many features of caecilian amphibians, including their elongate bodies, lack of limbs and girdles, and relatively heavily ossified skulls are interpretable as adaptations to a primarily fossorial lifestyle. In agreement with this, the adult caecilian eye is relatively small, is covered with skin and sometimes also with bone, lacks some features expected of a well-developed vertebrate eye and has several features apparently co-opted to novel sensory structures and functions. Current understanding of the timescale of amphibian diversification based on relaxed molecular clocks suggests that the earliest divergence between extant caecilian lineages occurred in the Triassic (Roelants et al. 2007) and that caecilians may therefore have had a reduced visual system for over 200 million years.

Reduction in the visual system of the Gymnophiona has long been recognised (see Noble 1931 and references therein) and has attracted the attention of several recent authors who have addressed the extent to which the caecilian eye is functional. (Wake 1980; 1985; Himstedt 1995; 1996; Dunker 1997; 1998a; b). Additionally, Wake (1985; 1993) surveyed the diversity of eye morphology within caecilians and explored the utility of 'non-traditional' characters drawn from the visual system for inferring phylogenetic relationships within the group. Wilkinson (1997) demonstrated many contradictions in previous reports of the eye morphology of caecilians that he was unable to resolve and

which undermined previous use of eye characters in phylogenetics, highlighting the need for additional morphological study. In this chapter I provide a first description of the eye of *Rhinatrema bivittatum* and compare it to the eyes of a diverse range of caecilians. This caecilian is a member of the Rhinatrematidae, which is considered to be the sister group of all other caecilians and is thus of considerable relevance to inferring the ancestral condition of the caecilian eye. As I will show, the eye of *Rhinatrema bivittatum* has many of the features typical of a vertebrate eye and is relatively well developed compared to other caecilians.

3.1.2 Previous research on caecilian eye

The eye is very small in caecilians compared to most other amphibians (Fig 3.1), both absolutely and in relation to head and body size (Himstedt 1995). The fossorial and possible nocturnal habits have led some to workers to consider the caecilian eye to be non-functional (e.g., Norris and Hughes 1918; Walls 1942). Storch and Welsh (1973) examined the ultrastructure of the choroid layer and the receptor cells of *Ichthyophis* cf. *kohtoaensis*. They found reduced numbers of organelles in receptor cells, a choroid layer with a smooth basal plasma membrane and few apical cellular processes affording only loose contact with the receptor cell outer segments. They interpreted these data as indicating 'markedly decreased functional activity'. There are further differences between caecilian eyes and other amphibian eyes including a lack of initiation of components of accommodation and the fusion of cornea to dermis that are expected to correlate with reduced function (Himstedt 1995).

More recent analyses do not agree that the eyes of caecilians are non-functional. Wake (1985) compared 18 caecilian species, including representatives of five of the six

recognised families, and found morphological evidence in all the species examined of functioning photoreceptor organs, such as an intact retina and optic nerve. Himstedt (1995) performed behavioural experiments on specimens of *Ichthyophis* cf. *kohtoaensis* and produced electroretinograms, finding that they responded to light at specific wavelengths and that blinded caecilians showed less sensitivity. These experiments suggest that *Ichthyophis* have a limited visual function, perhaps only detecting light from dark. In particular, specimens show a negative phototaxis after the first day of hatching and no visual response to prey (Himstedt 1995).

Based on her comparative studies, Wake (1985) listed seven evolutionary trends in caecilian eye morphology:

(1) The eye is overlain by increasingly thicker, more glandular skin, and sometimes bone also.

(2) Extrinsic muscles are attenuate, and some or all may be lost.

(3) Cell numbers in the retina are reduced and their organisation becomes net-like rather than stratal.

(4) The optic nerve is attenuate, perhaps represented only by glial cells.

(5) The lens, round in aquatic larvae and adults, becomes more 'spheroid' [sic]; less crystalline and more cellular (retention of the embryonic condition) or amorphous or entirely absent.

(6) The vitreous body is reduced or lost.

(7) The cornea adheres to the overlying dermis or periosteum.

Caecilians with less-heavily ossified skulls, such as *Ichthyophis* have better developed eyes. Those with heavier, stegokrotaphic skulls (e.g., *Boulengerula*), which are

seen as a derived adaptation to a dedicated burrowing lifestyle (e.g., Gower et al. 2004), show more rudimentary features of the eye, which is essentially a mass of undifferentiated cells. More opaque bone and glandular skin covers the rudimentary eye in *Boulengerula* whereas transparent skin covers the eye of *Ichthyophis*.

A diagnostic feature of caecilians is the presence of a sensory tentacle, which is associated with the vomeronasal organ and its glands, and is believed to be involved in tactile and chemoreceptive functions (Fig. 3.1, Himstedt and Simon, 1995). In most caecilians the tentacle migrates anteriorly from the eye in ontogeny, and in scolecomorphids the eye remains attached to the tentacle and migrates (out of the orbital chamber) with it (Nussbaum 1977). It is believed that the tentacle has co-opted several structures associated with the typical vertebrate eye such as the Harderian gland, the retractor and levator bulbi muscles, and their nerves (Table 3.1, Billo and Wake, 1987).

Fig 3.1 Anatomy of nasal cavities and tentacle apparatus in *Ichthyophis* cf. *kohtoaensis*. A Lateral view of the head; between eye and nostril, the tentacle is visible. B Cross section at the level of the tentacle ducts. C. Dorsal view of the nasal cavities. Abbreviations: E= Eye; LJ= lower jaw; N= nasal cavity; Nm=nasal cavity, medial part; Nl=nasal cavity lateral psrt; No nostril; T= tentacle; TD tentacle ducts; TS= Tentacle sac; VNO = vomeronasal organ: From Himstedt and Simon 1995

Table 3.1 Amphibian eye tissues hypothesised to have been incorporated into the caecilian tentacle by Billo and Wake (1987).

Other Terrestrial amphibians	Caecilians
Lower Eyelid	Tentacle fold
Interpalpebral space	Tentacle aperture
Conjunctival sac	Tentacle sheath
Conjunctiva of the eye	Tentacle sac
Lachrymal ducts	Tentacle ducts
Harderian gland	Harderian gland
Levator bulbi	Compressor tentaculi
Retractor Bulbi	Retractor tentaculi

Wake (1993 a; b; 1994 a; b) included characters of the visual system in her investigations of the role of 'non-traditional' characters in phylogenetics. She constructed 13 eye characters based primarily on the variation reported in Wake (1985) plus observations on some additional taxa. Although there is obviously value to increasing the number of informative characters in phylogenetic analysis, Wake's analyses proved controversial, in the methods used for individuating characters and character states, the specific coding of the included taxa, and the specific phylogenetic inferences that they supported (Wilkinson 1995; 1997). In particular, Wilkinson (1997) highlighted inconsistencies between the codings employed by Wake (1993a) and the morphologies described by Wake (1985) and internal inconsistencies in the descriptions, summaries and discussions within Wake (1985). These include inaccurate coding (Wake 1993) of the number of extrinsic muscles *Geotrypetes seraphini*, *Typhlonectes natans*, *Sylvacaecilia grandisonae* and *Idiocranium russeli*, the number of layers in the retina of *Boulengerula taitanus* and *B.boulengeri, Scolecomorphus uluguruensis and Uraeotyphlus narayani.* With *Geotrypetes seraphinii,* the vitreous body is incorrectly coded as absent and the lens is described as adherent to cornea and retina but coded as only attached to cornea. *G.seraphinii* is coded having a cellular lens but has a crystalline lens. The cornea is stated as being adherent to the dermis differing from the photograph in the publication (Wake 1985). The lens is stated as being round, however the distinction between spheroid and round is unknown. Wake's (1995) numerical phylogenetic results to be unrepeatable and there were inconsistencies in the character coding, ordering and weighting which compromised the analyses (Wilkinson 1997).

Wilkinson (1997) produced a revised eye character data employing more reductive character codings in which inconsistencies were resolved when possible but coded as missing data when inconsistencies could not be resolved. These results led Wilkinson (1997) to conclude that the neuroanatomical subset records the convergent loss of eye structures as different caecilian groups evolved rudimentary eyes in response to a fossorial lifestyle. A re-examination of Wake's morphological and phylogenetic studies on eyes (1985,1993,1994) is required because of the quality and inconsistencies of the data produced (Wilkinson, 1997).

Furthermore, detailed phylogenetic analyses and randomization tests by Wilkinson (1997) demonstrated significant incongruence between neuroanatomical and more traditional data, and highlighted that characters associated with rudimentation of the visual system appeared to provide a coherent but misleading phylogenetic signal. Subsequently, other researchers have used these data in studies of character construction (Forey and Kitching, 2000) and of methods for discovering non-phylogenetic correlation among characters (O'Keefe and Wagner, 2001). Recently, Caprette et al. (2004) used a phylogenetic analysis of eye characters to test alternative scenarios for the origin of snakes, finding that snakes grouped ecologically with caecilians and numerous fishes rather than phylogenetically with other squamates.

The controversies over the coding of caecilian eye characters indicate a need for a fresh look at caecilian eye morphology. The use of caecilian eye characters as a test data set for issues in phylogenetic theory and their use in studies of the origins of other groups serve to underpin the need for a re-evaluation. As a contribution to this, I present the first description of the eye of *Rhinatrema bivittatum*, a member of the one family

(Rhinatrematidae) that was not represented in Wake's (1985) survey, but which is of particular relevance to understanding the evolution of the caecilian eye because of the phylogenetic placement of the Rhinatrematidae as sister-group to all other caecilians, which is supported by diverse morphological and molecular data (Nussbaum 1977; Wilkinson 1992, 1996; Frost et al. 2006; San Mauro et al. 2004; Roelants et al. 2007). Current understanding of well-developed caecilian vision is based on extensive behavioural, physiological and morphological research on *Ichthyophis* cf. *kohtoaensis* (Himstedt 1995), and published information on the eye of rhinatrematids is limited to the scores for two species of *Epicrionops* provided by Wake (1993a) for her 13 eye characters. I also provide new comparative data on the morphology of the eyes of several other caecilian species.

3.2 Materials and Methods

I examined and photographed serial sections (8 μm) of the head of an adult female *Rhinatrema bivittatum* (Field number MW 3985, collected from Patawa, Kaw Mountains, French Guiana by Mark Wilkinson, David Gower, and Alex Kupfer in May 2007). Total length of the specimen was 176mm. The sections were alternately stained in Mayer's haemalum and eosin, Masson's green trichrome and Malory's PhosphoTungstic Acid Haematoxylin (PTAH). Sections of a variety of other caecilians prepared in the same way were also examined for comparison. Werner Himstedt of the Technical University, Darmstadt kindly donated a specimen of *Ichthyophis* cf. *kohtoaensis* for sectioning. The eye and surrounding area were decalcified in a saturated solution of EDTA then fixed in 4% Paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. The eye was embedded in resin stained in Toluidine blue, dehydrated with increasing concentrations of industrial methylated spirit (IMS). Sections were cut at 3 μm on a microtome, expanded on water, and mounted on glass slides. Observations and photographs were made using a Nikon stereoscopic SMZ-u Eclipse E600 microscope with an attached Nikon Coolpix digital camera.

3.3 Results

3.3.1 The eye of *Rhinatrema bivittatum*

3.3.1.1 Gross morphology

Although small, the eyes are clearly visible through transparent skin and, superficially appear as well developed as the best developed eyes of any other caecilians (Fig. 3.4). In contrast to most caecilians the eyes are slightly raised above the adjacent skin. The tentacular aperture can be seen as a small slit at the anterior margin of the eye.

Figure 3.3 shows the major features of the eye of Rhinatrema bivittatum that are visible in transverse serial sections. The skin over the eye of *Rhinatrema bivittatum* is thin and transparent lacking pigment and dermal glands. Table 3.2 summarises the dimensions of the eye and some of its components.

Table 3.2. Dimensions of the *Rhinatrema bivittatum* eye (all in μm).

Eye Width	Eye Height	Lens Width	Lens Height	Retina Thickness	Head Width	Head Height	Optic nerve width
255	320	130	165	100	4100	3250	25

Figure 3.2. Head of a preserved *Rhinatrema bivittatum* in dorsal view. Note transparent skin over eye. Grid = mm^2

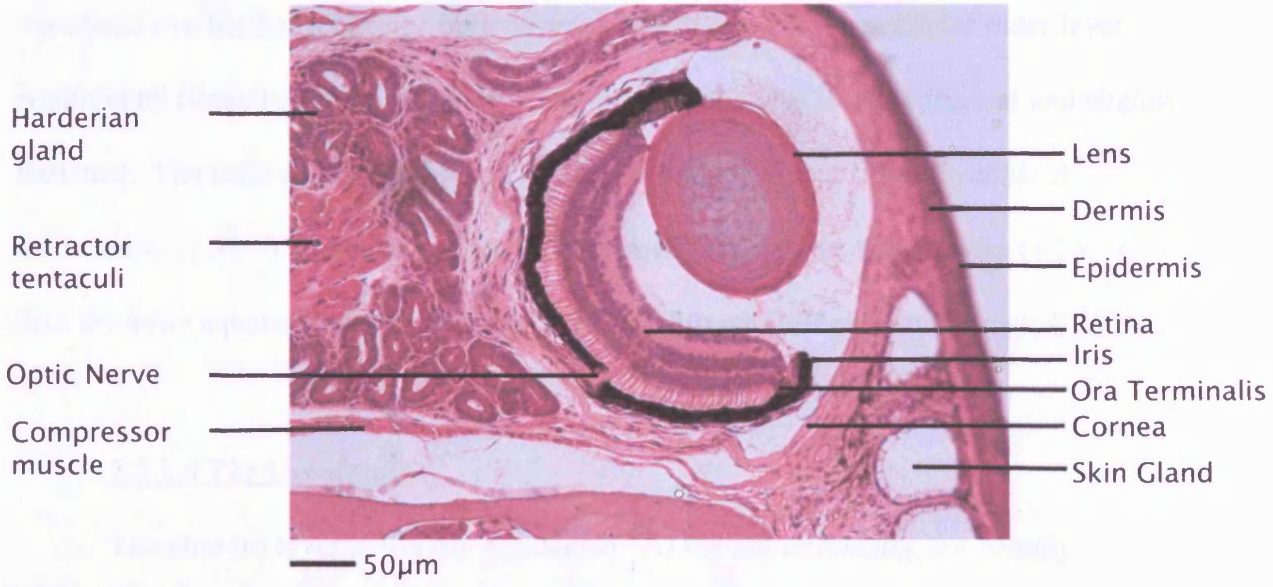


Figure 3.3. Transverse section of the right eye of *Rhinatrema bivittatum*.

3.3.1.2 Fibrous tunic

The sclera is loosely organised into ribbon like bundles. The limbus area between the disorganised sclera and smooth cornea can be seen. The cornea is adherent with the dermis above the eye but does not appear to fuse completely (Fig 3.3) with the partial fusion of the cornea with the overlying skin forming a secondary spectacle. The cornea bulges out from rest of the eyeball. In fishes, the cornea is essentially nonrefractive and flat, with the lens used for refraction of light. The terrestrial cornea is associated with a higher refractive power compared to aquatic forms, due to the difference in refractivity between air and the cornea (Walls 1942, Hoskins 1990). The degeneration appears to have been halted in the embryonic condition as, unlike in other amphibians (Walls 1942), the primary spectacle, the area of transparent skin above the eye, never becomes a part of the eye.

3.3.1.3 Lens

The lens has a mostly crystalline organization as expected in a visually orientated vertebrate eye but has a cellular outer layer shown in Fig 3.3. The cellular outer layer connects by fibres to the tip of the iris. The shape of the lens is sub-spherical and slightly flattened. The ratio of height to width (1.27:1) is similar to that of *Ichthyophis* cf. *kohtoaensis* (1.32:1) and more akin to the terrestrial salamander *Salamandra* (1.25:1), than the more aquatic *Triturus vulgaris* (1.06:1) (Grüsser-Cornehls and Himstedt 1976).

3.3.1.4 The Uveal tract

The choroid layer is heavily pigmented. At the ora terminalis, the heavily pigmented choroid layer extends forward to a rudimentary iris, considered as such

precisely because it is a continuation of the choroid layer. The extension of the non-photosensitive retina forward of the ora terminalis is observed to cover the entire iris.

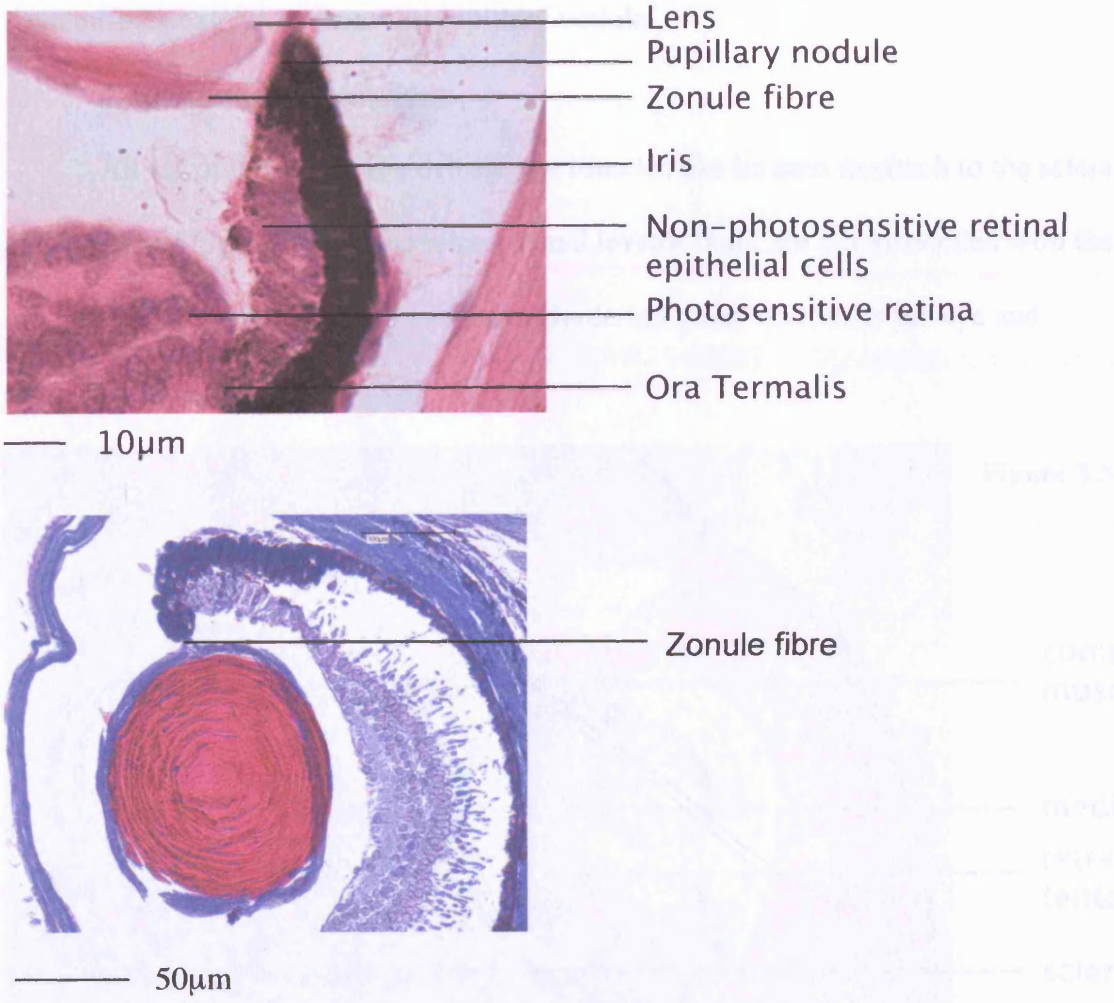


Figure 3.4. Sections showing features of the iris in *Rhinatrema bivittatum* (above) and *Ichthyophis* cf. *kohtoaensis* (below). Note the prominent zonule fibres extending from iris.

Similarly, a non-photosensitive continuation of the retina occurs anteriorly to cover the surface of the ciliary body and iris in human eyes. Only zonule fibres associated with the large pupillary nodule at the end of the ciliary process are present (Walls 1942). The ciliary muscle, Canal of Schlemm and protractor lentis muscle connected to the cornea are absent. The zonule fibres stemming from these heaviest uveal folds are the most

important for the suspension of the lens, and transmit the force that protracts it in the act of accommodation (Walls 1942). This iris has features of the ciliary body, with the zonule fibres extending from the pupillary nodule.

3.3.1.5 Extrinsic Muscles.

All six of the classical extrinsic eye muscles can be seen to attach to the sclera in *R. bivittatum* (Fig 3.5 a-d). The retractor and levator bulbi are not associated with the orbit, but with the tentacle. An extensive Harderian gland surrounds the eye and tentacular muscles.

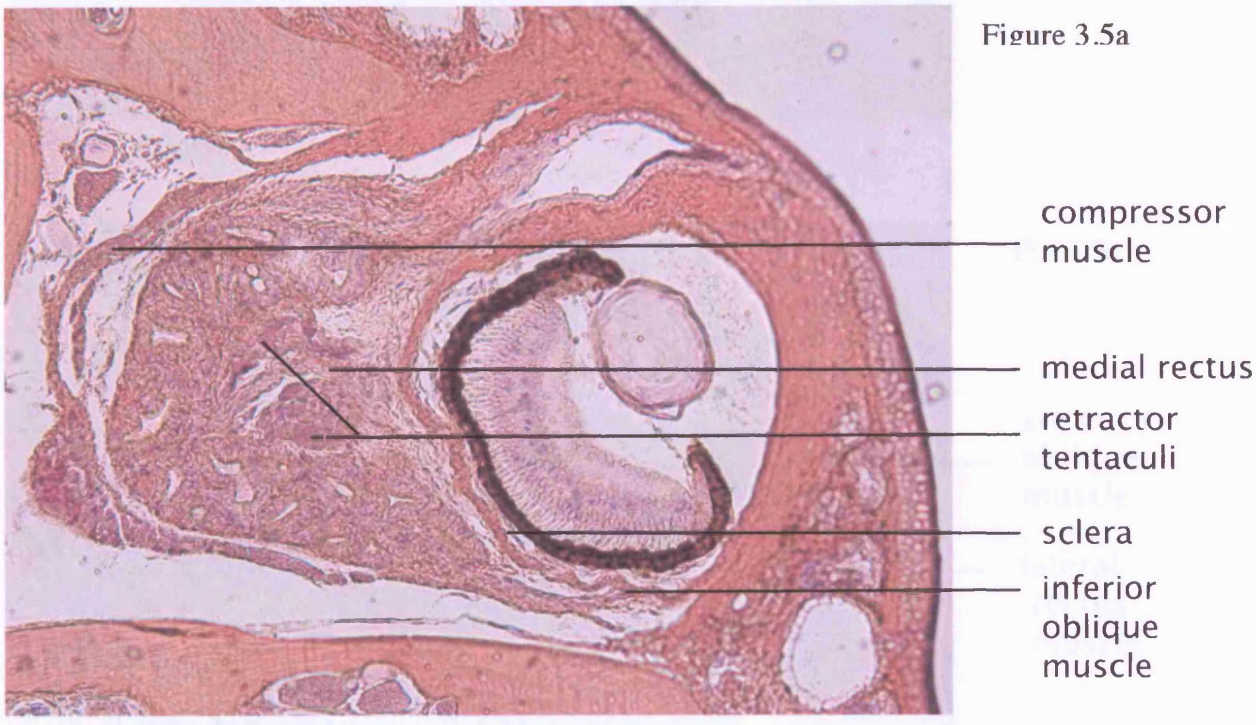


Figure 3.5a

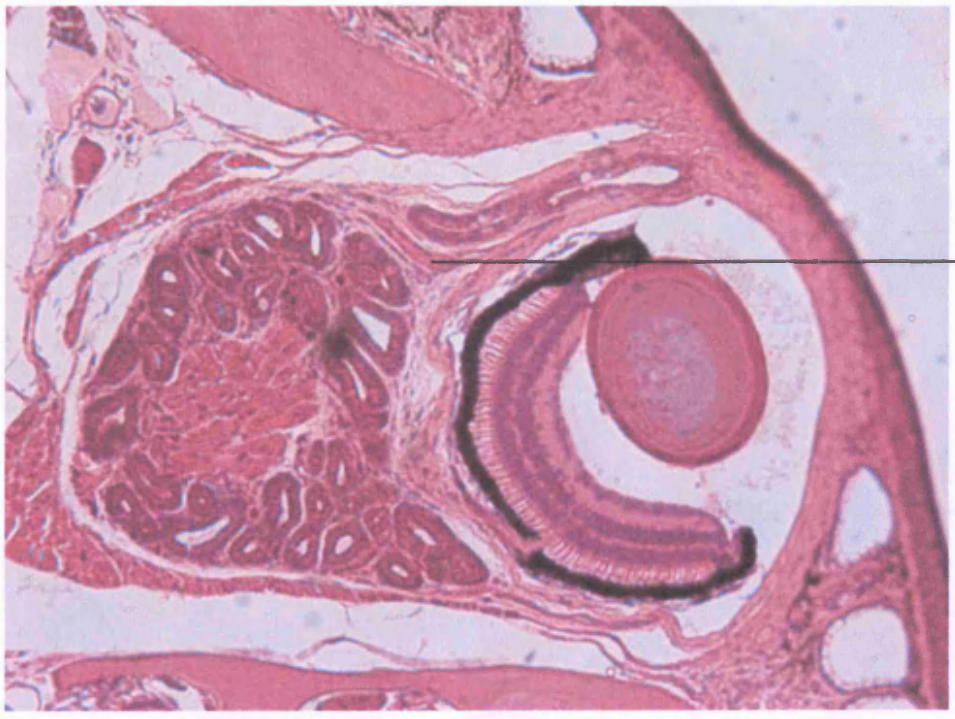


Fig 3.5b

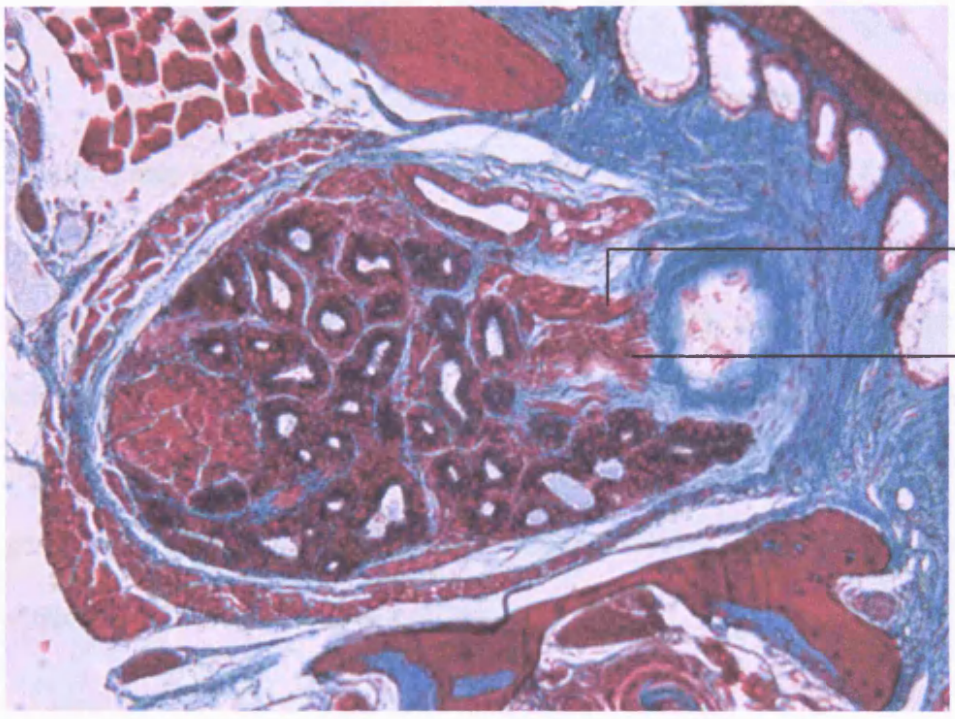


Fig 3.5c

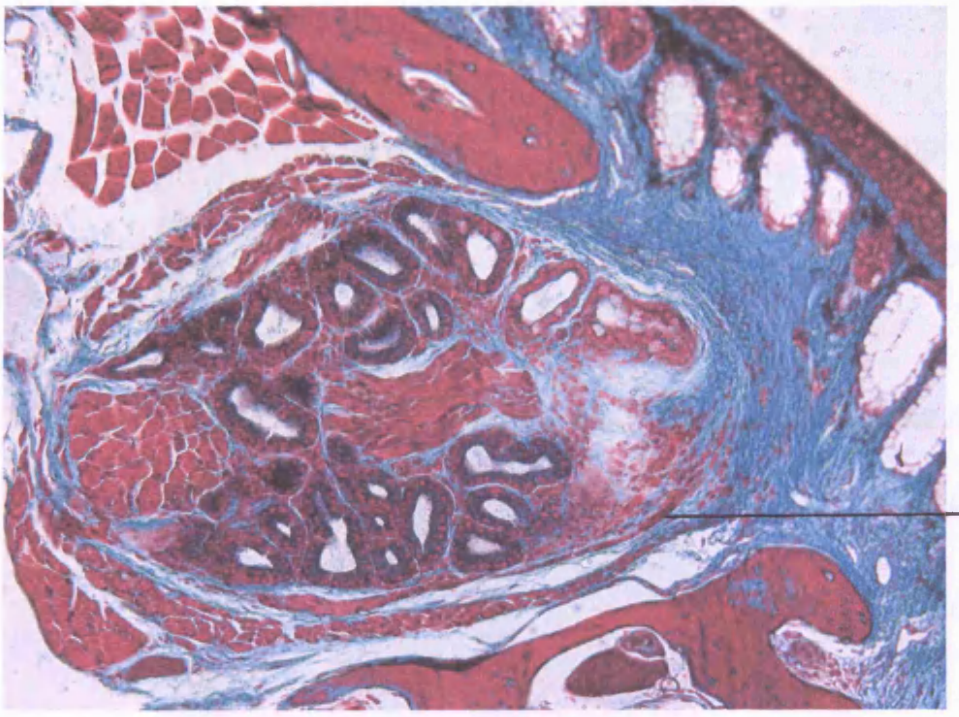


Figure 3.5 a-d. The extrinsic eye musculature of *Rhinatrema bivittatum*

3.3.1.6 Retina

The retina is well organised into seven densely packed layers shown in Figure 3.6 The thickness of each layer is shown in Table 3.3. There is some displacement of nuclei into the plexiform layers. As far as can be determined, no cone photoreceptor cells are present. The convergence of axons coming from ganglionic cells through the choroid and sclera is seen in Fig 3.3. The outer segment layer appears uniform with only the large 'red rod' photoreceptor type. A well-developed optic nerve leaves the retina from the ganglion cell layer through a fossa in the choroid layer (Fig 3.3). Aqueous humour and vitreous humour appear to be present.

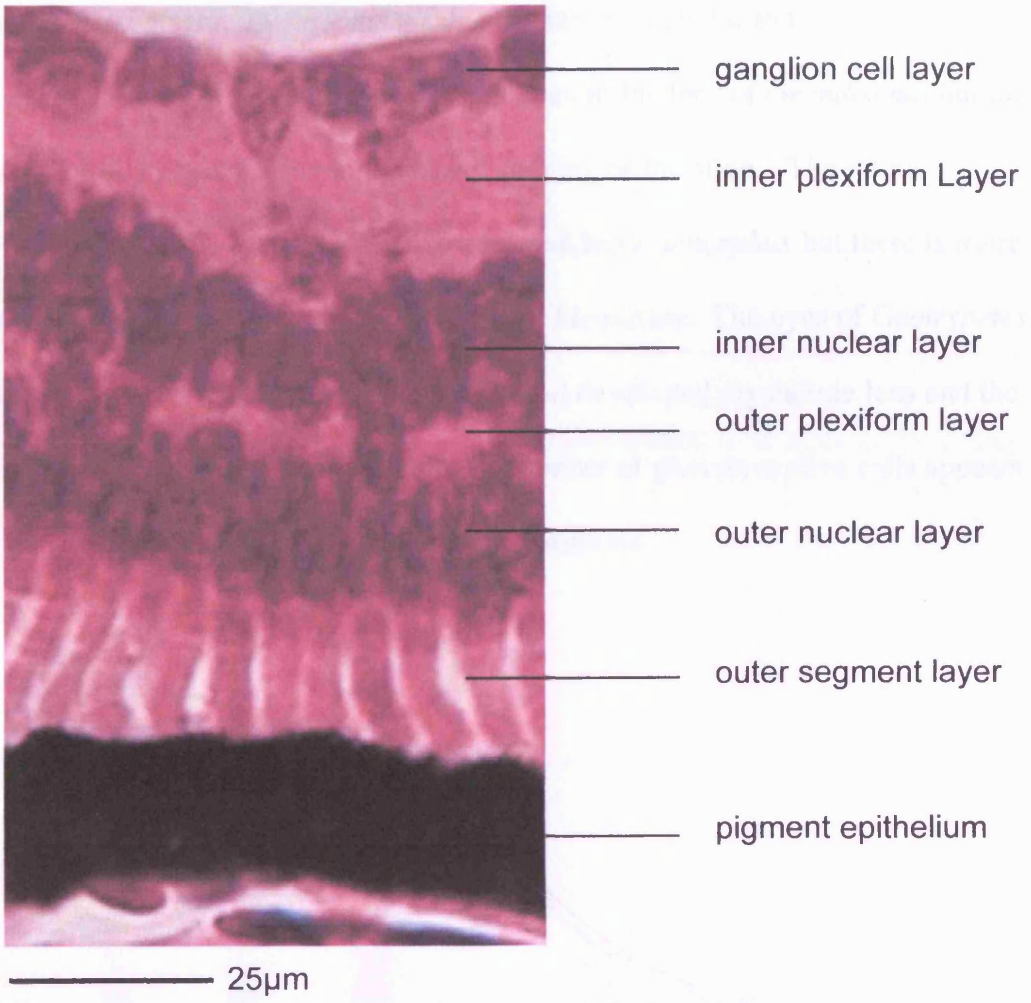


Figure 3.6. Retinal layers of *Rhinatrema bivittatum*

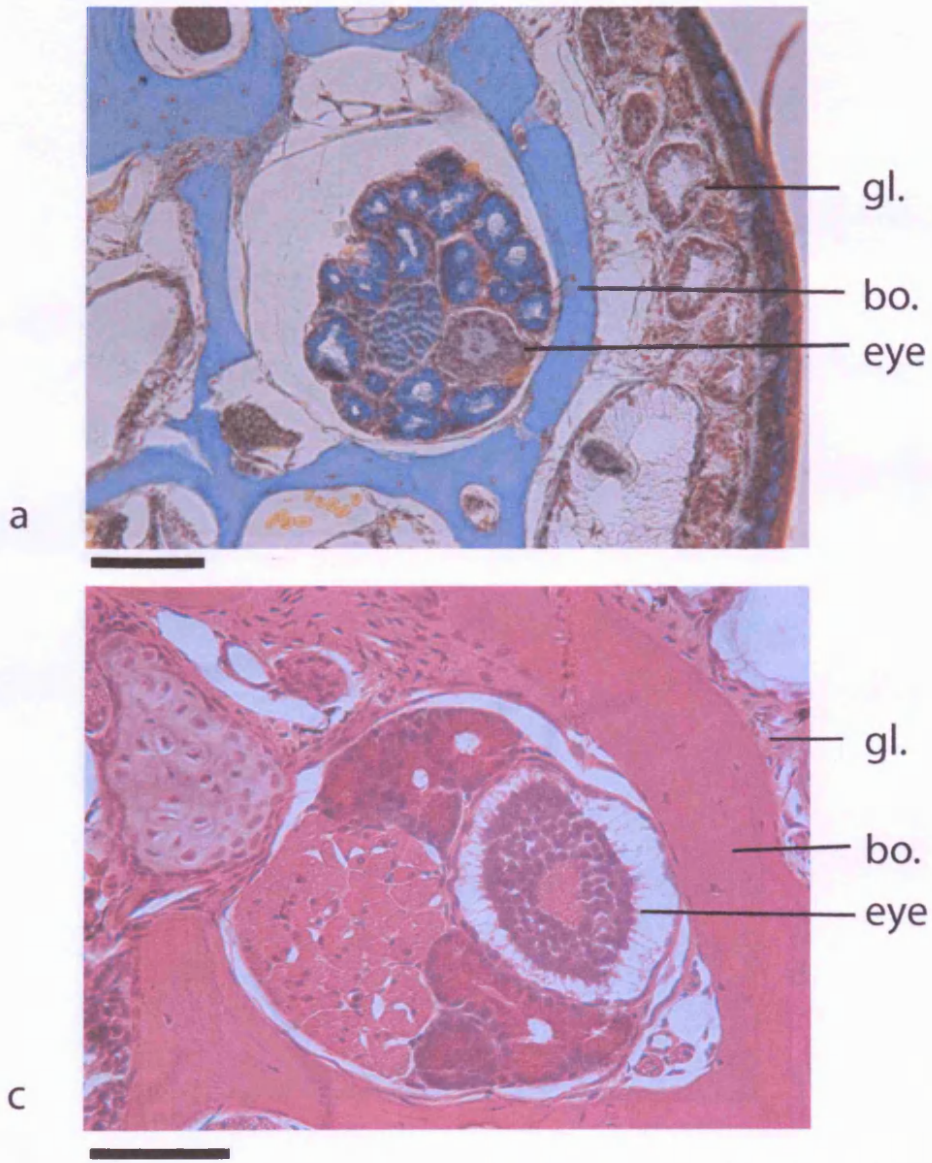
Table 3.3 Thicknesses of retinal layers of *Rhinatrema bivittatum* (in µm)

Ganglion cell layer	Outer segment layer	Outer nuclear layer	Inner nuclear layer	Outer plexiform layer	Inner plexiform layer	Pigment epithelial
7.5	25	17.5	17.5	5	10	10

3.3.2. Diversity of caecilian eyes

Figure 3.7 gives an overview of the diversity of caecilian eyes. *Herpele* and *Boulengerula* show a rudimentation of the eye with no lens and only

undistinguished retinal cells. *Scolecomorphus kirkii* has the most cellular lens with the eye attached to the tentacle. *Dermophis mexicanus* has less cellular than *Scolecomorphus*. There are some cells that can be seen in the lens of *Grandisonia* but the extent cannot be fully assessed due to the heavy staining of the slide. The retina is organized into layers in *Dermophis*, *Grandisonia*, *and Scolecomorphus* but there is more displacement of cell nuclei compared to *Rhinatrema bivittatum*. The eyes of *Geotrypetes*, *Ichthyophis*, *Typhlonectes*, and *Idiocranium* have well developed crystalline lens and the retina is also well stratified in these species. The number of photoreceptive cells appears to be drastically lower than in *Idiocranium* than *Rhinatrema*.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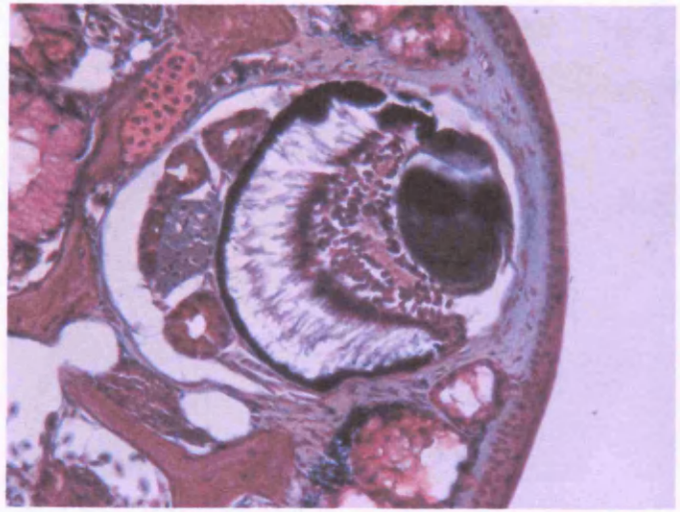

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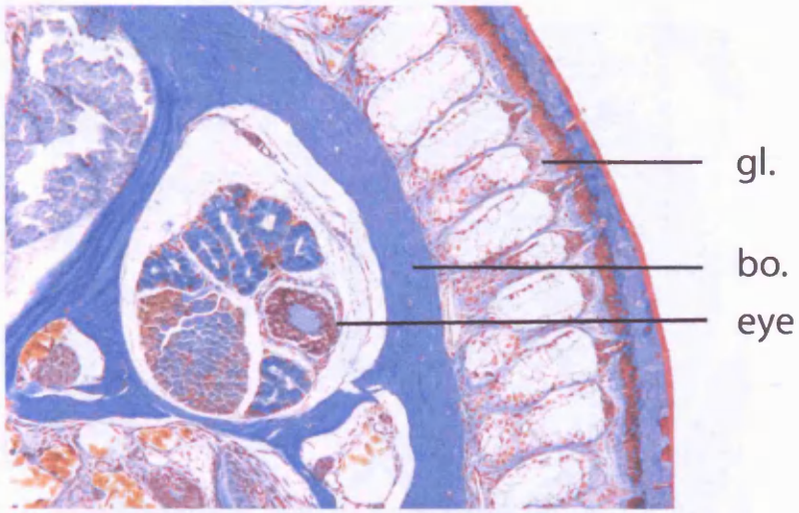

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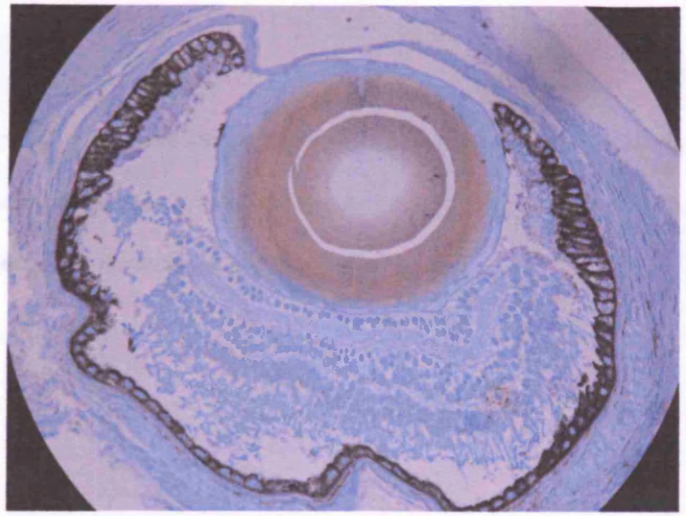

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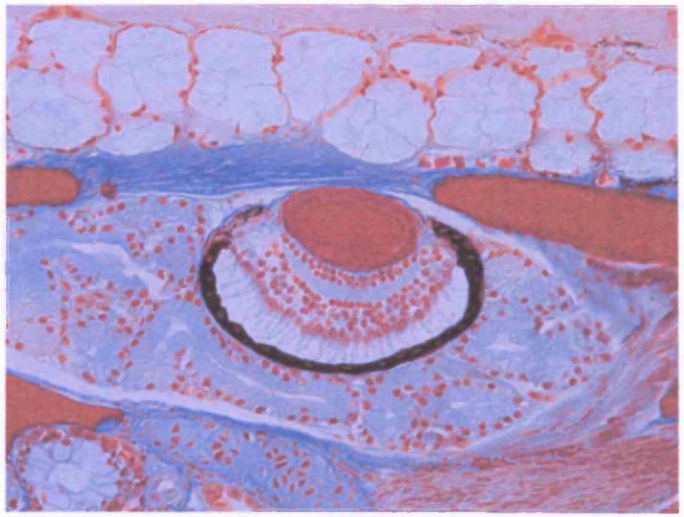


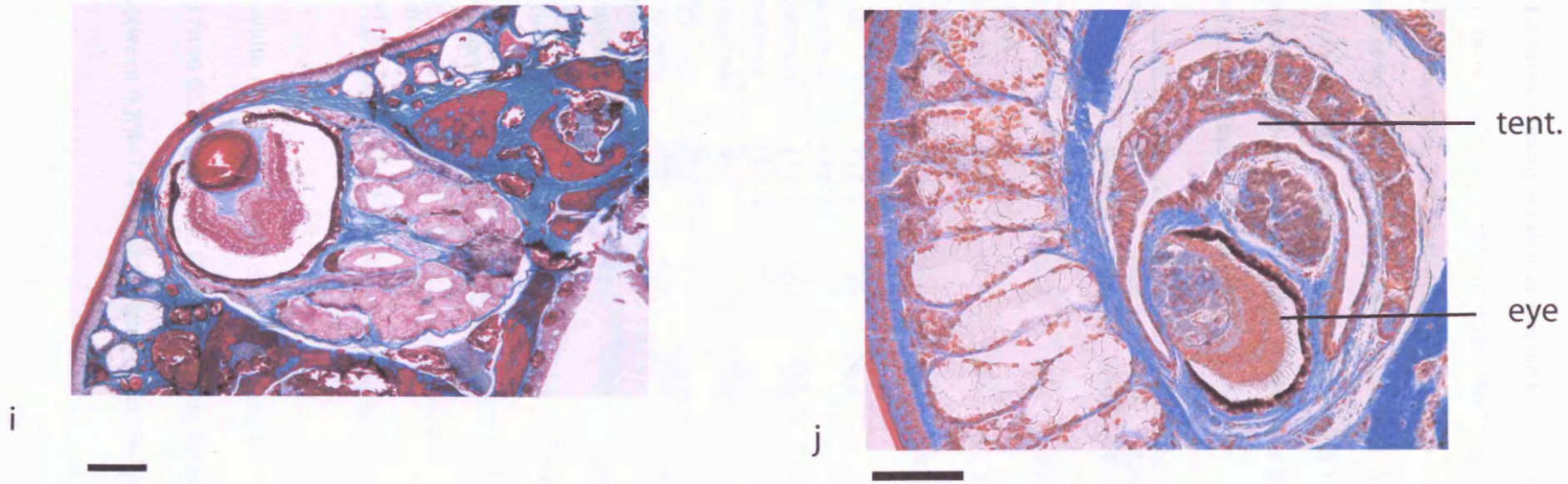


Figure 3.7. (Previous pages) Diversity of caecilian eyes. a. *Boulengerula taitanus*, b. *Dermophis mexicanus*, c. *Gegeneophis ramaswamii*, d. *Geotrypetes seraphinii*, e. *Grandisonia larvata*, f. *Herpele squalostoma*, g. *Ichthyophis* cf. *kohtoaensis*, h. *Idiocranium* russeli, i. *Typhlonectes natans*, j. *Scolecomorphus kirkii*. Scale bars = 100µm. gl.=glandular skin, bo.=bone.

Table 3.4 Measurements of caecilian eyes (all in μm). The maximum axial measurements of different features of the eye are listed in the table below. The relative sizes of the eye and optic nerves are in brackets. The relative size of the eye was obtained by dividing the width of the eye by the width of the head. The relative size of the optic nerve was obtained by dividing the optic nerve width by the eye width. *H.squalostoma, G. ramaswamii and B.taitanus* do not have lenses and stratified retinas. The eyes of *Ichthyophis* sections were isolated from the head so no measurements could be taken relating to the head size

Species	Eye Width	Eye Height	Lens Width	Lens Height	Retina Thickness	Head Width	Optic nerve width
Rhinatrema bivittatum	255 (6.2%)	320	130	165	100	4100	25 (9.8%)
Ichthyophis cf *kohtoaensis*	700 (?)	625	275	350	150	?	60 (8.6%)
Scolecomorphus kirkii	237 (7.4%)	260	62.87	98.46	85	3200	?
Herpele squalostoma	112.5 (2.6%)	125	N/A	N/A	N/A	4375	?
Gegeneophis ramaswamii	137.5 (4.5%)	180	N/A	N/A	N/A	3075	?
Boulengerula taitanus	87.5 (3.4%)	100	N/A	N/A	N/A	2550	?
Geotrypetes seraphinii	400 (10.1%)	435	210	235	90	3950	22.5 (5.6%)
Dermophis mexicanus	400 (4.8%)	545	142.5	200	110	8250	?
Typhlonectes natans	700 (10.1%)	770	380	300	120	6950	30 (4.2%)
Idiocranium russeli	212.5 (14.9%)	137.5	100	62.5	75	1425	10 (4.7%)
Grandisonia alternans	240 (15.2%)	250	75	140	135	1580	12.5 (5.2%)

The width of relatively well developed eyes (with a cellular lens and stratified retina) varied from 6.2% to 15.2%, the intermediate forms (with a cellular lens Fig.3.9 b and j) was between 4.8%-7.4% and the least developed eyes (no eyes or stratified retina) were

from between 2.6-4.5% of the head width. The development of the eye of *Ichthyophis* could be indicated by the relative size of the optic nerve which is similar to that of *R.bivitattum*. The width of the optic nerves of other caecilians is about 5% the width of the eye size.

Wake (1985) found the optic nerve in all caecilian species. However in these sections provided no optic nerve is detected in *S.kirkii*, *H.squalostoma*, *G. ramaswamii*, *B.taitanus*, *D. mexicanus*, and *H.rostratus*. The optic nerve would be especially small in *H. squalostoma G.ramaswamii* and *B.taitanus* and it is possible the nerve is too small to detect due to the thickness of the sections, which is between 8-12.5μm. No optic nerve is detected In *D.mexicanus and S.kirkii,* which have an intermediate development. These slides, donated by Werner Himstedt from Darmstadt had been sectioned to a thickness of 12.5μm. It is possible the sections, which contained the optic nerve were again too thick to show the nerve.

3.4 Discussion

3.4.1 Comparison with previous caecilian research

The eye of adult *Rhinatrema bivittatum* is as well developed as any caecilian, as might be anticipated from the phylogenetic position of the Rhinatrematidae and the retention of a greater proportion of presumed ancestral morphological traits in this family (Nussbaum 1977; Wilkinson 1996). It has several features expected of a well-developed terrestrial eye including the shape of the lens, the shape of the cornea and the shape of the eyeball. In these respects it agrees with the findings of Himstedt (1995) for *Ichthyophis* cf. *kohtoaensis*. In addition, caecilians have features associated with the tentacle that would have lubricated the eye ancestrally. These observations run counter to the claim by

Caprette et al. (2004) that caecilians are primitively aquatic. These authors further suggest an aquatic ancestry for snakes due to the grouping of snakes, caecilians and several fish groups in their 'phylogenetic' analysis of characters of the visual system. That caecilians appear to be a primarily terrestrial group undermines somewhat their broader conclusions.

The absence of an iris and of muscles of accommodation had been previously reported and accommodation presumed not to occur in caecilians (Himstedt 1995). However, that the position of the lens may be variable is suggested by the presence of zonule fibres, reported here for the first time in *Rhinatrema bivittatum*, and found, upon closer inspection, in *Ichthyophis* cf. *kohtoaensis* also. Zonule fibres are not apparent in any of the other caecilians examined and it is possible that their absence is a synapomorphy of the Teresomata. This hypothesis can be further tested through careful examination of additional taxa, particularly *Uraeotyphlus*, which, based on its phylogenetic relationships, would be predicted to have zonule fibres.

Some problems with the data reported by Wake (1985, 1993a) are apparent from the comparative images in Figure 3.9. For two pairs of closely related taxa, she reported the presence of the vitreous humour in one member of the pair and its absence in the other. One of the species pairs are *Scolecomorphus ulugeruruensis* and *Scolecomorphus kirkii* the other pair is *Chthonerpeton indistinctum* and *Typhlonectes natans* (Wake 1993a). This is surprising a priori since it would indicate multiple independent losses of an otherwise conservative feature that we might expect to accompany only the more extreme degeneration of the eye. It is more surprising since some of the species pairs involved, such as *Chthonerpeton indistinctum* and *Typhlonectes natans* have some of the

best developed eyes, and in none of the pairs is the eye as rudimentary as in, for example, *Boulengerula*. Figure 3.9 d and e shows the close proximity of the retina to the lens in some species with well-developed eyes, but in every case there is a large space between the outer segments of the retina and the pigmented epithelium of the choroid layer. The latter is not a normal feature of any vertebrate eye and must be interpreted as an artefact of preparation in which the retina has become detached. Evidence of such artefacts occurs in Wake's (1985) figures of, for example, *Gymnopis multiplicata*. Failure to recognise artefacts probably explains Wake's (1985, 1993a) surprising descriptions and codings of the presence or absence of the vitreous humour but do not explain failure to remark upon the equally artefactual presence of a retino-choroidal gap. The absence of vitreous humour is unlikely when a stratal retina and lens is present.

Another problematic character from Wake's (1993a) data set is the attachment of the lens to either the cornea or retina. It can be clearly seen, even with the cellular lens of *Scolecomorphus kirkii*, that there is a distinct outer layer present, with this lens capsule preventing fusion of the lens to other tissues. Such fusion was reported in *Ichthyophis* sp., *Dermophis mexicanus*, *Geotrypetes seraphinii*, *Idiocranium russeli* and *Typhlonectes natans* but observations of sections of the eyes of these species (Fig. 3.9), most especially of the large crystalline lenses of *Geotrypetes* and *Typhlonectes*, refute the claim. The mistaken coding can be attributed to the displacement of tissues during preparation of the slides. It is possible for the lens and cornea, both of which are epidermal, to fuse, as seen in Wake's (1985) figure of *Gymnopis multiplicata*. However it is unlikely that the retina and the lens will fuse due to their separate origins, and reports to the contrary probably result from a detachment and displacement of the retina.

Previous descriptions and codings of the shape of the lens are also problematic. Lens shape has been ambiguously described in various caecilians as spheroid, spherical, round, and slightly flattened in Wake (1985). It is quite unclear as to how round differs from spheroid despite their having being used as alternative character states by Wake (1993a). The sections in this study show all lens shapes to be slightly flattened as expected in a terrestrial vertebrate (Fig. 3.9).

As noted by Wilkinson (1997), there are many contradictions between the various sources of information of caecilian eye morphology. The new observations presented here strongly suggest a need to re-evaluate all the eye character data that have been used in phylogenetic analysis, not just the subsets over which contradictions have been shown to exist by Wilkinson (1997). Due to time constraints it has not been possible to evaluate new characters based on caecilian eye morphology observations in a phylogenetic analysis.

Rods are believed to be more sensitive to light than cones. The eye morphology of *Rhinatrema bivittatum* have features that are associated with nocturnality such as a rod-based retina, large lens and a thick and dark pigment epithelial. This suggests that the eye is adapted to reflect as much light onto the retina with a reduction of obscuring light signals that could potentially reflect within the eyeball (Wall 1942). The presence of red or green rods as found in other amphibians cannot be determined at present.

3.4.2 Comparison with other vertebrates with degenerate visual systems

There has been considerable research on the eyes of other vertebrates that live in darkness, whether the creatures are living underground, are cave dwelling, nocturnal, or

inhabit the light-poor deep sea. For example, there has been extensive work on the Mexican cavefish *Astyanax* (Porter et al. 2007; Yamamoto and Jeffery 2000) and on the blind mole rat *Spalax* (Nevo 1999; Peichl et al. 2004). These studies may help to shed light on the evolution of eyes in caecilians, however it must be noted that the timescale over which rudimentation of the visual system has occurred in caecilians is considerably longer than for some of the other species. Some commonalities are found with nocturnal vertebrates, such as geckos that, like caecilians, have a rod based visual system (Kojima et al. 1992). Rods are more sensitive to light than cones but higher visual acuity is achieved in cones. Rods are thought to be younger in an evolutionary sense than cones (but see Pisani et al. 2006 in appendix) with a possible intermediate type or a cone photoreceptor cell being the ancestral type. Rods came later as a means of extending the day for the vertebrate. This 'transmutation theory' is supported by studies on nocturnal geckoes with rod-based retinas that found three types of opsin expressed (Taniguchi et al 1999). One visual pigment was related to long wave sensitive, one to rod opsin (Kojima et al. 1992) and a U.V. sensitive opsin (Loew 1994). In the nocturnal Tokay gecko, *Gecko gecko*, there are three types of rod; single rod, and primary and accessory rods of double rods. The longwave sensitive opsin 521nm is found in all the rods except for the thin accessory rod outer segments (Kojima et al.1992). The shortwave sensitive opsin, 467nm, is found in accessory outer segments and so is the U.V sensitive opsin in 20% of the accessory cones. This would suggest that the two opsins are conserved through the transmutation from cones to rods. It appears that the morphological changes are independent of the types of visual pigments that occur.

Other morphological adaptations have occurred in nocturnal animals. These

vertebrates may have a larger pupil, or a slit shaped pupil, a developed tapetum lucidum (which gives the mirror effect at the back of vertebrate eyes such as cats, fish and owls), a broader iris and large or spherical lens, as seen with owls (Walls 1942). As well as these elements of the eye that are disproportionately large the absolute size of the eye can be large. For example in owls and the nocturnal primate *Tarsius* the eye is so large that they are locked in a close-fitting orbit and cannot be turned. They have evolved to turn their heads through an extraordinary amount, up to 270° in owls. Extension of the visual field happens also by having a spherical lens and extensive cornea so the object viewed is not distorted (Walls, 1942). These features are in contrast to what is seen in burrowing vertebrates such as caecilian, amphisbaenids and blind mole rats and in cave dwelling fish such as *Astyanyx*. In an extreme confinement to dark underground environments, the importance of vision is reduced. Lightless subterranean ecotopes lead commonly to differential regression evolution or disappearance of the vertebrate and invertebrate eye (Walls 1942) and their replacement with other sensory systems.

Concealed beneath the skin, the small regressed eyes of *Spalax* are further embedded within a hypertrophied Harderian gland similar to the condition in caecilians (Nevo 1999). Thus, light can only penetrate to the retina with difficulty and the thick covering skin precludes proper image formation. The eye is extremely reduced in size compared to other mammals (700μm diameter), although the neuroretina appears to be normally structured with thin inner and outer plexiform and nuclear cell layers. Development of the eye is initiated normally but at an early stage displays a number of degenerative features. The optic fissure fails to close, the anterior chamber collapses and the iris-ciliary body becomes hypertrophied and heavily pigmented. The lens remains

vascularized. In contrast, neurogenesis follows a normal course of development, but the ganglion cell and photoreceptor layers remain relatively thin. Photoreceptors have been shown to form normal synapses in the outer plexiform layer.

The evolution of an atrophied eye and reduced visual system is an adaptively advantageous response to the unique subterranean environment. Factors favouring regression include mechanical aspects, metabolic constraints, and competition between sensory systems (Nevo 1999). The primary advantage of sensory atrophy is the metabolic economy gained by the reduction of visual structures that do not contribute significantly to the animal's fitness (Nevo 1999). The retention of a rudimentary visual system in those caecilians that have a heavily ossified skull and appear to be the most dedicated burrowers (e.g., *Boulengerula*, *Herpele*, *Gegeneophis*) suggests a similar persistent function with respect to photoperiodic perception and entrainment.

Other structures than the eye are used for photoreception. Together with the eye, pineal complex and deep brain, the Harderian gland, participates in photoperiodic perception (Avivi et al. 2004). The adult pineal pattern of Gymnophiona differs to the pineal complex found in other amphibians. The pineal of adult caecilians is described to be like that of a juvenile salamander (Leclercq et al. 1995). Furthermore, there is some behavioural evidence of light detection in the skin of caecilians (Ross 1959). *Ichthyophis* show negative phototaxis when light is shone on the tail. In a typical photoresponse, skin illumination is followed by locomotor activity after a latency of several seconds (Ronan and Bodznick 1991). Behavioural studies dating from early 20th century indicate that several anamniotic vertebrates respond to illumination of the skin (reviewed by Steven, 1963). Dermal melanophores of *Xenopus laevis*, like retinal photoreceptors, are

photosensitive (Provencio et al. 1998). When maintained *in vitro*, the melanosomes in dermal melanophores migrate to the cellular periphery in response to illumination (Provencio et al. 1998). The melanophore response to these agents has led to the development of innovative systems for studying functional interactions between ligands and their receptors. Melanophore photosensitivity is reversibly activated by retinaldehydes (and has an action spectrum characteristic of opsin-like photopigments (Bellingham et al. 2006)).

Light perception structures within the lateral line of lampreys has also been isolated and identified (Ronan and Bodznick 1991, Deliagina et al 1995). Lateral line systems have been observed in larval ichthyophids, rhinatrematids, *Uraeotyphlus oxyurus*, *Sylvacaecilia*, some species of *Grandisonia* and the embryos of *Hypogeophis rostratus* (Wilkinson 1992). There is no evidence of a lateral line system in viviparous caecilians within the Caeciliidae, Scolecomorphidae and Typhlonectidae.

The opsins associated with non-visual photoreception include Vertebrate Ancient opsin, pineal opsin, parapineal opsin, the recently isolated *Ciona intestinalis* (Tunicata) Ci-Opsin1 (Terakita 2005, Kusakabe et al. 2001) the tmt (teleost multiple tissue), and the panopsin (encephalopsin) subfamily (Terakita 2005). These opsins play a role in non-spatial directional vision (Land and Nilsson 2002), or in non-visual photoreception (e.g. circadian entrainment).

The presence of these other opsin-like pigments could be detected by molecular analysis and associated structures should be examined in the future to gain knowledge on non-visual photoreception and periodicity functions in caecilians.

Himstedt (1995) considered whether the *Ichthyophis* eye degenerated over time compared to other cave dwelling vertebrates such as the cavefish *Astyanax mexicanus* that show rudimentation through degeneration. To understand the evolution of eye degeneration in caecilians it is necessary to determine the molecular and cellular mechanisms of the degenerative process, and whether the same or different genes and mechanisms are involved in the loss of vision as found in other organisms.

The genes involved in rudimentation have been studied in the cavefish *Astyanyx*. Lens transplantation studies by Yamamoto and Jeffery (2000) between closely related surface dwelling fish with cavefish showed how the lens affected the development of the eye. When a cavefish lens was transplanted into a surface fish optic cup the eye died on schedule. Whereas, when a surface fish lens was transplanted into a cavefish optic cup it continued to grow and differentiate, as it would have in the surface fish host. These results suggest that the cavefish lens is autonomously fated for apoptosis by time of transplantation. After obtaining a surface fish lens, the cornea and iris developed, which are normally missing in cavefish, and the retina enlarged and became more organised. Further growth resulted in the presence in the presence of a highly developed eye containing all of the expected eye tissues, including the cornea, iris and photoreceptor cells. The cornea and iris are derived in part from optic neural crest cells, indicating that cavefish neural crest cells are present and located in the proper positions to be induced by the lens.

Changes in the expression of specific developmental genes have been observed when comparing the surface dwelling fish with cave-dwelling fish. Pax6, one of the genes controlling cavefish eye degeneration, is down regulated in cave-dwelling fish (Jeffery

2001). Sonic hedgehog gene (SHH) expression appears to have an affect on Pax6 (Jeffery 2001). Gene expression of SHH is hyperactive along the anterior embryonic midline of the neural plate in early cavefish embryos (Yamamoto et al. 2004). The developmental and gene expression studies have revealed a negative relationship between midline signalling, which promotes enhancement of other sensory organs that may be advantageous to the survival of blind cavefish. In cavefish the taste buds appear to increase in number due to the increase in (Sonic HedgeHog) SHH expression (Jeffery 2001). A scenario is proposed for the loss of vision in these cavefish (Jeffery 2001). The developing lens normally produces a factor that is responsible for inducing differentiation of the anterior eye segment (e.g. cornea and iris) and sustaining retinal growth by suppression of apoptosis. Although generation of new cells in the retina is not prevented, cell death triggered by the absence of lens signal prohibits net growth, degeneration begins, and the cavefish eye is overwhelmed by the growth of the body (Jeffery 2001).

This does not occur in *Ichthyophis,* which appears to reach a stage of development at hatching then does not degenerate when considering number of retinal cells (Himstedt, 1995). This would suggest again possible functionality of the eye. It would be difficult to do transplantation studies in caecilians due to the number of animals required. However possible ways to study developmental genes is to obtain gene sequences from a cDNA library constructed from caecilian eyes and perform some in-situ hybridisation studies to see the expression of the developmental genes. It would be also informative if these developmental genes affect tentacle development as well as other sensory systems, due to the evolutionary history of the tentacle, and to see if similar developmental genes are involved in eye degeneration in the Gymnophiona.

Based on ganglion cell numbers and lens shape, Himstedt (1995) stated that all structural elements in the eye of *I.* cf. *kohtoaensis* remain unchanged during post-hatching development. No sign of anatomical degeneration could be observed, and in all developmental stages the eye has the structure of a functional sense organ. The only relevant changes are that the cornea becomes more fused with the dermis and the skin above the eye, though remaining translucent, also thickens from between 25 - 30 μm in larvae up to 100 μm in adults. The distribution and number of transmitter-expressing cells also changes but no general reduction of the visual system is detectable (Dunker1998 a; b). Seratonergic cells increase steadily during embryogenesis but then declined dramatically during larval life and this is similar to other amphibians.

3.4.3 Further research

A comprehensive re-evaluation of eye characters, based on larger sample sizes and more taxa is required to underpin more comprehensive understanding of the evolutionary trends in the caecilian visual system and the extent to which similar changes have occurred independently in different lineages and in the same order. More detailed study of the retina, and the diversity of photoreceptor types (if any) would benefit from transmission electron microscopy.

There is also some evidence that eye development may differ between species in relation to life history differences that would be interesting to examine further. Thus, Wake (1995) found that the eye of the viviparous *Dermophis mexicanus* continues to grow after birth whereas with in *Ichthyophis* the eye remains unchanged from hatching to

adulthood, and the lens does not alter its size or shape (Himstedt 1995). The latter is unlike other amphibians that change lens shape at metamorphosis. The number of retinal cells also did not increase or decrease. Unfortunately, the potential for such additional studies is limited by the availability of suitable material, although in recent years there has been an upsurge in discoveries of populations of many caecilian species from which suitable material might be obtained.

Sequences of developmental genes such as Pax6 and SHH could be compared between caecilians to observe possible changes that might be responsible for the rudimentation of the eye. In-situ hybridization experiments could be made to determine expression of genes for the different sensory organs, similar to the experiments described in Chapter 6 on *Astyanyx*.

It could be possible to repeat the experiments of lens replacement as done in *Astyanyx*. However a number of challenges that could be envisaged when working with caecilians that would have to be overcome. They are relatively hard to breed compared to fish and are comparatively rare compared to amphibians such as *Xenopus laevis*. The eye of degenerate forms such as *Boulengerula* are tiny and so there would be difficulty in transplanting lens from another caecilian such as *Rhinatrema*. If these difficulties could be overcome it would be interesting to observe if a well-developed lens would trigger further development of the rest of the degenerate eyes of caecilians.

3. 5 Conclusion

Some features of the eye of *Rhinatrema bivittatum* suggests complex visual functionality, including the crystalline lens, the presence zonule fibres, the presence of six extrinsic

muscles, and the retinal organization. However the lack of iris muscles for accommodation, and the covering of the eye by skin suggest that a sharp image is not formed and that the eye serves principally for distinguishing between light and dark. The persistence of a visual system in even the most dedicated burrowers suggests the possibility that the rudimentary visual system has a role in photoperiodic entrainment. The newly described features of caecilian eyes show that a comprehensive re-examination of previous eye character data sets are required, especially if these data are to continue to serve as an important test data set for methodologists interested in character construction or detecting correlated sets of characters.

Chapter 4

Identification and Characterization of Visual Pigments in Caecilians

4.1. Introduction

4.1.1 Overview

It is widely assumed that variations in spectral tuning and absorbance capabilities represent adaptations to specific visual needs associated with particular habitats or lifestyles (Lythgoe 1979; Peichl 2005). Examples of apparent adaptations of the visual system of vertebrates to habitat or lifestyle are numerous, e.g. in deep-sea fish that shift their spectral sensitivity to match the narrow bandwidth of light (Hunt et al. 2001), or lampreys which appears to spectrally tune to a marine or freshwater environment (Davies et al. 2007a), the loss of opsin function in mammals associated with nocturnality, in the order Rodentia (Carvalho et al. 2006), the family Procyonidae (Peichl and Pohl 2000), and in marine mammals (Griebel and Peichl 2003) The spectral sensitivity is adjusted by changes in the visual pigment; the chromophore or tertiary structure of the opsin protein (see Introduction; Bowmaker and Hunt 2006. Hart and Hunt 2007).

It could be predicted that caecilians which are subterranean, with obvious rudimentation of the eye (see Chapter 3) and may have nocturnal habits, will have a detectable change in their spectral sensitivity compared to more visually oriented amphibians, observed in the properties of the visual pigments present.

4.1.2 Frog and salamander visual pigments

Amphibian retinas have been used extensively in the study of photoreception. Advantages of using amphibians for research is the large size of the principle rods, making studies of anatomy, molecular biology and electrophysiology relatively easy. Also the duplex nature of the retina with many morphological photoreceptor types means there is a large scope for study of cone cell biology and neurobiology of colour vision (Rohlich and Szel 2000). Further, species of *Xenopus*, for example, are frequently used as experimental animals and are therefore readily available for study.

One rod class and three cone classes, have been found in frogs and salamanders. The primary structure of an amphibian rod opsin was first elucidated by Pittler et al. (1992) in the frog *Rana pipiens*. The sequence isolated had about 85% identity at the amino acid level with mammalian rod opsin and is longer than mammalian and fish opsins (Pittler et al. 1992). Other amphibian rod opsin sequences have been found to be the same length as that of *Rana pipiens*. Subsequent complete sequences of rod opsin have been published from *Bufo bufo*, *Rhinella marina*, *Rana temporaria,* (Fhyrquist et al. 1998), *Lithobates catesbeianus* (Kayada et al. 1995), *Xenopus laevis* (Batni et al. 1996), *Silurana tropicalis* (Klein et al. 2004) and the salamanders *Ambystoma tigrinum* (Chen et al. 1996) and *Cynops pyrrhogaster* (Sakakibara et al. 2002). Partial fragments of rod opsin have been used in phylogenetic study for taxonomic purposes of amphibians including 4 species of caecilian (Frost et al. 2006 ; Venkatesh et al. 2001) Full length cone opsin sequences from the SWS2, SWS1, and LWS opsin classes have been isolated from ranid frogs (Liebman and Entine 1968), *Xenopus laevis* (Batni et al 1996;Harosi

1983), and the salamanders *Ambystoma tigrinum* (Harosi 1975) and *Cynops pyrrhogaster* (Sakikabara et al. 2002; Takahashi et al. 2001). The RH2 opsin appears to have been lost, but a visual pigment was identified by MSP with maximal absorbance value 502nm in a cone reported by Liebman and Entine (1968). No further evidence of the presence of RH2 in subsequent research on amphibian visual pigments. Opsin classes are strongly associated with specific photoreceptor types in amphibian retinas. The nomenclature of 'green' and 'red' rods is confusing and is attributed to the original identification of a second class of rod in amphibian retinas (Denton and Pirenne 1952) that appeared green under microscopy. The rod opsin is found within "red" also known as "principle" rods. The SWS2 is associated with smaller "green" rods, SWS1 with small single cones, LWS with large cones (Rohlich et al. 2000). However exceptions occur. No green rod is apparent in the Japanese common newt, *Cynops pyrrhogaster* but the SWS2 gene has been identified in this species (Takahashi et al. 2001) SWS1 violet detection has also been identified in *Xenopus laevis* (Hunt et al. 2007). The proportions of each class of opsin and their photoreceptors vary in the retina as measured in *Xenopus* (Rohlich et al. 2000). The greatest percentage (up to 53% in *Xenopus*) of the retina is made up of rods with 97-98% being 'red' rods with the proportion of 'green' rods being much lower. The proportion of LWS cone photoreceptors is greater than SWS2 rods and individual SWS1 cones with the lowest concentration. The various photoreceptor types are distributed evenly across the retina (Rohlich et al. 2000).

Immunohistochemical analysis on subspecies of proteus salamander, *Proteus anguinus*, showed a varying degree of reaction with opsin antibodies depending on the

morphological rudimentation of the eye (Kos et al. 2001). The black proteus, *Proteus anguinus parkelj,* with a well developed 'normal' eye structure, has principle (red) rods (40-50% of photoreceptors) , no green rods, and two types of cone, potentially M/LWS (50-58% of photoreceptors) and VS/UVS. Blind cave salamanders, *Proteus anguinus anguinus*, from the Planina cave contained red-sensitive cone opsin and rod opsin. However, only rod opsin could be detected in *Proteus anguinus anguinus* from the Otovec doline. The eye of salamanders from this region has regressed and has no discernable photoreceptor outer segment (Kos et al. 2001).

Porphyropsins can occur in amphibians, that appear to vary in proportion depending on ontogeny. Larger proportions of porphyropsin are found in larval stages (Crescitelli 1958 Wilt, 1959; Reuter, 1969). During metamorphosis the relative amount of porphyropsin decreases until the photoreceptors contained mostly the rhodopsin. This phenomenon has been measured in *Rana temporaria* (Reuter 1969). In contrast the paedomorphic mudpuppy, *Necturus* sp., which reaches sexual maturity whilst retaining larval characteristics, has mainly porphyropsin in the retina (Brown et al. 1968). There were contradicting reports over whether *Xenopus laevis* had porphyropsin throughout its life or whether rhodopsin is present in the larval forms (Crescitelli 1973, Wald 1955). Consensus in the latest research states that only porphyropsin is present (Bridges et al. 1977, Witkovsky 2000). In contrast *Bufo bufo* has predominately rhodopsin at the larval tadpole stage and retains this for the adult stage (Muntz and Reuter, 1966). Porphyropsins will have a λmax shifted towards longer wavelengths compared to rhodopsins (Crescitelli 1972).

4.1.3 Caecilians visual pigments.

Electroretinograms have detected one class of opsin (Himstedt 1995) in the caecilian *Ichthyophis* cf. *kohtoaensis*. This is to be assumed to be rod opsin due to the λmax value at 502nm. The skin that covers the eye was shown also to absorb maximally at 410nm, shifting the peak spectral absorbance to longer wavelengths of near 520-530nm(Himstedt 1995).

Fragments of rod opsin sequence from four species of caecilian have been published. The species are *Rhinatrema bivitattum*, *Herpele squalostoma*, *Schistometopum thomense* (Frost et al. 2006) and *Typhlonectes natans* (Venkatesh et al. 2001). This species sampling represent derived and primitive caecilians that fragments have been identified from, it suggests rod opsin is present throughout the Gymnophiona. Immunohistochemical measurements were made on wide ranging species showing differing degrees of rudimentation in the eye using antibodies to rod opsin, transducin, and cone opsin (Nguyen 2003). The species examined are *Ichthyophis* cf. *kohtoaensis*, *Typhlonectes* sp. *Dermophis mexicanus*, *Geotrypetes seraphinii*, *Herpele squalostoma* and *Boulengerula taitanus*. Strong reactions were observed with rod antibodies CERN 886 and 858 and weaker reactions occurred with one cone antibody CERN 874. However CERN 874, an antibody raised against chicken cone opsin, can react with rod opsin (Nguyen 2003; Foster et al. 1993). This cross-reaction occurs because of similarities in the structure of opsins, the strength of reaction depending on the species involved (Rohlich and Szel 2000). CERN 9412, an antibody to bovine transducin, reacted positively suggesting that the phototransduction pathway is intact in the retina of

caecilians. No clear evidence of cone photoreception was therefore obtained using this method.

4.2 Objectives

1.To determine which visual pigments are expressed in the retina of caecilians.

2. To determine the nucleotide sequences of opsins expressed in the retina of Gymnophiona.

3. To determine the λmax of such pigments using in situ methods.

4.3. Results

4.3.1. Microspectrophotometry (MSP)

4.3.1.1. Microspectrophotometry (MSP) of photoreceptor cells in retina

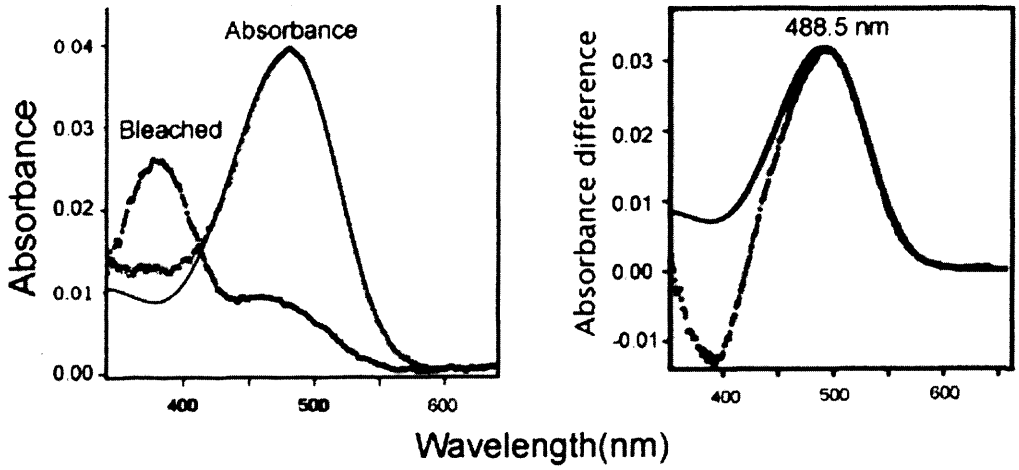
MSP measures directly from the retina the λmax of visual opsins expressed in the outer segments of photoreceptors.

Figure 4.1 (next page) shows similar peak absorbance measurements for the three caecilian. The values 488.5nm ± 0.9nm from 31 photoreceptor cells of *Typhlonectes natans*, for *Rhinatrema bivittatum* 487.5nm ± 1.0 nm from 13 cells, and 486.6nm ± 2.2nm from 8 cells of *Geotrypetes seraphinii* were measured. The species were specifically selected from different families and diversity of Gymnophiona. *Rhinatrema bivittatum* is considered a primitive caecilian and is regularly used as the sister group of other caecilians in analyses. *Typhlonectes natans* is an aquatic caecilian that is readily available through the pet trade. *Geotrypetes seraphinii* is a member of the caecilid family of caecilians. Both *T. natans* and *G. seraphinii* are considered relatively derived

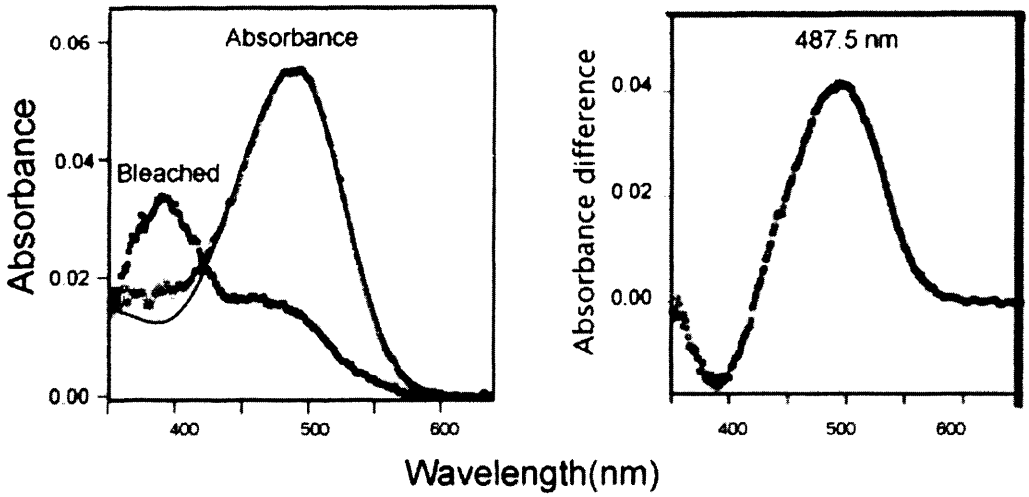
caecilians with well developed eyes but not closely related. The MSP results demonstrate there are functional visual pigments in the caecilians analysed.

Figure 4.1 (next page) MSP absorbance spectra values for the caecilian species *Typhlonectes natans*, *Rhinatrema bivitattum* and *Geotrypetes seraphinii*. Upon illumination, bleaching, the absorbance peak disappears and is replaced by a peak at 365nm. This represents the oxime (stabilised form of the bleached photopigment chromophore) of the liberated retinal. The difference spectra between bleached and absorbance spectra for each species is shown on the right.

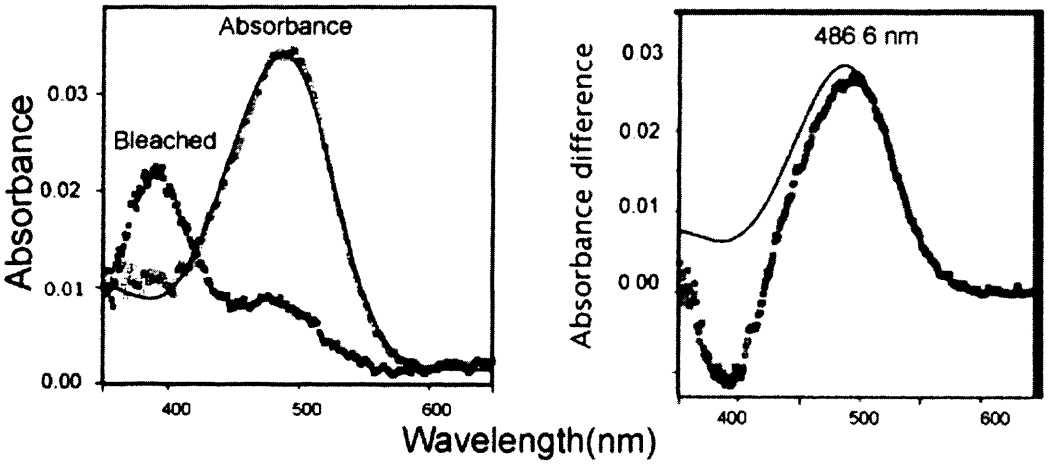
Typhlonectes natans



Rhinatrema bivatattum



Geotrypetes seraphinii



4.3.1.2 MSP to Measure Transmission of Light through Skin Covering the Eye in *Typhlonectes natans*

Fig 4.2 (below) shows the transmission of light through the skin above the eye of *Typhlonectes natans*. The skin was dissected from the area above the eye that appears transparent (see Chapter 3).

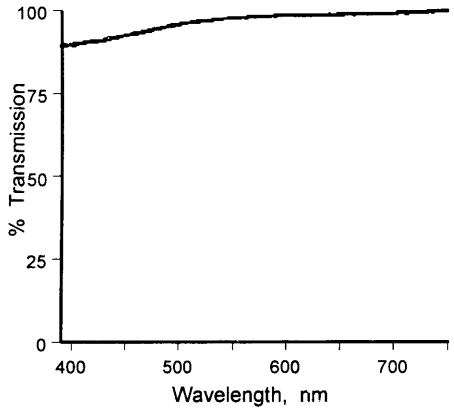


Figure 4.2 MSP transmission spectrum values through skin covering eye of *Typhlonectes natans*. This shows that the skin above the eye is almost transparent but there is a small amount of absorbance at shorter wavelengths..

4.3.2 Isolation of opsin sequences from cDNA

To identify the pigment responsible for the MSP results the opsin sequences expressed in the retina were isolated from *Typhlonectes natans*, and from *Ichthyophis* cf. *kohtoaensis*, a relatively primitive caecilian for which cDNA was available. The λmax had been measured in *Typhlonectes natans*. Rod opsin was determined to be the causal visual pigment by measurement of the expression of the protein in two species of the caecilian,

Typhlonectes natans. The opsin sequence was also found to clade with other vertebrate rod opsins as shown in chapter 5. The results for *Ichthyophis* cf. *kohtoaensis* was similar suggesting that throughout caecilians the opsin expressed in the retina is rod opsin.

The details of the method of how the opsin sequences are in the Materials and Methods chapter but a summary is described here.

To isolate the visual pigments responsible for the MSP results, cDNA was created from mRNA from the eyes of two species of caecilian, *Ichthyophis* cf. *kohtoaensis* and *Typhlonectes natans*. Fragments of opsin were amplified from the cDNA using degenerate primers designed from an alignment of vertebrate opsins. These have picked up all classes of visual pigments in a wide range of vertebrates from lamprey (Davies et al. 2007a)to platypus (Davies et al. 2007b). A nested protocol was used in two rounds to pick up sequences from exon 1 to exon 4 (see Fig. 4.3 gel photos) From the sequences obtained (Table 4.2) specific primers were designed for 5' and 3' RACE to isolate the untranslated ends of the rod opsin sequence. These PCR experiments utilized degenerate primers used successfully in other vertebrates (Carvalho et al. 2006; Davies et al. 2007a; b). Further PCR experiments used primers designed from amphibian cone pigment sequences in attempts to isolate cone fragments from different classes.

Table 4.1 Degenerate primers pairs used to isolate visual pigments.

	Primer sequence(5'-3')
All Opsin F1	CCGCGAGAGATACATNGTNRTNTGYAARCC
All Opsin F2	ATTTTAGAAGGTCTGCCRGWSNTCNTGYGG
All Opsin R1	ATTGGTCACCTCCTTYTCNGCYYTYTGNGT
All Opsin R2	CCCGGAAGACGTAGATGANNGGRTTRWANA

Table 4.2 Primers used for 3' and 5' RACE.

Primer sequence(5'-3')	
RACE 5' R1	AACCAAGAATAATCAACAAAAACATGTAGG
RACE 5' R2	AAGTTGCTCATGGGTTTACAGACCACTACG
RACE 3' F1	CGATGGTCCAGGTATATCCCAGAGGGTATG
RACE 3' F2	TTCCACTGCTCATCATTTTCTTCTGCTACG

Figure 4.3 Electrophoresis gel photos of the amplification of caecilian rod opsin genes.

a-d *Ichthyophis* cf. *kohtoaensis*

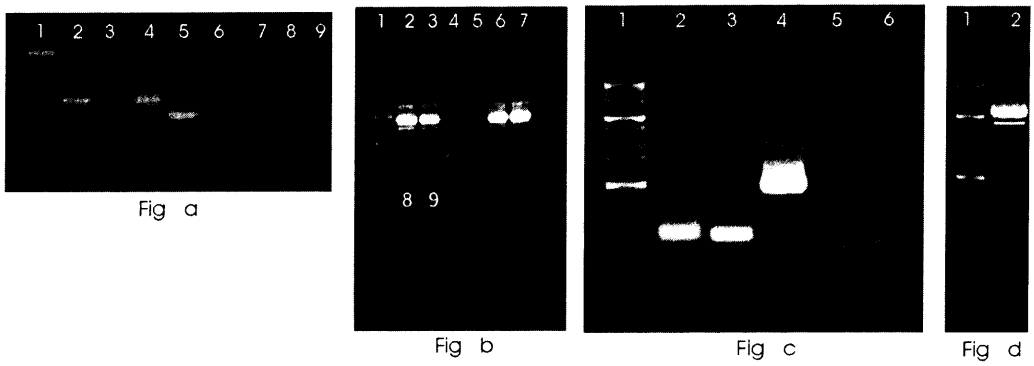


Figure 4.3a. *Ichthyophis* cf. *kohtoaensis* Second round PCR products of opsin using degenerate Allopsin primer pairs from Table 1. annealing temperature 50°C. Lanes 1-9 1.Hyperladder 1(Bioline), 2. 1st round F1 and R2 2nd round F1 and R1 (363bp) 3.1st round F1-R2: 2nd round F2 and R2 (393bp) 4.1st round F1-R1 2nd round F1-R1 (363bp) 5. 1st round F2-R2 and 2nd F2-R1 (223 bp.) 6.-ve of reaction 1st round F1 and R2 2nd round F1 and R1 7. –ve 1st round F1-R2 2nd round F2 and R2 8. –ve of 1st round F1-R1 2nd round F1-R1 9. –ve (no DNA) 1st round F2-R2 and 2nd F2-R1

Figure 4.3b. 3' RACE PCR of *Ichthyophis* cf. *kohtoaensis* 1.Hyperladder 2 (Bioline) 2.1st round 50°C 2nd 55°C 3.1st round 50°C 2nd 60°C 4. –ve of 1st round 50°C 2nd 55°C 5. –ve 1st round 50°C 2nd 60°C 6.1st round 55°C 2nd 55°C 7. 1st round 55°C 2nd 60°C 8. –ve 1st round 55°C 2nd 55°C 9.-ve 1st round 55°C 2nd 60°C

(Previous page)Figure 4.3c. 5' RACE PCR of *Ichthyophis* cf. *kohtoaensis* 1st round 50°C 2nd round 55°C 1.Hyperladder 2 (Bioline) 2. *I.kohtoaensis* 1st round 3. –ve 1st round 4. *I.kohtoaensis* 2nd round 5. –ve 1st and 2nd round 6. –ve 2nd round

Figure 4.3d PCR using KOD Taq polymerase to isolate complete coding region of rod opsin *Ichthyophis* cf. *kohtoaensis*. Annealing temp 60°C 1. Hyperladder 2 (Bioline). 2. *I.* cf. *kohtoaensis*

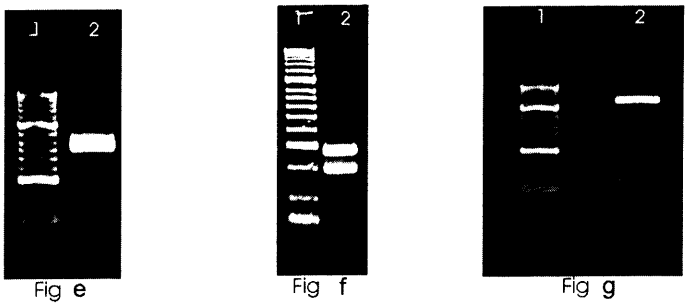


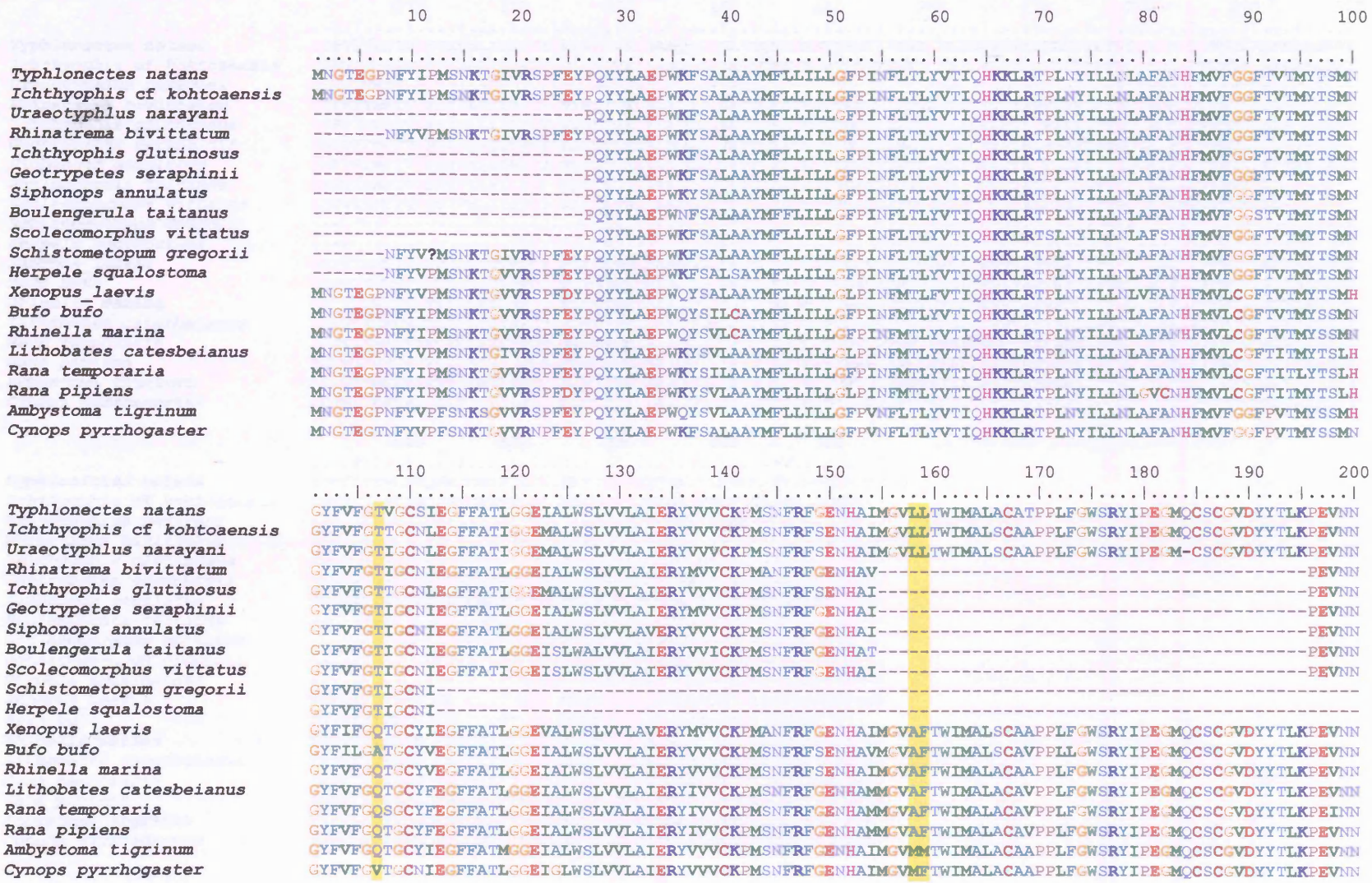
Figure 4.3 e-g *Typhlonectes natans*

Figure 4.3e 3'RACE 1. Hyperladder 2 (Bioline) 2.*T. natans*

Figure 4.3f 5'RACE 1. Hyperladder 2 (Bioline) 2.*T. natans*

Figure 4.3g PCR using KOD Taq polymerase to isolate complete coding region of rod opsin

Figure 4.4 (next page) Alignment of caecilian amino acid sequences with amphibian opsins. Sequences from *Ichthyophis* cf. *kohtoaensis* and *Typhlonectes natans* isolated as stated in section 4.3.2. Fragments of rod opsin sequences from caecilians *U. narayani*, *R. bivittatum*, *I. glutinosus*, *G. seraphinii*, *S. annulatus*, *B. taitanus* and *S. vittatus* isolated as stated in 5.3.2 (Chapter5). Rod opsin sequences from frogs; *Bufo bufo*, *Xenopus laevis*, *Rhinella marina*, *Rana catesbiena*, *Rana temporaria*, *Rana pipiens*, salamanders; *Ambystoma tigrinum* and *Cynops pyrrhogaster*, and caecilians *S. gregorii* and *H. squalostoma* were downloaded from NCBI database (Accession numbers in appendix) Sites 107, 158, 159, 337, 352 highlighted in yellow are conserved in caecilian opsins but differ from other amphibians and therefore may be responsible for the spectral tuning shift observed.


10 20 30 40 50 60 70 80 90 100
Typhlonectes natans
Ichthyophis cf kohtoaensis
Uraeotyphlus narayani
Rhinatrema bivittatum
Ichthyophis glutinosus
Geotrypetes seraphinii
Siphonops annulatus
Boulengerula taitanus
Scolecomorphus vittatus
Schistometopum gregorii
Herpele squalostoma
Xenopus_laevis
Bufo bufo
Rhinella marina
Lithobates catesbeianus
Rana temporaria
Rana pipiens
Ambystoma tigrinum
Cynops pyrrhogaster
110 120 130 140 150 160 170 180 190 200
Typhlonectes natans
Ichthyophis cf kohtoaensis
Uraeotyphlus narayani
Rhinatrema bivittatum
Ichthyophis glutinosus
Geotrypetes seraphinii
Siphonops annulatus
Boulengerula taitanus
Scolecomorphus vittatus
Schistometopum gregorii
Herpele squalostoma
Xenopus laevis
Bufo bufo
Rhinella marina
Lithobates catesbeianus
Rana temporaria
Rana pipiens
Ambystoma tigrinum
Cynops pyrrhogaster

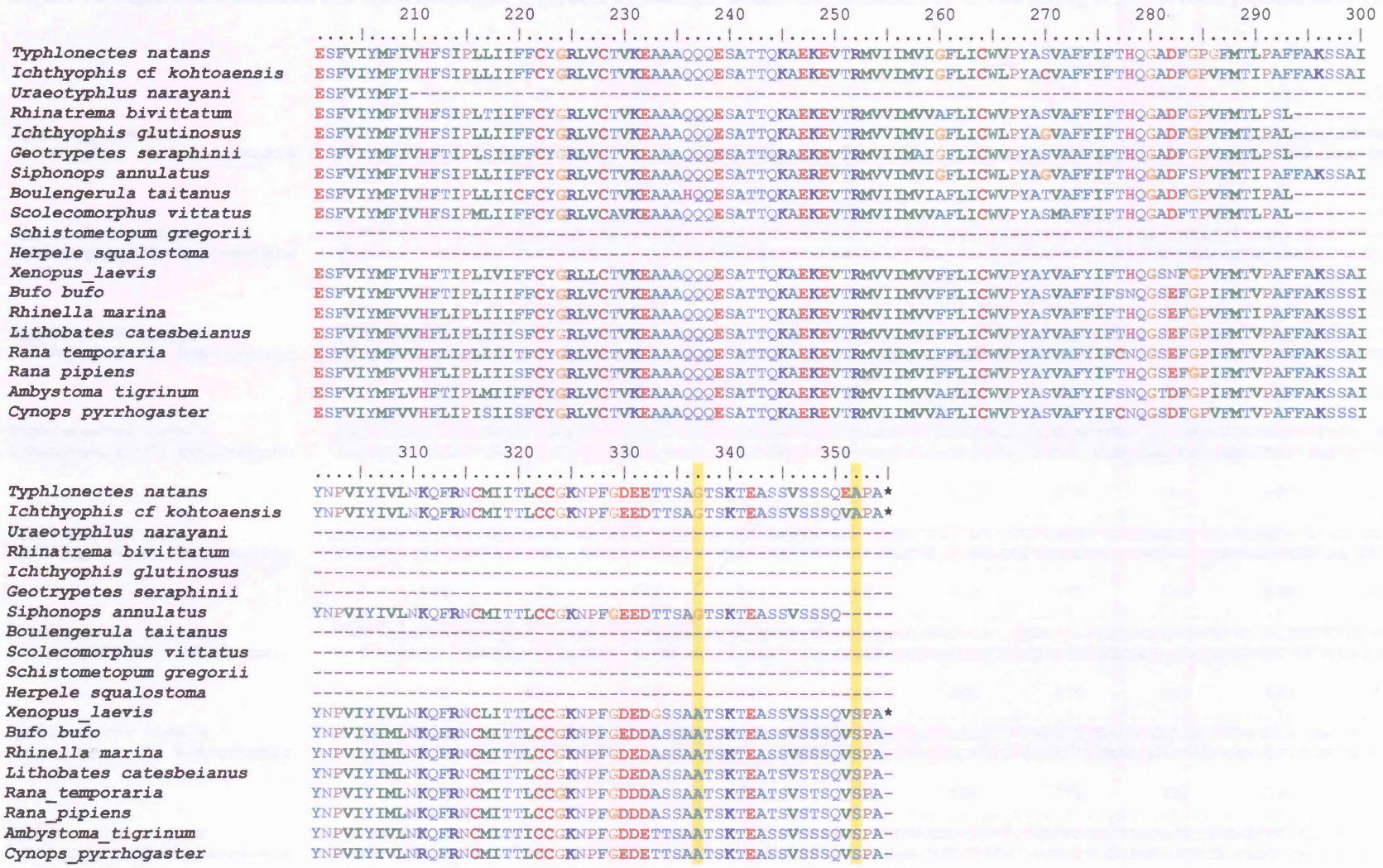
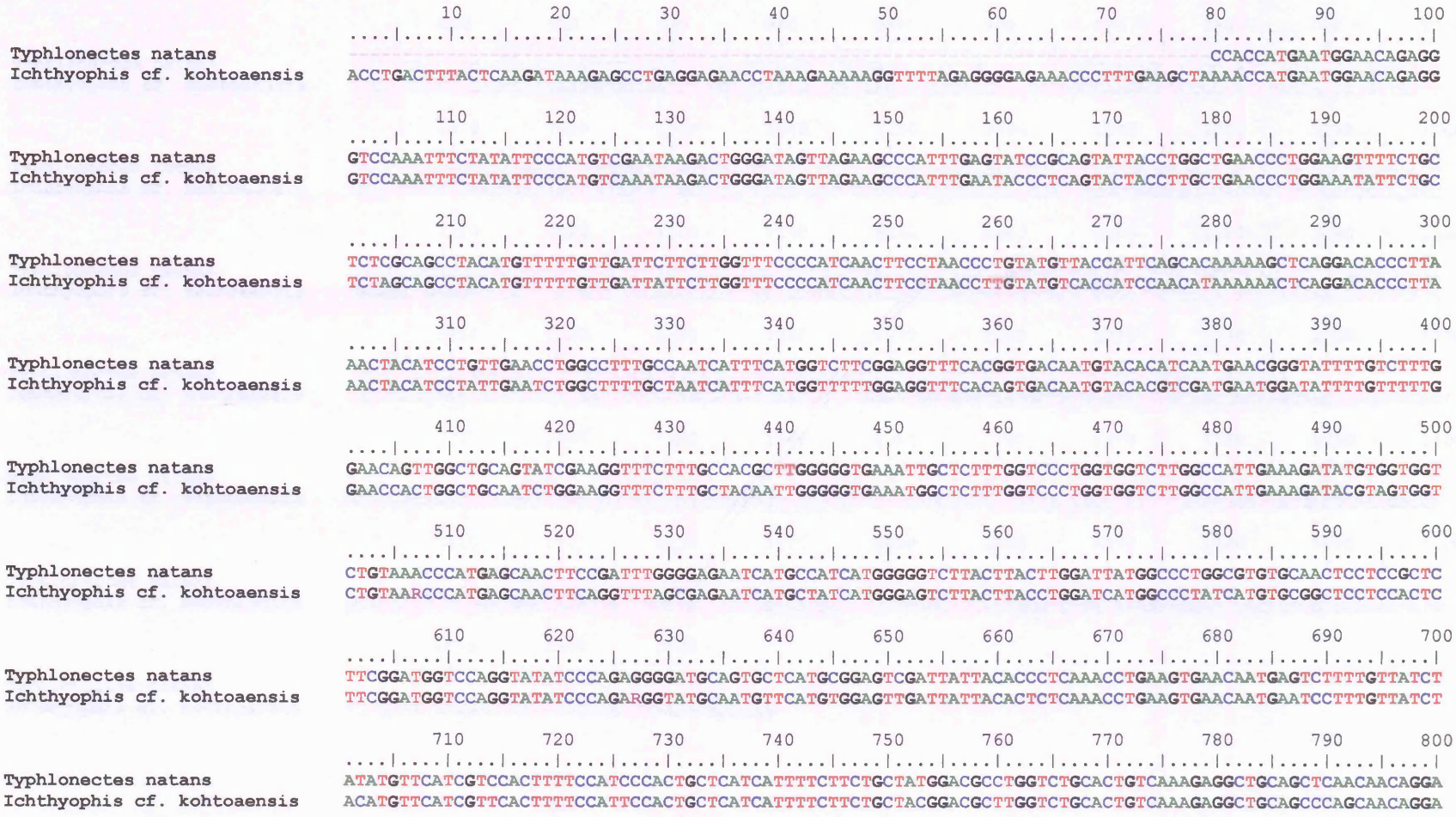
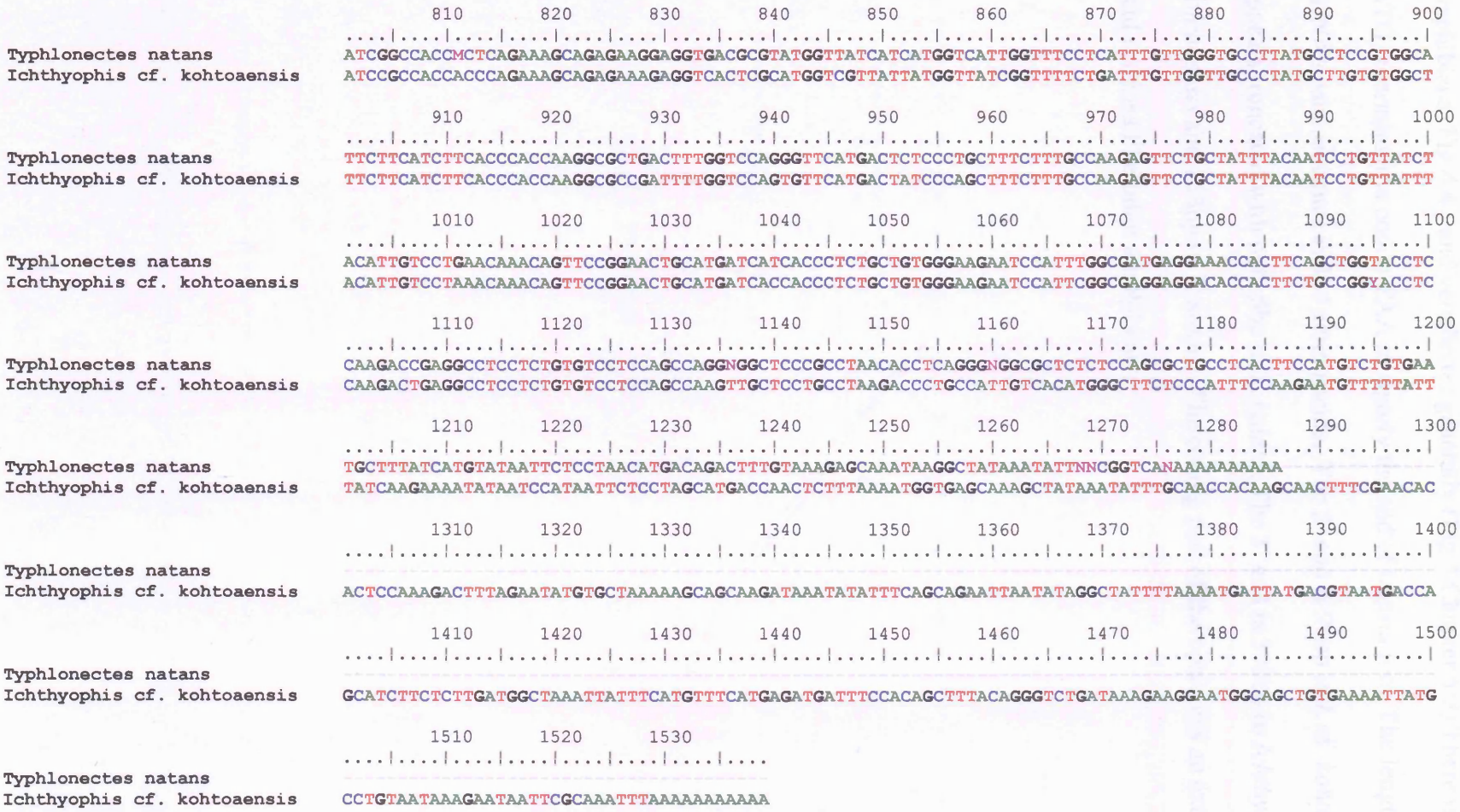

210 220 230 240 250 260 270 280 290 300
Typhlonectes natans ESFVIYMFIVHFSIPLLIIFFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVIIMVIGFLICWVPYASVAFFIFTHQGADFGPGFMTLPAFFAKSSAI
Ichthyophis cf kohtoaensis ESFVIYMFIVHFSIPLLIIFFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVVIMVIGFLICWLPYACVAFFIFTHQGADFGPVFMTIPAFFAKSSAI
Uraeotyphlus narayani ESFVIYMFI----------
Rhinatrema bivittatum ESFVIYMFIVHFSIPLTIIFFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVVIMVVAFLICWVPYASVAFFIFTHQGADFGPVFMTLPSL-------
Ichthyophis glutinosus ESFVIYMFIVHFSIPLLIIFFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVVIMVIGFLICWLPYAGVAFFIFTHQGADFGPVFMTIPAL-------
Geotrypetes seraphinii ESFVIYMFIVHFTIPLSIIFFCYGRLVCTVKEAAAQQQESATTQRAEKEVTRMVIIMAIGFLICWVPYASVAAFIFTHQGADFGPVFMTLPSL-------
Siphonops annulatus ESFVIYMFIVHFSIPLLIIFFCYGRLVCTVKEAAAQQQESATTQKAEREVTRMVVIMVIGFLICWLPYAGVAFFIFTHQGADFSPVFMTIPAFFAKSSAI
Boulengerula taitanus ESFVIYMFIVHFTIPLLIICFCYGRLVCTVKEAAAHQQESATTQKAEKEVTRMVIIMVIAFLICWVPYATVAFFIFTHQGADFGPVFMTIPAL-------
Scolecomorphus vittatus ESFVIYMFIVHFSIPMLIIFFCYGRLVCAVKEAAAQQQESATTQKAEKEVTRMVIIMVVAFLICWVPYASMAFFIFTHQGADFTPVFMTLPAL-------
Schistometopum gregorii ----------
Herpele squalostoma ----------
Xenopus_laevis ESFVIYMFIVHFTIPLIVIFFCYGRLLCTVKEAAAQQQESATTQKAEKEVTRMVVIMVVFFLICWVPYAYVAFYIFTHQGSNFGPVFMTVPAFFAKSSAI
Bufo bufo ESFVIYMFVVHFTIPLIIIFFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVIIMVVFFLICWVPYASVAFFIFSNQGSEFGPIFMTVPAFFAKSSSI
Rhinella marina ESFVIYMFVVHFLIPLIIIFFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVIIMVVFFLICWVPYASVAFFIFTHQGSEFGPVFMTIPAFFAKSSSI
Lithobates catesbeianus ESFVIYMFVVHFLIPLIIISFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVVIMVIFFLICWVPYAYVAFYIFTHQGSEFGPIFMTVPAFFAKSSAI
Rana temporaria ESFVIYMFVVHFLIPLIIITFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVIIMVIFFLICWVPYAYVAFYIFCNQGSEFGPIFMTVPAFFAKSSAI
Rana pipiens ESFVIYMFVVHFLIPLIIISFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVIIMVIFFLICWVPYAYVAFYIFTHQGSEFGPIFMTVPAFFAKSSAI
Ambystoma tigrinum ESFVIYMFLVHFTIPLMIIFFCYGRLVCTVKEAAAQQQESATTQKAEKEVTRMVIIMVVAFLICWVPYASVAFYIFSNQGTDFGPIFMTVPAFFAKSSAI
Cynops pyrrhogaster ESFVIYMFVVHFLIPISIISFCYGRLVCTVKEAAAQQQESATTQKAEREVTRMVIIMVVAFLICWVPYASVAFYIFCNQGSDFGPVFMTVPAFFAKSSSI
310 320 330 340 350
Typhlonectes natans YNPVIYIVLNKQFRNCMIITLCCGKNPFGDEETTSAGTSKTEASSVSSSQEAPA*
Ichthyophis cf kohtoaensis YNPVIYIVLNKQFRNCMITTLCCGKNPFGEEDTTSAGTSKTEASSVSSSQVAPA*
Uraeotyphlus narayani ----------
Rhinatrema bivittatum ----------
Ichthyophis glutinosus ----------
Geotrypetes seraphinii ----------
Siphonops annulatus YNPVIYIVLNKQFRNCMITTLCCGKNPFGEEDTTSAGTSKTEASSVSSSQ-----
Boulengerula taitanus ----------
Scolecomorphus vittatus ----------
Schistometopum gregorii ----------
Herpele squalostoma ----------
Xenopus_laevis YNPVIYIVLNKQFRNCLITTLCCGKNPFGDEDGSSAATSKTEASSVSSSQVSPA*
Bufo bufo YNPVIYIMLNKQFRNCMITTLCCGKNPFGEDDASSAATSKTEASSVSSSQVSPA-
Rhinella marina YNPVIYIMLNKQFRNCMITTLCCGKNPFGDEDASSAATSKTEASSVSSSQVSPA-
Lithobates catesbeianus YNPVIYIMLNKQFRNCMITTLCCGKNPFGDEDASSAATSKTEATSVSTSQVSPA-
Rana_temporaria YNPVIYIMLNKQFRNCMITTLCCGKNPFGDDDASSAATSKTEATSVSTSQVSPA-
Rana_pipiens YNPVIYIMLNKQFRNCMITTLCCGKNPFGDDDASSAATSKTEATSVSTSQVSPA-
Ambystoma_tigrinum YNPVIYIVLNKQFRNCMITTICCGKNPFGDDETTSAATSKTEASSVSSSQVSPA-
Cynops_pyrrhogaster YNPVIYIVLNRQFRNCMITTLCCGKNPFGEDETTSAATSKTEASSVSSSQVSPA-

Figure 4.5Alignment of caecilian rod opsin nucleotide sequences including 5' and 3' untranslated ends.. The coding region is from position 85 to 1128.




810 820 830 840 850 860 870 880 890 900
Typhlonectes natans ATCGGCCACCMCTCAGAAAGCAGAGAAGGAGGTGACGCGTATGGTTATCATCATGGTCATTGGTTTCCTCATTTGTTGGGTGCCTTATGCCTCCGTGGCA
Ichthyophis cf. kohtoaensis ATCCGCCACCACCCAGAAAGCAGAGAAAGAGGTCACTCGCATGGTCGTTATTATGGTTATCGGTTTTCTGATTTGTTGGTTGCCCTATGCTTGTGTGGCT
910 920 930 940 950 960 970 980 990 1000
Typhlonectes natans TTCTTCATCTTCACCCACCAAGGCGCTGACTTTGGTCCAGGGTTCATGACTCTCCCTGCTTTCTTTGCCAAGAGTTCTGCTATTTACAATCCTGTTATCT
Ichthyophis cf. kohtoaensis TTCTTCATCTTCACCCACCAAGGCGCCGATTTTGGTCCAGTGTTCATGACTATCCCAGCTTTCTTTGCCAAGAGTTCCGCTATTTACAATCCTGTTATTT
1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
Typhlonectes natans ACATTGTCCTGAACAAACAGTTCCGGAACTGCATGATCATCACCCTCTGCTGTGGGAAGAATCCATTTGGCGATGAGGAAACCACTTCAGCTGGTACCTC
Ichthyophis cf. kohtoaensis ACATTGTCCTAAACAAACAGTTCCGGAACTGCATGATCACCACCCTCTGCTGTGGGAAGAATCCATTCGGCGAGGAGGACACCACTTCTGCCGGYACCTC
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
Typhlonectes natans CAAGACCGAGGCCTCCTCTGTGTCCTCCAGCCAGGNGGCTCCCGCCTAACACCTCAGGGNGGCGCTCTCTCCAGCGCTGCCTCACTTCCATGTCTGTGAA
Ichthyophis cf. kohtoaensis CAAGACTGAGGCCTCCTCTGTGTCCTCCAGCCAAGTTGCTCCTGCCTAAGACCCTGCCATTGTCACATGGGCTTCTCCCATTTCCAAGAATGTTTTTATT
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
Typhlonectes natans TGCTTTATCATGTATAATTCTCCTAACATGACAGACTTTGTAAAGAGCAAATAAGGCTATAAATATTNNCGGTCANAAAAAAAAAA
Ichthyophis cf. kohtoaensis TATCAAGAAAATATAATCCATAATTCTCCTAGCATGACCAACTCTTTAAAATGGTGAGCAAAGCTATAAATATTTGCAACCACAAGCAACTTTCGAACAC
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
Typhlonectes natans
Ichthyophis cf. kohtoaensis ACTCCAAAGACTTTAGAATATGTGCTAAAAAGCAGCAAGATAAATATATTTCAGCAGAATTAATATAGGCTATTTTAAAATGATTATGACGTAATGACCA
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
Typhlonectes natans
Ichthyophis cf. kohtoaensis GCATCTTCTCTTGATGGCTAAATTATTTCATGTTTCATGAGATGATTTCCACAGCTTTACAGGGTCTGATAAAGAAGGAATGGCAGCTGTGAAAATTATG
1510 1520 1530
Typhlonectes natans
Ichthyophis cf. kohtoaensis CCTGTAATAAAGAATAATTCGCAAATTTAAAAAAAAAAA

The alignments shows the caecilian rod opsin sequences are consistent with that of other amphibians (Fig.4.4) and vertebrates generally (Fig 5 Chapter 5.3) There is a start codon ATG, a termination codon TAA to signify the end of translation. The length of the cDNA opsin sequence is 354 amino acids. The 5' end is 99bp in *I.* cf. *kohtoaensis* and appears truncated with only 5bp in *T. natans*. The 3' end is 390bp in *Ichthyophis* cf. *kohtoaensis* and 133bp in *T. natans*. The coding part of the opsin has an extra 5 amino acid residues like other amphibians.

Table 4.3 Table of amino acid residues of structural importance in rod opsin gene and presence in caecilian sequences. Bovine rhodopsin numbering. ✓ = site is present ?=site has not been sequenced. ***Tn=Typhlonectes natans, Ik=Ichthyophis*** **cf.** ***kohtoaensis, Rb=Rhinatrema bivitattum, Gs=Geotrypetes seraphinii, Un=Uraeotyphlus nayarani, Ig=Ichthyophis glutinosus, Sa=Siphonops annulatus, Bt=Boulengerula taitanus, Sv=Scolecomorphus vitattus, Sg=Schistometopum gregorii*** **and** ***Hs=Herpele squalostoma.***

Site Sp.	Lys 296	Glu 113	Glu-Arg-Tyr 134-136	His 65	His 152	His 211	Gly 121	Pro 171	Pro 215	Pro 267	Pro/Gly 291	Pro 303	Cys 110	Cys 185 187	Cys 322-323
Tn.	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Ik.	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rb.	✓	✓	✓	✓	✓	✓	✓	?	✓	✓	✓	✓	✓	?	?
Gs.	✓	✓	✓	✓	✓	✓	✓	?	✓	✓	✓	✓	✓	?	?
Un.	?	✓	✓	✓	✓	✓	?	✓	?	?	?	?	✓	✓	?
Ig.	✓	✓	✓	✓	✓	✓	✓	?	✓	✓	✓	✓	✓	?	?
Sa.	✓	✓	✓	✓	?	✓	?	?	✓	✓	✓	✓	✓	?	✓
Bt.	✓	✓	✓	✓	✓	✓	✓	?	✓	✓	✓	✓	✓	?	?
Sv.	✓	✓	✓	✓	✓	✓	✓	?	✓	✓	✓	✓	✓	?	?
Sg.	?	?	?	✓	?	?	?	?	?	?	?	?	✓	?	?
Hs.	?	?	?	✓	?	?	?	?	?	?	?	?	✓	?	?

The Lys-296 residue is the site for the Schiff's base linkage with the chromophore. Site

Glu-113 serves as a counterion forming a salt bridge with Lys-296. The ERY (Glu-134, Arg –135, Tyr-136) motif thought to be critical for G-protein (transducin) activation is present. Histidine residues at sites His-65, His-152 and His 211 are thought to be critical in regulation of the transition from metarhodopsin I to metarhodopsin II are conserved. Other sites are found to be invariant proline and glycine sites Gly-121, Pro 171, Pro 215, Pro 267, Pro/Gly 291 and Pro 303. Additionally the carboxyl region of this protein is extremely rich in serines (7 residues) and threonines (four residues) these residues are the potential sites of phosphorylation by rhodopsin kinase and are required for inactivation of photoactivated rhodopsin, metarhodopsin II, and for binding of a regulatory protein arrestin. The coding region has been completed for *Ichthyophis cf. kohtoaensis* and *Typhlonectes natans*. These species have the amino acid residues expected in functioning rod opsin gene. In the third transmembrane region, Glu-113 serves as a counterion to stabilize the inactive opsin molecule forming a salt bridge with Lys-296. Conserved cysteines are found at sites 110, 185, 187, 322 and 323. Sites 110, 185 and 187 are involved in disulphide bond formation and sites 322 and 323 are thought to be involved in a palmitoylation regulatory role. These structurally critical residues are present and no nonsense mutations occur in all caecilians sequenced suggesting a functioning visual pigments are present.

4.3.3 Expression of visual pigment gene

The complete rod opsin sequence of *Typhlonectes natans* and *Ichthyophis* cf. *kohtoaensis* was amplified using a proofreading Taq polymerase in the PCR reaction (Fig 4.3d and g). The primer pairs were designed for the 5' and 3' ends with restriction site tags for EcoR1 and Sal1. The digested PCR products were then directionally cloned into a derivative of

the mammalian expression vector pMT4 carrying the sequence of bovine rod opsin 1D4 epitope (including the stop codon) at the 3'end of the coding sequence. HEK293T cells were transfected with vector containing the opsin insert. These cells were harvested after 48 hours and washed with PBS buffer. Pigments were generated by suspending the cells in PBS buffer and incubating with 40 μM 11-cis-retinal in the dark. The 11-cis-retinal is dissolved in ETOH. The pigment was solubilized from cell membranes by incubating with buffer containing detergent, then purified by immuno affinity chromatography using a monoclonal antibody directed against the 1D4 epitope of bovine rod opsin. The dark and the bleached absorption spectra were measured and the difference between these two peaks obtained. The data was fitted to a Govardovski A1 template using an Excel program. The absorption spectra were measured and found to be of a same value 493nm for both caecilians (Fig. 3).

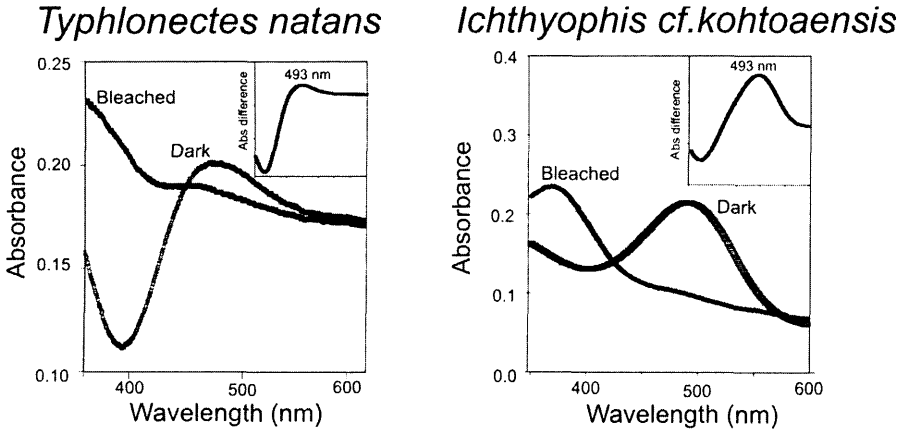


Figure 4.6 Expression absorbance spectrum values for the caecilian species *Typhlonectes natans* and *Ichthyophis cf. kohtoaensis*. Top right inset is difference spectra between bleached and absorbance spectra for each species.

A spectral shift has occurred from an average of 500nm for rod opsin other amphibians to an average of 487.5nm for caecilians. The maximum wavelength of absorbance is similar to that of deep sea dwelling fish. Rhodopsins form of rod opsin are around 500nm. The porphyropsins are long wave or red shifted with values around 523nm. The proportions of porphyropsins and rhodopsins can vary with development and can also be co-expressed in a single rod (Chen et al. 1996). Referring to the alignment of amphibian rod opsin sequences (Fig 4.4) specific amino acid residues is consistent in caecilians but not other amphibians. These are potential spectral tuning sites. However only one of these sites is within the retinal binding site at 158 in helix 4 (Fig 4.7).

The amino acid sites, 83, 122, 207, 211,265, 292, 295, known to be spectral tuning sites for RH pigments see fig 4.7(Davies et al. 2007a), are identical in all amphibians including caecilians and are not responsible for the apparent spectral shift detected. The sites 107, 158, 159, 337 and 352 (highlighted in yellow in Fig 4.4 and red circles in Fig 4.7) are conserved in caecilians measured in vitro or in vivo, *Geotrypetes seraphinii, Rhinatrema bivittatum, Ichthyophis* cf. *kohtoaensis and Typhlonectes natans*, but differ from other amphibians and are potential spectral tuning sites.

Figure 4.7 (Modified from Davies et al. 2007b). Diagram of potential spectral tuning sites in rod opsin molecules expressed in the retina of caecilians. A schematic representation of the three-dimensional structure of a visual pigment with residues conserved in caecilians shown in red. The figure shows the opsin seven transmembrane domains (TMD I-VII), 3 extracellular domains (ECD I-III), 3 intracellular domains (ICD I-III), amino- and carboxy-termini (N or C), and the retinal chromophore. Amino acids are represented by circles. The black circles represent residues shown to influence the spectral sensitivity of vertebrate pigments. The green circles in particular show sites that influence RH visual pigments (Davies et al. 2007b, Yokoyama 2000). The red circles represent the changes non-synonymous amino acid changes in caecilians compared to other amphibians. Grey residues are critical for maintaining protein structural integrity.

4.3.4 In situ-hybridization.

Visualising the expression of the rod opsin gene in the eye of *Typhlonectes natans* was attempted using in situ-hybridization techniques. In situ hybridization used targeted probes constructed using the specific opsin nucleic acid sequence to label the photoreceptor cell (Rohlich et al. 2000). This unfortunately did not reveal any results as no staining was obtained by the NBT/BCIP method (see Materials and Methods chapter). The Cryosections were probed using a 300bp fragment of rod opsin of *Typhlonectes natans* did not reveal positive results for the control. No inferences can be made about visual pigment localization and subsequent tests using amphibian probes from cones were not attempted.

4.3.5 Detection of cone visual opsin classes by construction of cDNA library, PCR and Southern blot techniques.

PCR techniques on cDNA and genomic DNA of *Typhlonectes natans*, *Ichthyophis cf. kohtoaensis* and on genomic of *Rhinatrema bivittatum,* considered to be a basal caecilian and most likely to have a full complement of visual pigments, isolated no cone opsin sequences. Primers were designed from alignments of all the cone opsins primers were class specific.

A southern blot was also attempted in order to identify cone opsin in the caecilian genomic DNA. However no positive reactions were visible using probes of *Ichthyophis* and *Typhlonectes* rod opsin and subsequent testing using probes constructed using *Xenopus* cone opsin fragments were not then attempted.

The presence of cone visual pigments cannot be ruled out because of the negative results from the Southern blot and PCR techniques.

A poor quality cDNA library was made using *Ichthyophis* cf. *kohtoaensis* eyes and brain, It was possible to identify from sequencing that caecilian eye genes were present but after screening of plates, no opsin was detected.

4.4 Discussion

4.4.1 Summary of findings.

Rod opsin is the only visual pigment found to be expressed and in the caecilian genome and it does appear that the cone opsins have been lost. The λmax is greater in the reconstituted opsin results than from the MSP in *Typhlonectes natans* by 4.5nm. However, small differences between *in situ* and *in vitro* values are not uncommon (Pointer et al. 2007). The sequence identity of the protein used in the reconstitution and the fragments from PCR of genomic DNA, determined that the only opsin sequences obtained from the caecilians were that of rod opsin. The sequences were identified using BLAST against published opsin sequences from the NCBI database and in the phylogenetic analysis with other vertebrate opsins (Chapter 5). Only one class of opsin was found using MSP and therefore the opsin responsible is confirmed to be RH1. It is possible that the MSP could have been from an RH2 class of opsin that can have a λ_{max} value between 490-510nm. (Yokoyama 2000) However, since no RH2 sequences were isolated using degenerate or class specific primers on caecilian cDNA and genomic DNA, and RH2 sequences not being found in other amphibians (Sakakibara et al. 2002) reduces the possibility that an RH2 opsin was responsible for the MSP result.

Measurement of the rod opsin absorbance reveals a spectral tuning shift compared to other amphibians. Sites known to cause a spectral shift in RH pigments are conserved in the Lissamphibia including caecilians. A different tuning mechanism is therefore responsible for this shift. Sites 107, 158, 159, 337 and 352 are conserved in caecilians rod opsin measured in vitro or in vivo, *Geotrypetes seraphinii, Rhinatrema bivittatum, Ichthyophis* cf. *kohtoaensis and Typhlonectes natans*, but differ to other amphibians and are potential spectral tuning sites. Fragments isolated from other caecilian species have these conserved sites when the potential tuning residue had been sequenced. Site 158 is within the retinal binding site and changes within this area are most likely to effect a spectral shift. Leucine is present at this site in caecilians, in the other amphibians is either a histidine seen in the frog sequences or alanine as seen in salamanders. Leucine and alanine have similar properties and are hydrophobic and charged but histidine is positively charged which might cause a change in spectral tuning. It is possible that one site or the overall effect of the 5 residues non-synonymous change has caused the change in visual pigment absorbance value. The way to test if these residues together or individually are to perform site directed mutagenesis, which incorporates the change in a primer then regeneration technique to measure the absorbance of the protein.

Rod opsin fragments are found in all species even in those that have a reduced eye morphology e.g. *Boulengerula*. No nonsense mutations appear to have been introduced into *B. taitanus*rod opsin sequence and suggests that it may be functional but like mole rats may have a photoperiodic function rather than a visual function (Nevo 1999).

The spectral shift may be due to the nocturnal habits but there is no evidence for a blue shift towards shorter wavelengths in nocturnal animals (Peichl 2005). The skin

above the eye appears to absorb slightly at shorter wavelengths which agrees with the analysis of Himstedt (1995) where the skin that covers the eye was shown also to absorb maximally at 410nm, shifting the peak spectral absorbance to longer wavelengths. A similar shift towards 500 nm may therefore occur in caecilians.

4.4.2 Comparison with other vertebrates

Vertebrates that live in dark conditions exhibit similar morphology, physiological and behavioural characteristics as seen in the caecilian visual system. Subspecies of the cave salamander *Proteus anguinus* show a similar morphological diversity and rudimentation of eyes (Kos et al. 2001). One population of *P. anguinus* in particular has appeared to have lost cone opsins but retained the rod opsin for scotopic vision. Similarly, most species of deep-sea fish have lost cone opsins and photoreceptors (Douglas et al 1998). Other blue shifted rod opsins are found in deep-sea fish (Hope et al. 1997). This degree of shift in caecilians has not been observed in any other terrestrial animal. As all caecilian species measured have a similar value, mutation in the nucleotide sequence occurred early in caecilian evolution to result in the observed absorbance. There are 4 amino acid changes conserved within the two complete caecilian rod opsin, and fragments of *Geotrypetes seraphinii* and *Rhinatrema bivittatum* sequences compared to other amphibians. None occur within the retinal-binding pocket that would be expected, for a single mutation to cause a significant shift. Therefore a new mechanism is being used. It is possible that these 4 amino acids together or one or two amino acid changes cause the spectral shift but exist outside the retinal binding pocket (Yokoyama 2000).

4.4.3 Further Research

Confirmation of the absence of cone opsin sequences should be attempted using cDNA isolated from *Rhinatrema bivittatum* eyes. If cone opsins are present then it is most likely to be present in this species of caecilian as it is considered primitive. However it is possible that opsins associated with diurnal and colour vision have been lost in these amphibians. Other opsins of interest include those associated with periodicity. It would be interesting to find if there is a similar loss or decrease in maximum absorption spectral tuning values are observed in burrowing reptiles. Site-directed mutagenesis of the opsin of caecilians at the four sites mentioned could reveal which are responsible for the spectral shift.

4.5 Conclusion

Caecilians have a functioning rod opsin for the detection of light. No nonsense mutation are apparent and structurally important amino acids are present in caecilians with the most rudimentary eyes. No cone opsins have been detected to suggest colour or diurnal vision. A blue shifted rod opsin is observed in caecilians and is probably caused by non-synonymous changes in the amino acid residue compared to other amphibians. These potential tuning sites have not been reported to be responsible for spectral shifts in previous research on rod opsins. Future analysis will determine if the absorbance of the almost transparent skin above the eye may be a reason for the spectral shift.

Chapter 5

Phylogenetic Analysis of Caecilian Rod Opsin Sequences

5.1 Introduction

Understanding the evolution of the visual system of caecilians requires an understanding of the phylogeny of the Gymnophiona. Morphological and recent molecular phylogenetic studies have resulted in a greater understanding of the relationships of caecilians to other amphibians and of relationships among the main caecilian lineages (e.g., San Mauro et al. 2005; Ruta et al. 2007; Frost et al. 2006; Roelants et al. 2007). However, some relationships are not yet compellingly resolved and there is still a need for new molecular markers that may contribute to answering outstanding phylogenetic questions.

Opsin, in particular RH1 opsin, sequences have been used in phylogenetic analyses to help resolve problematic relationships in several vertebrate clades (e.g., amphibians - Bossuyt et al. 2000; Che et al. 2007; Glaw et al. 2006; Faivovich et al. 2005; Frost et al. 2006; Venkatesh et al. 1999, 2001; fish -; Turtles - Fujita et al. 2004; and Archosaurs - Birks et al. 2002). Rhodopsin may be an ideal genetic system for exploring both practical and methodological issues (such as the effects of selection and adaptive evolution) in phylogenetic reconstruction because it has been cloned from a variety of species and much is known about its function and molecular evolution (Chang

et al. 2000). However only very few sequences, four in total, have been isolated from caecilians (Venkatesh et al.1999; Frost et al. 2006). In this chapter, amino acid sequences of rod opsin from two species of caecilian, and fragments of non-coding and coding nucleotide sequences from eight caecilian species are analysed phylogenetically. Analyses of amino acid sequences are used to confirm their identity and to examine the relationships of the Gymnophiona to other amphibians. Analyses of the nucleotide sequence data are used to investigate the potential of these data to help resolve relationships among caecilians.

5.1.1 Relationships of Caecilians to other amphibians

Living amphibians consist of three extant crown clades the Gymnophiona, Caudata, and Anura, which together form the Lissamphibia. Most researchers accept the monophyly of the Lissamphibia (Frost et al. 2006, Ruta et al. 2007) but controversy remains on the relationships of the extant taxa to various fossil groups. The Lissamphibia is generally accepted to be within the Temnospondyli (Ruta et al. 2007). An alternative hypothesis is that the frogs are temnospondyls and that salamanders and caecilians are lepospondyls (Carroll et al. 1975; Carroll 2000; Anderson 2001). Another hypothesis is that the Lissamphibia are within the Lepospondyli (Laurin 1998).

There are two opposing views of how the three orders of extant Lissamphibia are interrelated. Based on some molecular analyses using mitochondrial and nuclear data it has been suggested that the caecilians and salamanders are more closely related to each other than they are to frogs (Hedges et al. 1993; Larson1991; Feller et al. 1998; Zhang et al. 2003), with the clade comprising caecilians and salamanders termed the Procera. The alternative is that salamanders and frogs are sister taxa, comprising the clade Batrachia.

The Batrachia hypothesis is supported by both morphological and by substantial molecular evidence (e.g. Duellman et al. 1994; Frost et al. 2006; Rage et al. 1982; Roelants et al. 2007; San Mauro et al. 2004, 2005; Zhang et al. 2005). The most recent molecular studies that provide good support for the Batrachia hypothesis include the analysis of 21 complete mitochondrial genomes from representatives of the major groups of tetrapods, including one from each order of amphibian (Zhang et al. 2005) and the two largest, in terms of both taxon sampling and combination of nuclear and mitochondrial genes, phylogenetic analysis of living amphibia conducted to date (Frost et al. 2006; Roelants et al. 2007).

5.1.2 Caecilian interrelationships

Progress in understanding phylogenetic relationships within the Gymnophiona has been summarised recently by Wilkinson and Nussbaum (2006). Caecilian interrelationships were first investigated using morphological data by Nussbaum (1979). Subsequent analyses of Duellman et al. (1986) and Hillis (1991) that used taxa and a subset of Nussbaum's (1979) characters identified a clade comprising the Caeciliidae, Typhlonectidae, and Scolecomorphidae that Nussbaum (1991) referred to informally as the 'higher' caecilians and that Wilkinson and Nussbaum (2007) named Teresomata. In the earliest studies the Uraeotyphlidae, Ichthyophidae, and Rhinatrematidae were successively more distant outgroups to the Teresomata. In more recent morphological and molecular analyses, the monophyly of Teresomata has been strongly supported, and the single major change in understanding of family-level relationships has been the hypothesised sister-grouping of the the Ichthyophiidae and Uraeotyphlidae (Wilkinson and Nussbaum 1996; Gower et al. 2002; Wilkinson, 1997; Wilkinson et al. 2002; San

Mauro et al. 2004; Frost et al. 2006; Roelants et al. 2007) into a clade termed Diatriata by Wilkinson and Nussbaum (2007).

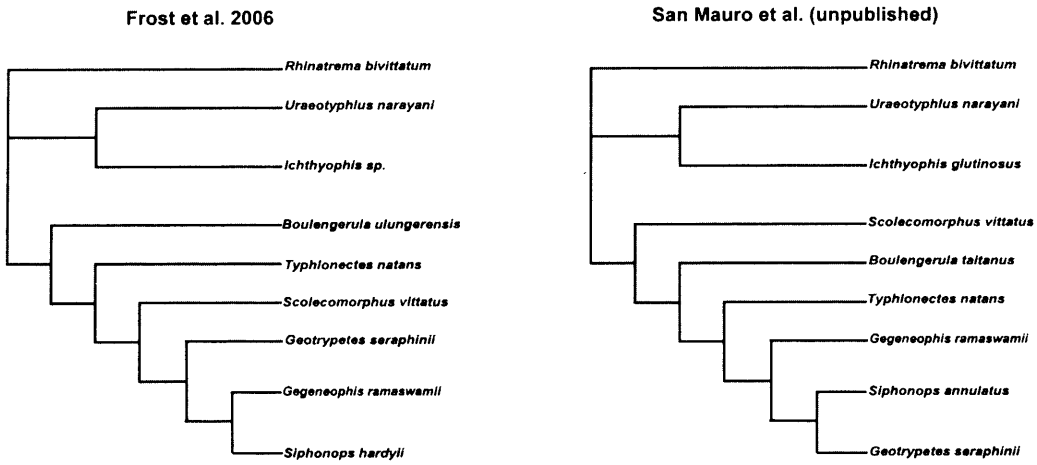


Figure 5.1. Phylogenies depicting alternative hypothesis of caecilian interrelationships from Frost et al. 2006 and San Mauro et al. unpublished. The Roelants et al. (2007) tree is identical to that of San Mauro et al.

Previous molecular analyses, both of relatively small data sets cytochrome b, 12S and 16S rRNA mitochondrial gene sequences (Hay et al. 1995; Hedges et al. 1993, Wilkinson et al. 2002, 2003) and of more substantial concatenations of mitochondrial and nuclear markers (Frost et al. 2006; Roelants et al. 2007) have added further support to findings based on morphology (Nussbaum 1979; Wilkinson and Nussbaum 1996; Wilkinson, 1997) that the Caeciliidae is paraphyletic with respect to the Typhlonectidae and perhaps also with respect to the Scolecomorphidae. Additionally, Gower et al. (2002) and Frost et al. (2006) found the Ichthyophiidae to be paraphyletic with respect to the Uraeotyphlidae. Within the Teresomata relationships remain somewhat uncertain. In Frost et al. (2006) the relationships (*Boulengerula* (*Typhlonectes* (*Scolecomorphus*

(*Geotrypetes* (*Gegeneophis, Siphonops*))))) are supported (Fig.1), whereas the analysis of Roelants et al. (2007) of concatenated mitochondrial and nuclear markers, and an as yet unpublished study by San Mauro et al. using a combined data set of complete mitochondrial complete genomes and RAG1 nuclear gene from 9 caecilian species strongly supported an alternative set of relationships within Teresomata: (*Scolecomorphus* (*Boulengerula* (*Typhlonectes* (*Gegeneophis* (*Siphonops*, *Geotrypetes*))))) (Fig 1.).

5.1.3 Using rhodopsin as a phylogenetic marker

Vertebrate opsins are distinguished into five phylogenetic or orthologous groups RH1 (rod opsin), RH2 (rod opsin-like), SWS1 (short wave sensitive), SWS2 (short wave sensitive), and LWS (long wave sensitive). This diversity of visual pigment genes results from a series of independent duplication and diversification events (Yokoyama 2000). Phylogenetic analyses of orthologous opsins reflect the deeper level relationships of the vertebrates (Chang et al. 2000) in addition to providing information on functionality of the genes. Phylogenetic analyses of vertebrate opsin amino acid sequences have been used to infer the complement of visual pigments in early vertebrates and whether true dim light vision occurred ancestrally (Pisani et al. 2005; Trezise et al. 2005; Collin et al. 2004). Complete rod opsin sequences have been isolated from a large variety of divergent vertebrates and used in studies of phylogenetic relationships (Chang et al. 2000). Additionally, the presence and absence of intron sequences were also used as a character to distinguish vertebrate clades (Venkatesh et al. 2001).

Although mitochondrial genomes provide large markers for phylogenetics, some workers have reservations about deeper-level results produced from data where high

bootstrap support may occur for unusual relationships (Cotton 2002). Various reasons have been given such as a low signal-to-noise ratio and wide differences in substitution rates between lineages, between classes of amino acids and between sites. Thus phylogenetic results from recent analyses of whole mitochondrial genomes need to be confirmed with data from nuclear genes (Cotton 2002; Springer et al. 2001) with different rates of evolution and substitution dynamics, such as opsins. Nuclear genes have been used with mitochondrial DNA as molecular markers in recent analyses of caecilian and amphibians (San Mauro et al. 2004; Frost et al. 2006; Roelants et al., 2007) but thus far only limited use has been made of any opsin sequences. Frost et al. 2006 used rhodopsin coding nucleotide sequences as one of the four nuclear markers used in the most comprehensive study to date of amphibian phylogeny. However, only three caecilian opsin sequence fragments were successfully amplified for that study.

5.2 Aims

5.2.1 To include the first complete amino acid sequences of caecilian rod opsin in an analysis using vertebrate visual opsins in order to provide phylogenetic assessment of the identity and orthology of the caecilian sequences.

5.2.2 To explore the potential of caecilian rod opsin sequences for inference of phylogenetic relationships of the amphibian orders.

5.2.2 To explore, for the first time, the use of newly isolated nucleotide sequences of both non-coding and coding segments of rod opsin from diverse caecilian taxa for determining caecilian interrelationships.

5.3 Phylogenetic methods

5.3.1 Analyses of amino acid sequences

Full-length rod opsin sequences were isolated from cDNA using PCR methods from *Ichthyophis* cf. *kohtoaensis* and *Typhlonectes natans* (Chapter 2, section 2.2.3). These taxa were chosen because of both the availability of suitable fresh material and because they span a considerable portion of the phylogenetic diversity within the Gymnophiona. The sequences were generated by two different PCRs, selecting three cloned genes for each species, and sequencing in the forward and reverse directions.

An alignment was created manually in MacClade 4.08 (Maddison and Maddison, 2000) using the caecilian sequences and 68 vertebrate visual opsin sequences downloaded from the NCBI database (Appendix) representing the five classes of visual opsins and including all amphibian and multiple representatives (typically up to three where available and including any with rudimentary eyes) of major vertebrate lineages. Bayesian inferences used Markov Chain Monte Carlo (MCMC) implemented in MRBAYES 3.1.2 (Huelsenbeck et al. 2001, Ronquist et al. 2003) sampling every 1000 generations from two parallel runs (each of four chains) of 1000000 generations. Convergence was established by comparing the standard deviations of split frequencies) and burn-ins determined by visual inspection of likelihoods using TRACER 1.3 (A. Rambault, http://tree.bio.ed.ac.uk/software/). Model jumping was used within MRBAYES 3.1.2 to estimate the fixed-rate model for amino acid data during the tree-search, this chose the BLOSUM62 *(BLOck SUbstitution Matrix)* model of amino acid substitution that was produced from multiple alignments of evolutionarily divergent proteins (Henikoff et al. 1992). An alternative, more parameter rich model, WAG with a

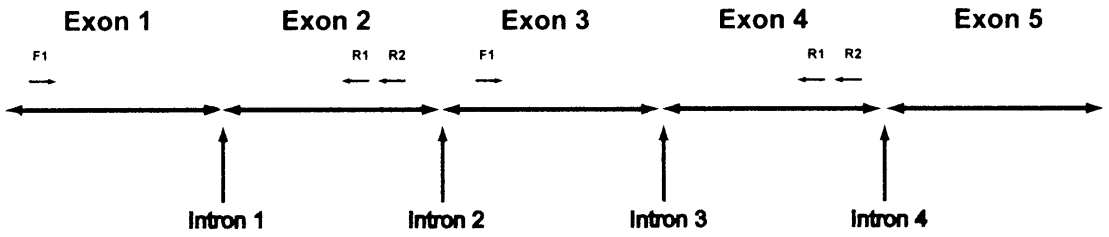
gamma distributed substitution rate and a proportion of invariant characters (WAG +G+I) was chosen by the program MultiPhyl (Keane et al. 2006). Trees were rooted using the LWS opsin sequences as outgroups to the sequences from the other classes of opsin.

5.3.2 Caecilian interrelationships

Genomic DNA was isolated from ethanol preserved tissues using a modified phenol chloroform method (Chapter 2, section 2.2.2.4). Taxa selected represented each of the 6 currently recognised families of caecilians (Wilkinson and Nussbaum 2006). Gene fragments were amplified using PCR methods with nested primers and annealing temperatures from 55°C-60°C (Chapter 2, section 2.2.3.1). Each sequence was generated by two different PCRs and selecting 3 cloned genes for each species and sequenced in the forward and reverse directions. Rod opsin coding and non-coding sequences from the first exon to the second and from the third exon to the fourth exon were isolated including the introns. Fig 5.1 and Table 5.1 show primers and diagram of annealing points on the gene of the primers.

DNA sequences from the PCR were partioned into coding and non-coding regions for analysis. The introns were aligned using Clustal X v1.81 (default parameters) and the coding regions were aligned manually. For each partition a different model was selected according to the Akaike Information Criterion (AIC) calculated using Modeltest version 3.4 (Posada et al. 1998). The models chosen are given in Table 5.2. Best-fit models in the coding regions were selected for each codon position and model parameters were estimated independently in MRBAYES v3.1.2. Aside from model selection Bayesian inferences were made as for the analyses of amino acid sequences described above. Trees were rooted with *Rhinatrema bivittatum*.

Figure 5.2 Table 5.1 Hemi-nested primers pairs used to isolate rod opsin fragments.



	Primer sequence (5′-3′)
Exon 1 F1	TTTYTATRTTCCCATGTCAAAYAAGACYGG
Exon 2 R1	GGCCATVATCCAVGTAARYAMGACHCCCAT
Exon 2 R2	GGACCAYCCRAAGAGSGGAGGMGYYGCACA
Exon 3 F1	GYTCATGYGGAGTYGATTATTACACHCTCA
Exon 4 R1	GGRTTGTAAATWGCRGAACTCTTGGCAAAG
Exon 4 R2	GCCACTCACCTGTTTRTTHAGSACAATGTA

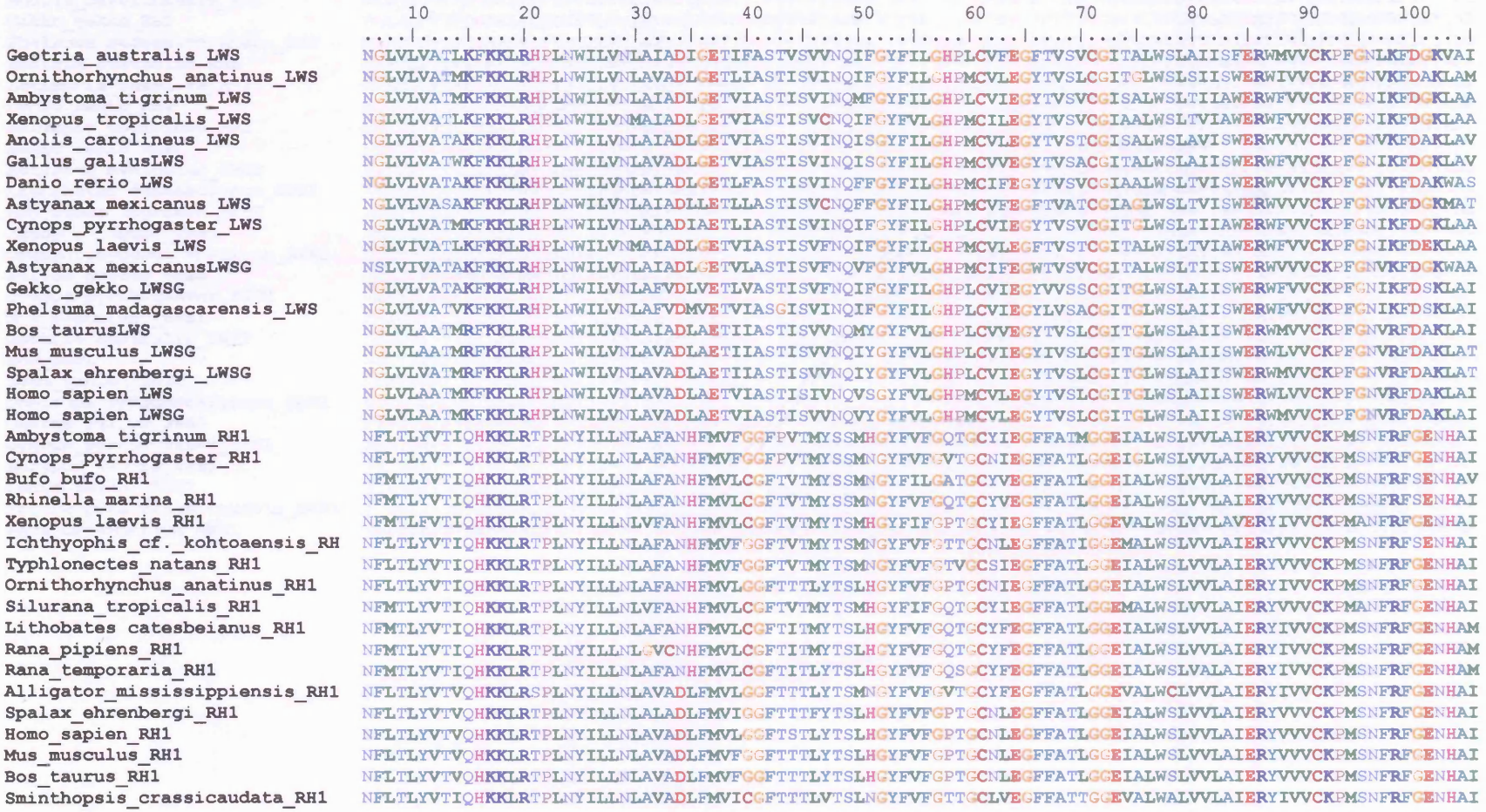
Table 5.2 of Model chosen for each partition using Modeltest. G - gamma correction for rate heterogeneity across sites. I = proportion of invariant sites.

Partition	Model suggested by Modeltest
Coding EX1+2	K81uf + G
Non-coding intron 1	HKY + G
Coding Exon 3-	HKY + I
Non-coding intron 3	TVMEF

5.4 Results

5.4.1 Phylogenetic analysis of amino acid sequences

Figure 5.3 Alignment of vertebrate opsin sequences




Gallus_gallus_RH1 NFLTLYVTIQHKKLRTPLNYILLNLVVADLFMVFGGFTTTMYTSMNGYFVFGVTGCYIEGFFATLGGEIALWSLVVLAVERYVVVCKPMSNFRFGENHAI
Astyanax_mexicanus_RH1 NFLTLYVTIEHKKLRTPLNYILLNLAVADLFMVFGGFTTTMYTSLNGYFVFGRLGCNLEGFFATFGGINSLWCLVVLSIERWVVVCKPMSNFRFGENHAI
Danio_rerio_RH1 NFLTLYVTIEHKKLRTPLNYILLNLAIADLFMVFGGFTTTMYTSLHGYFVFGRLGCNLEGFFATLGGEMGLWSLVVLAIERWMVVCKPVSNFRFGENHAI
Latimeria_chamulae_RH1 NFLTLFVTIQHKKLRTPLNYILLDLAVADLCMVFGGFFVTMYSSMNGYFVLGPTGCNIEGFFATLGGQVALWALVVLAIERYVVVCKPMSNFRFGENHAI
Anolis_carolinensis_RH1 NFLTLFVTIQHKKLRTPLNYILLNLAVANLFMVLMGFTTTMYTSMNGYFIFGTVGCNIEGFFATLGGEMGLWSLVVLAVERYVVICKPMSNFRFGETHAL
Geotria_australis_RhA NFLTLFVTVQHKKLRTPLNYILLNLAVSNLFMILFGFTTTMYTSMNGYFVFGPTMCSIEGFFATLGGEVSLWSLVVLAIERYIVICKPMGNFRFGNTHAI
Petromyzon_marinus_RH1 NFLTLFVTVQHKKLRTPLNYILLNLAVANLFMVLFGFTLTMYSSMNGYFVFGPTMCNFEGFFATLGGEMSLWSLVVLAIERYIVICKPMGNFRFGSTHAY
Gallus_gallus_RH2 NLLTLLVTFKHKKLRQPLNYILVNLAVADLFMACFGFTVTFYTAWNGYFVFGPVGCAVEGFFATLGGQVALWSLVVLAIERYIVVCKPMGNFRFSATHAM
Anolis_carolinensis_RH2 NILTLLVTFKHKKLRQPLNYILVNLAVADLFMACFGFTVTFYTAWNGYFIFGPIGCAIEGFFATLGGQVALWSLVVLAIERYIVVCKPMGNFRFSATHAL
Gekko_gekko_RH2 NGLTLFVTFQHKKLRQPLNYILVNLAAANLVTVCCGFTVTFYASWYAYFVFGPIGCAIEGFFATIGGQVALWSLVVLAIERYIVICKPMGNFRFSATHAI
Phelsuma_madagascarensis_RH2 NGLTLFVTFQHKKLRQPLNYILVNLAVANLLMVICGFTVTFYTSWYGYFVFGPMGCAFEGFFATIGGQVALWSLVVLAIERYIVICKPMGNFRFSSSHAM
Geotria_australis_RHB NFMTLFVTFKLKKLRQPLNFILVNLCVADLLMIMFGFTTTFYTAMNGYFVFGPTGCNIEGFFATLGGEVSLWSLVMLAIERYIVVCKPMGNFRFATTHAA
Latimeria_chamulae_RH2 NFLTLLVTFKHKKLRQPLNYILVNLAVASLFMVVFGFTVTFYSSLNGYFVLGPMGCAMEGFFATLGGQVALWSLVVLAIERYIVVCKPMGNFRFASSHAI
Danio_rerio_RH2 NGLTLLVTAQHKKLRQPLNFILVNLAVAGTIMVCFGFTVTFYTAINGYFVLGPTGCAIEGFMATLGGEVALWSLVVLAVERYIVVCKPMGSFKFSASHAF
Astyanax_mexicanus_RH2 NGFTLFVTAQHKKLQQPLNFILVNLAVAGMIMVCFGFTITISSAVNGYFYFGPTACAIEGFMATLGGEVALWSLVVLAIERYIVVCKPMGSFKFSASHAL
Danio_rerio_SWS2 NVLTIVCTIQYKKLRSHLNYILVNLAISNLWVSVFGSSVAFYAFYKKYFVFGPIGCKIEGFTSTIGGMVSLWSLAVVALERWLVICKPLGNFTFKTPHAI
Astyanax_mexicanus_SWS2 NVLTIVCTVQYKKLRSHLNYILVNLAISNLLVSTVGSFTAFVSFLNRYFIFGPTACKIEGFVATLGGMVSLWSLSVVAFERWLVICKPVGNFSFKGTHAI
Lithobates_catesbeianus_SWS2 NVLTVICTIKYKKLRSHLNYILVNLAVANLIVICFGSTTAFYSFSQMYFALGTLACKIEGFTATLGGMVSLWSLAVVAFERFLVICKPMGSFTFRENHAI
Ambystoma_tigrinum_SWS2 NLLTVICTIKYKKLRSHLNYILVNLAIANLLVVSVGSTVAFYSNAQMYFALGPLACKVEGFTATLGGMVGLWSLAVVAFERFLVICKPLGSFTFRESHAI
Xenopus_laevis_SWS2 NLLTIICTVKYKKLRSHLNYILVNLAVANLIVICFGSTTAFYSFSQMYFSLGTLACKIEGFTATLGGIIGLWSLAVVAFERFLVICKPMGNFTFRESHAV
Ornithorhynchus_anatinus_SWS2 NLLTVICTIKYKKLRSHLNYILVNLAVSNMLVVCVGSATAFYSFAHMYFVLGPTACKIEGFAATLGGMVSLWSLAVIAFERFLVICKPLGNLSFRGTHAI
Gallus_gallus_SWS2 NTLTIFCTARFRKLRSHLNYILVNLALANLLVILVGSTTACYSFSQMYFALGPTACKIEGFAATLGGMVSLWSLAVVAFERFLVICKPLGNFTFRGSHAV
Cynops_pyrrhogaster_SWS2 NFLTILCTFLNKKLRTHFNYILVNMAVANLLVIFIGPTLSFYSNSQMYFALGPLACKIEGFAATLGGIVGLWCLAVVAFERYLVICKPVGGFTFRESHAI
Mus_musculus__SWS1 NAIVLVATLHYKKLRQPLNYILVNVSLGGFLFCIFSVFTVFIASCHGYFLFGRHVCALEAFLGSVAGLVTGWSLAFLAFERYVVICKPFGSIRFNSKHAL
Geotria_australis_SWS2 NFLTVFVTIKYKKLRSHLNYILVNLAIANLIVVCCGSTLAFYSFMHKYFILGPLFCKMEGFTATLGGMLSLWSLAVLAFERCLVICKPFGNIAFRGTHAL
Bos_SWS1 NATVLVATLRYRKLRQPLNYILVNVSLGGFIYCIFSVFIVFITSCYGYFVFGRHVCALEAFLGCTAGLVTGWSLAFLAFERYIIICKPFGNFRFSSKHAL
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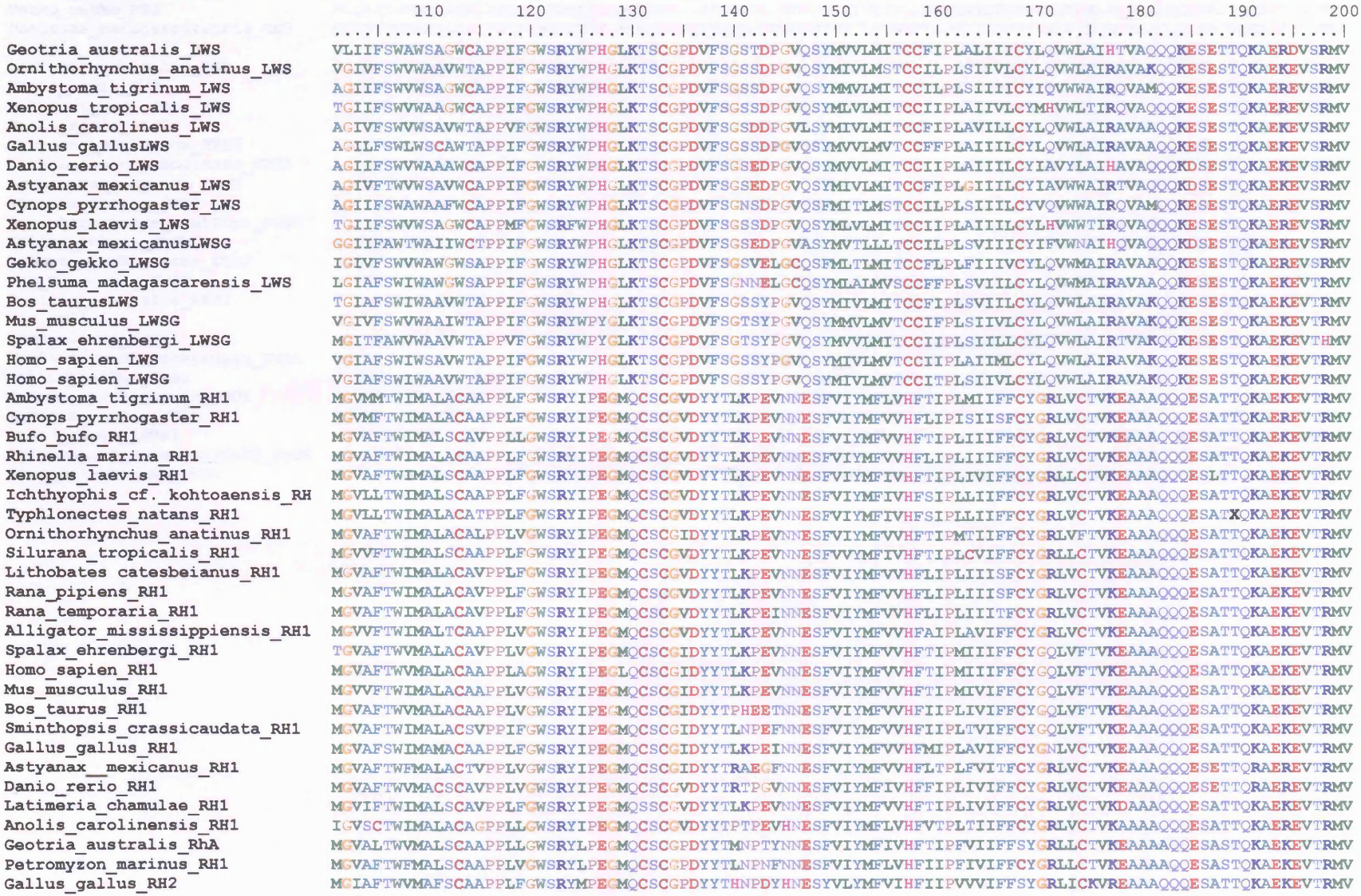
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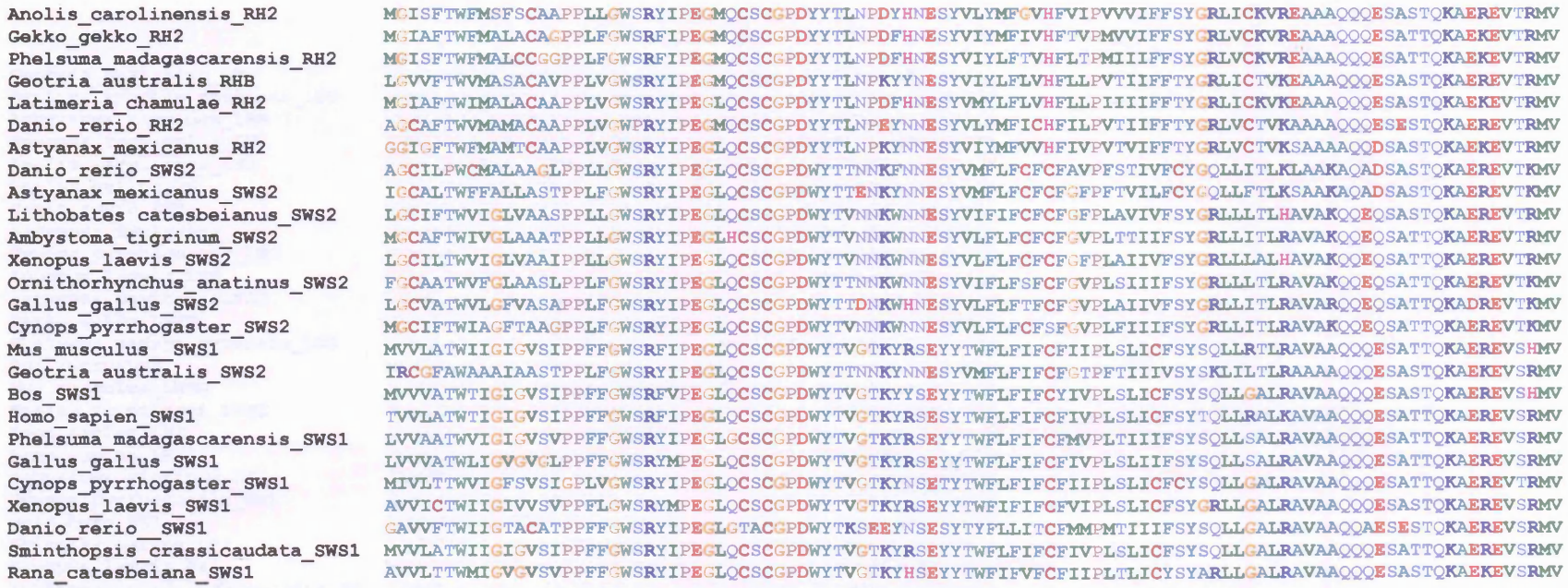
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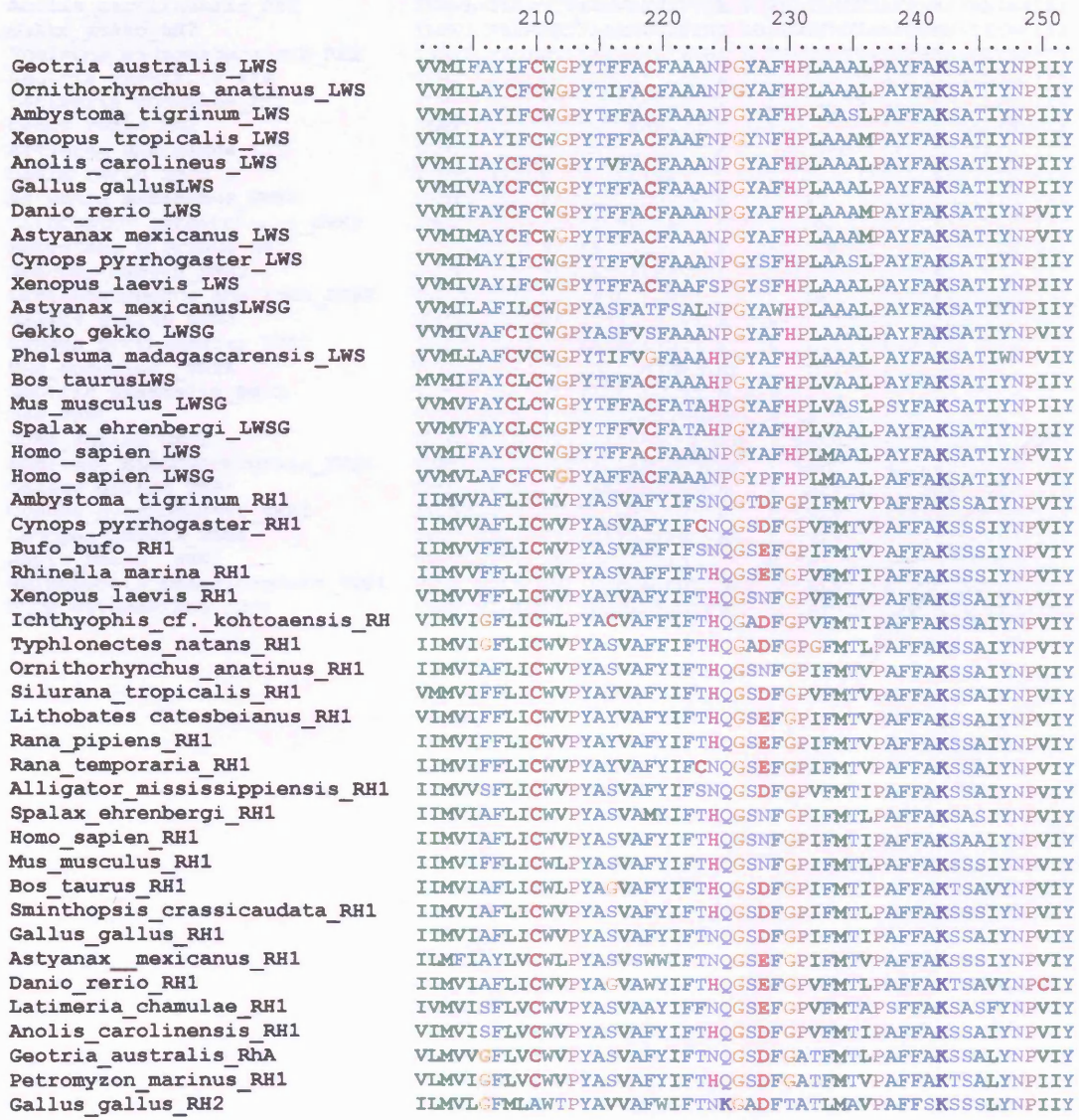
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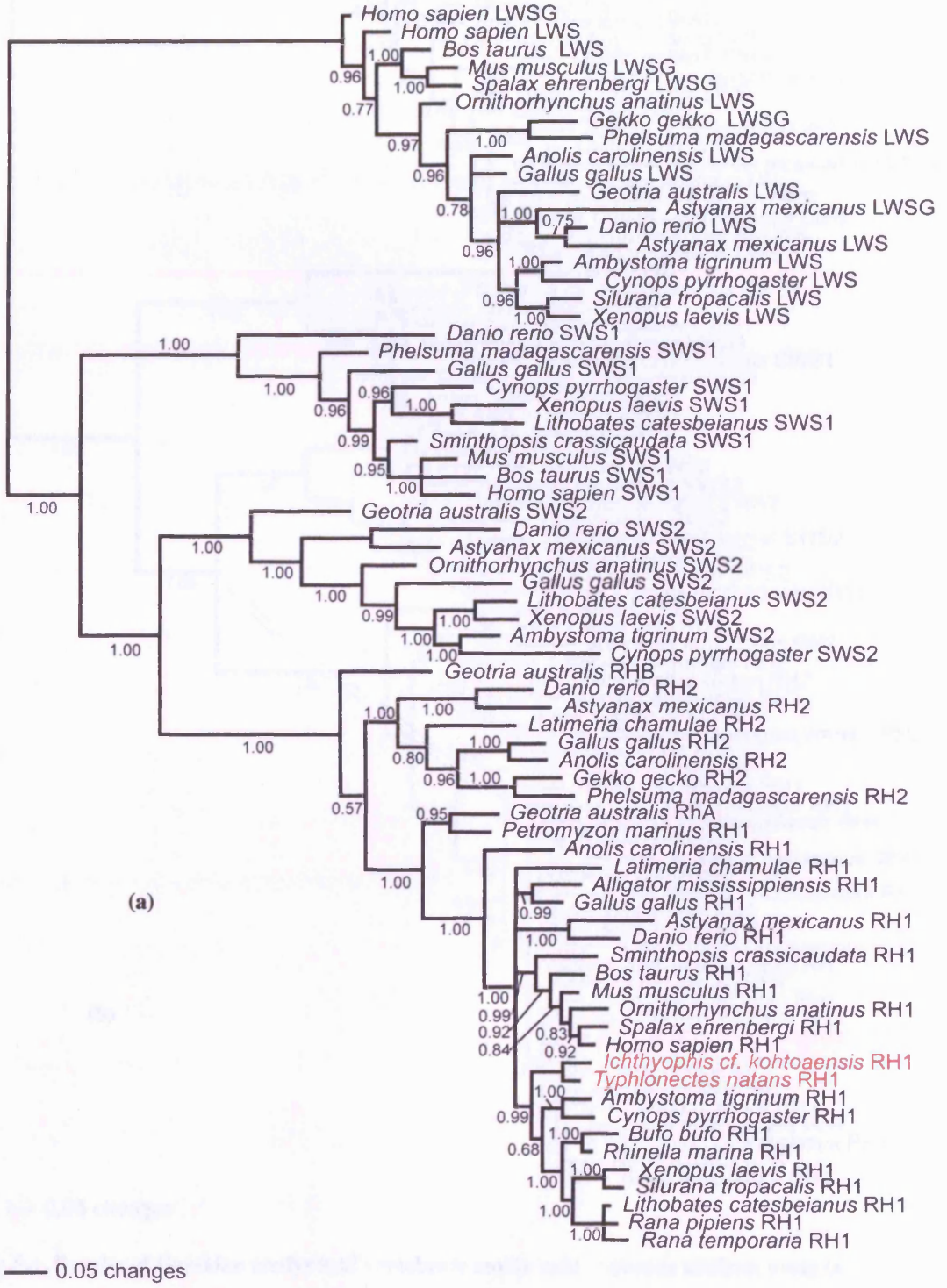
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Cynops pyrrhogaster SWS2
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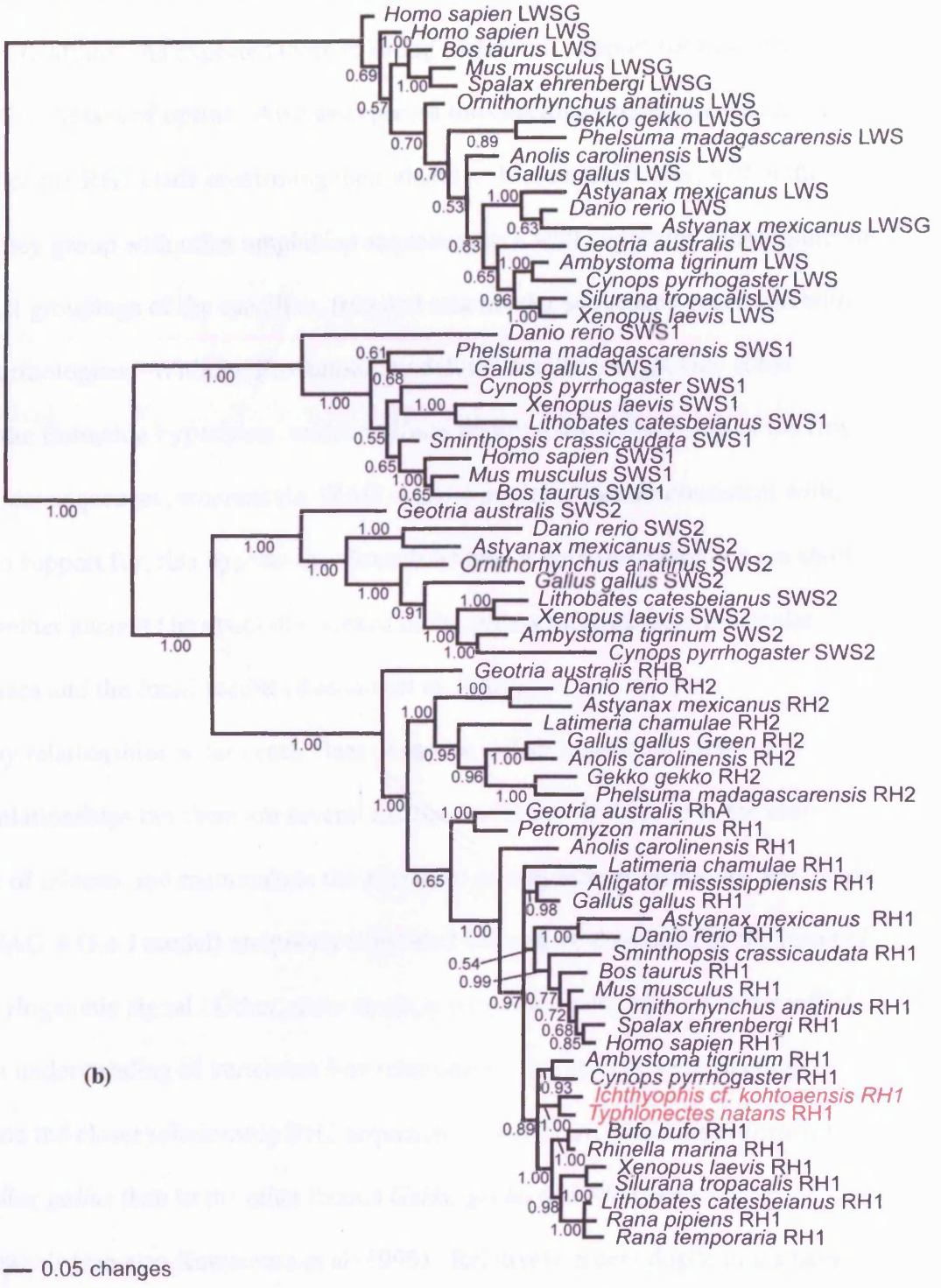


Fig.5.4. Results of Bayesian analyses of vertebrate amino acid sequence analysis using (a) Blosum62 and (b) WAG + G + I. Numbers indicate posterior probabilities. Caecilians are shown in red.

Alternative models resulted in very similar Bayesian inferences (Fig 5.4) of phylogenetic relations. As expected there is strong (maximal) support for monophyly each of the five classes of opsins. Also as expected the caecilian sequences are recovered as members of the RH1 clade confirming their identity. More specifically, within the RH1 clade they group with other amphibian sequences in a well supported Lissamphibian clade, ordinal groupings of the caecilian, frog and salamander sequences consistent with their being orthologues. With the Blossum62 model, the results also provide some support for the Batrachia hypothesis, with caecilians forming the sister group of the frog and salamander sequences, whereas the WAG + G + I model is merely consistent with, but offers no support for, this hypothesis. Branch lengths within the Amphibia are short despite the rather ancient (Jurassic) divergence of the orders inferred from molecular dating analyses and the fossil record (Roelants et al. 2007).

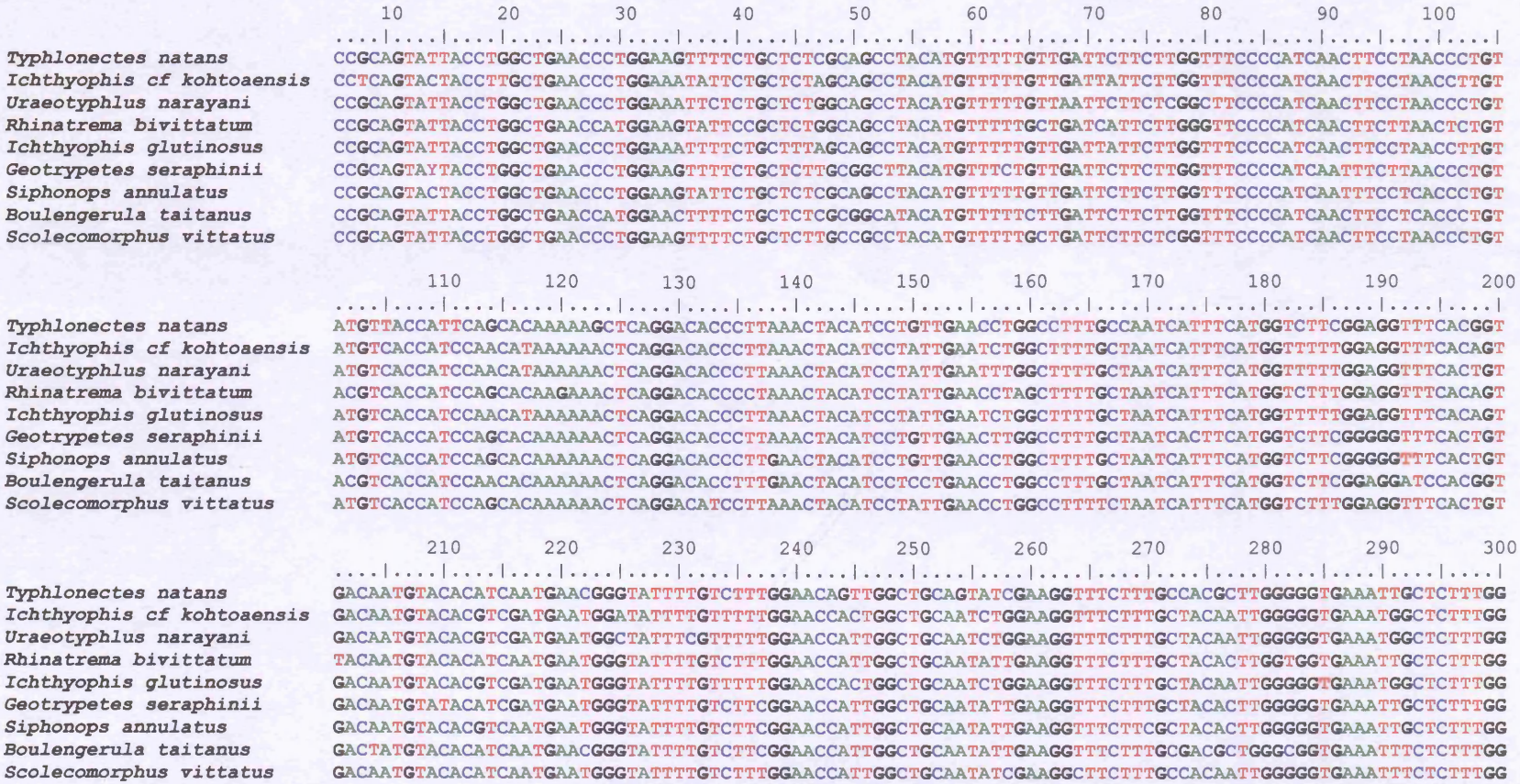
Many relationships within each class of opsins reflect traditional views of vertebrate relationships but there are several exceptions. Some of these (e.g. the close relationship of teleosts and mammals to the exclusion of amphibians within the RH1 class using the WAG + G + I model) are poorly supported and can be dismissed as the result of a lack of phylogenetic signal. Other, more strongly supported relationships that conflict with current understanding of vertebrate interrelationships are indicative of paralogy. These include the closer relationship RH2 sequences of the lizard *Anolis carolinensis* to the bird *Gallus gallus* than to the other lizards *Gekko gecko* and *Phelsuma madagascarensis* (see also Kawamura et al. 1995). Relatively recent duplications have produced multiple copies of RH2 in fishes and reptiles (Register et al. 2004; Kawamura et al. 1995). For example, *Astyanax mexicanus* has more than one copy of RH2 (Register

et al. 2004) each sequence maximally absorbing at different wavelengths, and one more longwave shifted than other indicated by the sequence *Astyanax* LWSG.

The well-supported placement of *Anolis carolinensis* RH1 as sister to the Gnathostomata RH1 is unexpected. Supporting the suggestion that this sequence is paralogous is that it was isolated from the brain and not the retina of *Anolis carolinensis* (Kawamura et al. 1994). Spurious grouping of the *A. carolinensis* sequence has been found in previous analyses (Chang et al. 2000; Kawamura et al. 1995). Unusual well-supported topologies are associated with the RH1 and SWS2 sequences of *Ornithorhynchus antinus,* the Platypus, placing it within Eutherian mammals (RH1) or as sister to *Gallus gallus and the amphibians* (SWS2). Other anomalies include the relationships of tetrapods within the SWS1 group, also possible due to paralogy, and the rooting within the LWS group which is most probably an analytical artefact.

5.4.2 Analysis of rod opsin fragments

The hemi-nested primers from exon1 to exon 2 amplified a single fragment in all taxa. The primers from exon 3 to 4 amplified a single fragment from all species except *Uraeotyphlus narayani*. The Exon 1&2, Intron 1, Exon 3&4 and Intron 3 PCR fragments from genomic material were analysed separately and an additional combined analysis was performed of the data sets that included *Uraeotyphlus narayani*. Intron lengths varied across caecilian species (Table 5.3) due to indels but are of identical length in the two species of *Ichthyophis* indicating some phylogenetic information in intron length. (Alignments in Appendix). Table 5.4 lists the number of characters used in each data partition and the number and percentage of informative characters.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Ichthyophis cf kohtoaensis CCTCAGTACTACCTTGCTGAACCCTGGAAATATTCTGCTCTAGCAGCCTACATGTTTTTGTTGATTATTCTTGGTTTCCCCATCAACTTCCTAACCTTGT
Uraeotyphlus narayani CCGCAGTATTACCTGGCTGAACCCTGGAAATTCTCTGCTCTGGCAGCCTACATGTTTTTGTTAATTCTTCTCGGCTTCCCCATCAACTTCCTAACCCTGT
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Ichthyophis glutinosus CCGCAGTATTACCTGGCTGAACCCTGGAAATTTTCTGCTTTAGCAGCCTACATGTTTTTGTTGATTATTCTTGGTTTCCCCATCAACTTCCTAACCTTGT
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Boulengerula taitanus CCGCAGTATTACCTGGCTGAACCATGGAACTTTTCTGCTCTCGCGGCATACATGTTTTTCTTGATTCTTCTTGGTTTCCCCATCAACTTCCTCACCCTGT
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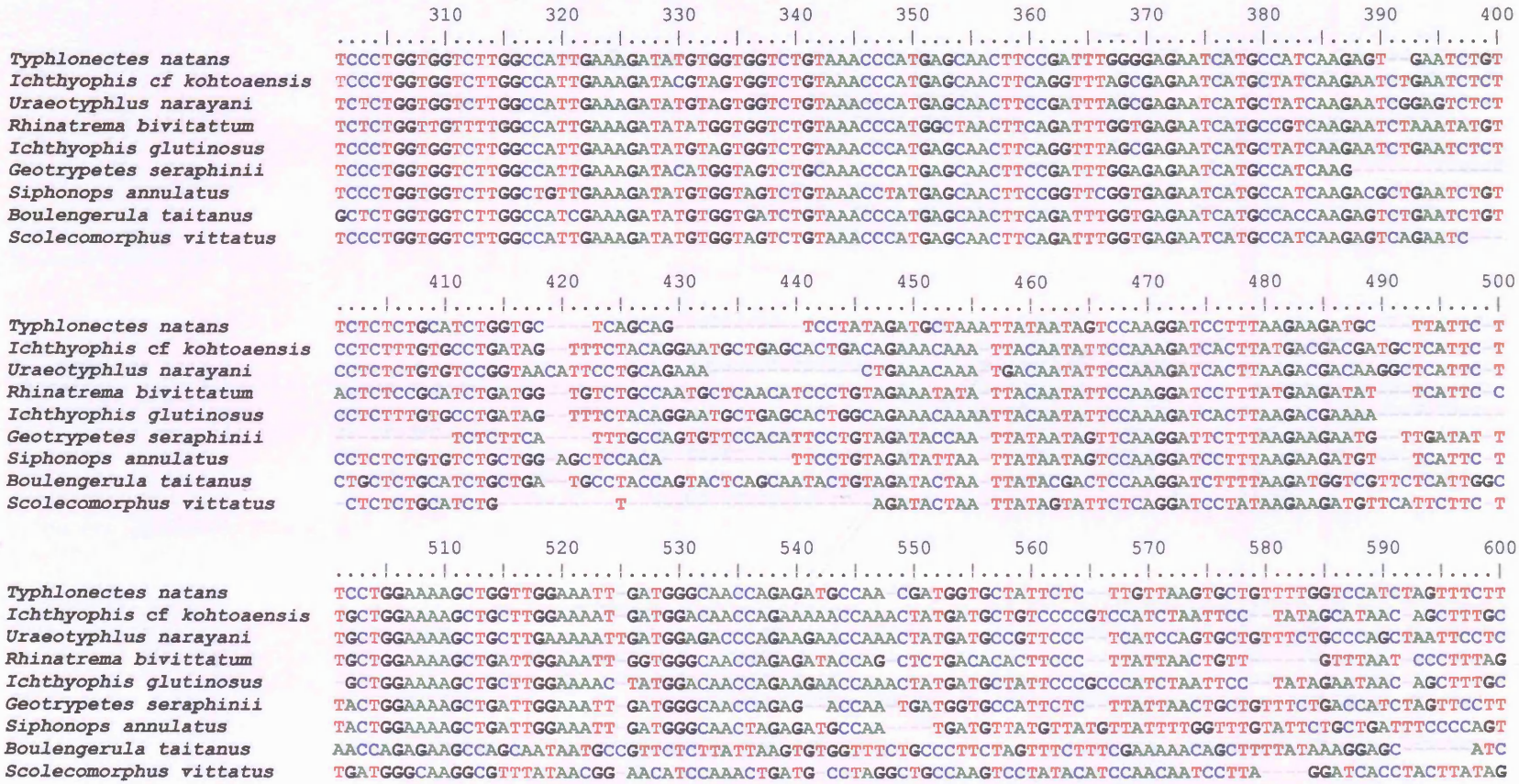
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Siphonops annulatus TCCCTGGTGGTCTTGGCTGTTGAAAGATATGTGGTAGTCTGTAAACCTATGAGCAACTTCCGGTTCGGTGAGAATCATGCCATCAAGACGCTGAATCTGT
Boulengerula taitanus GCTCTGGTGGTCTTGGCCATCGAAAGATATGTGGTGATCTGTAAACCCATGAGCAACTTCAGATTTGGTGAGAATCATGCCACCAAGAGTCTGAATCTGT
Scolecomorphus vittatus TCCCTGGTGGTCTTGGCCATTGAAAGATATGTGGTAGTCTGTAAACCCATGAGCAACTTCAGATTTGGTGAGAATCATGCCATCAAGAGTCAGAATC
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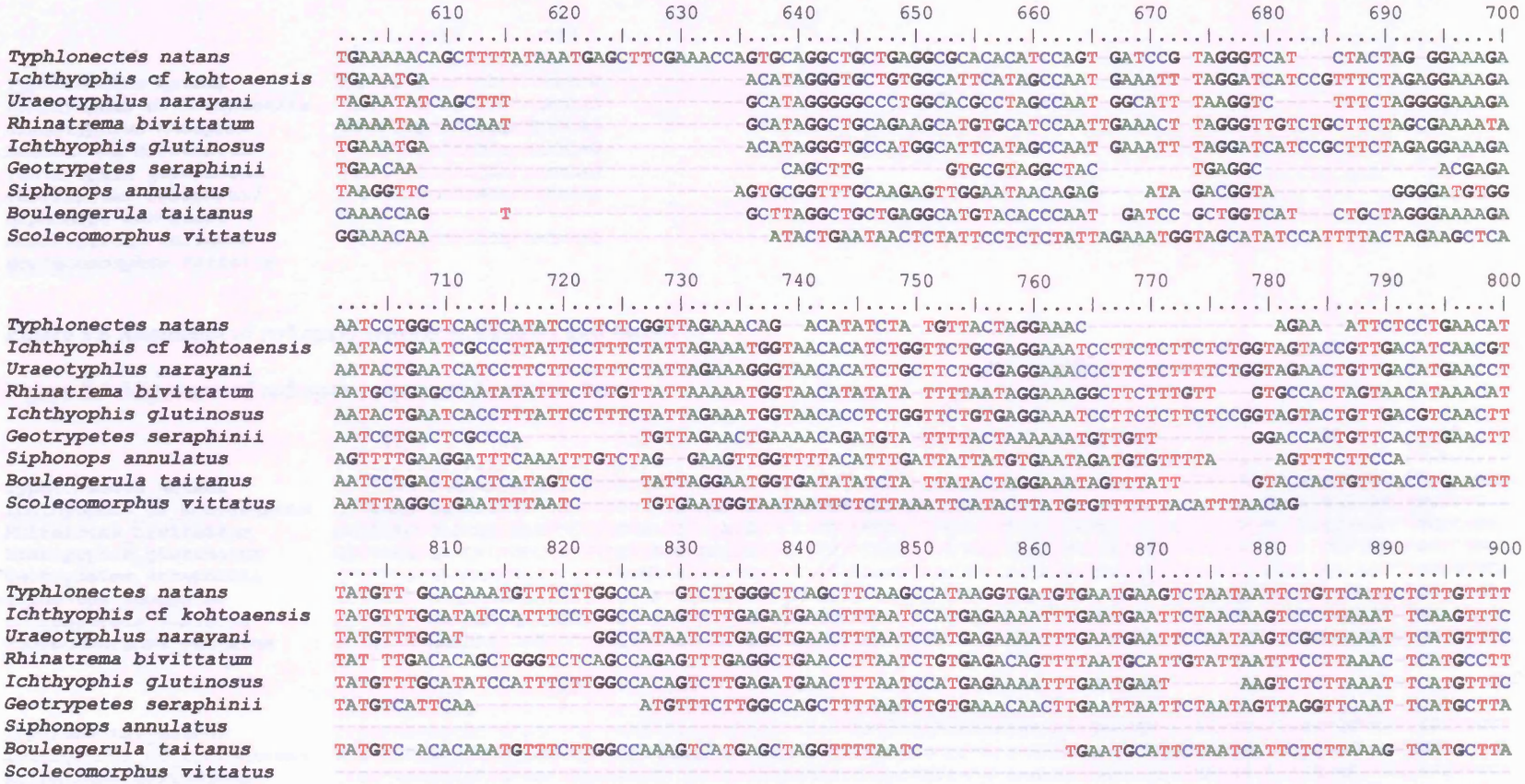
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Siphonops annulatus
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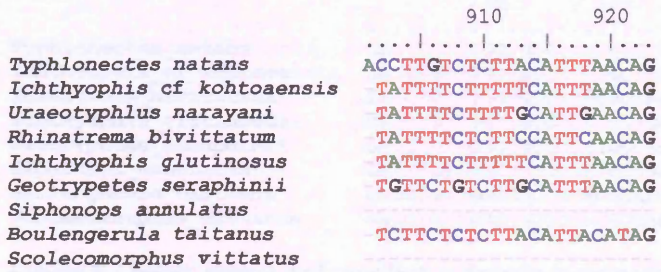
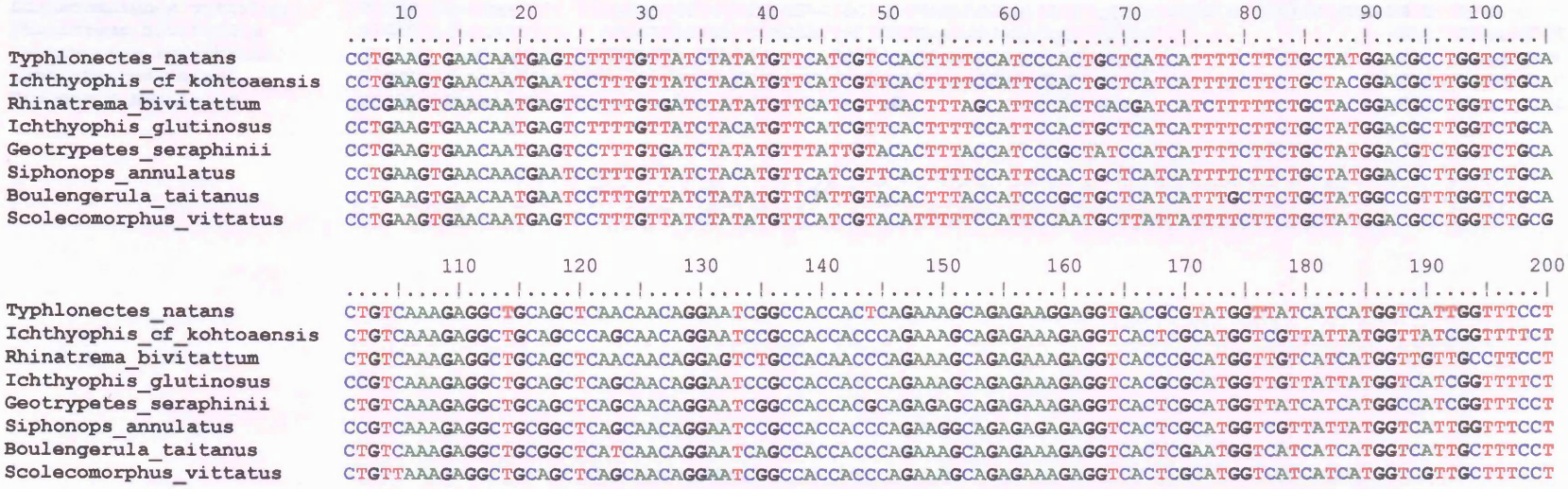


Figure 5.5 Alignment of rod opsin sequences Exon1-2 and Intron 1.

Figure 5.6 Alignment of rod opsin sequences Exon 3-4 below



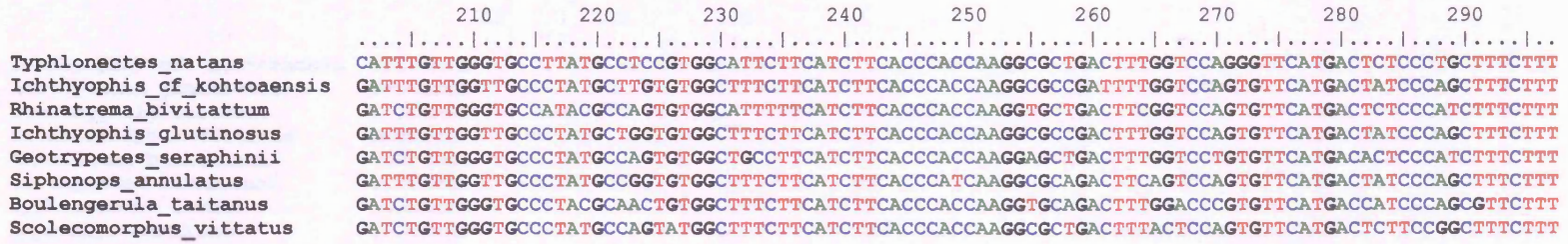
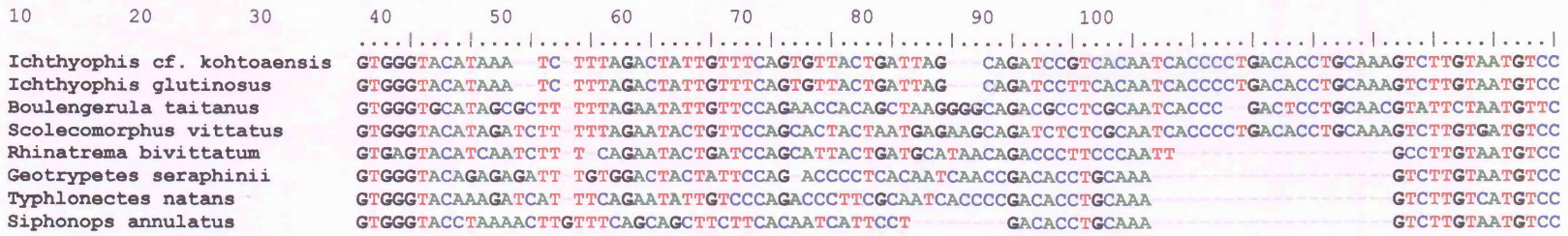
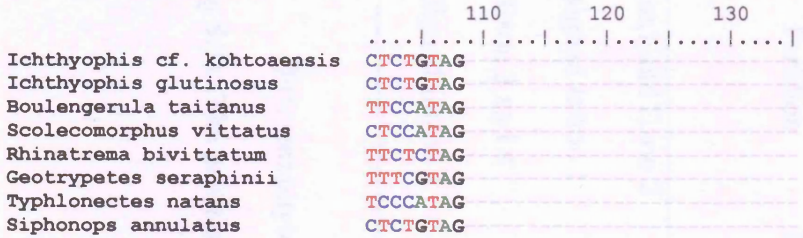


Figure 5.7 below Intron 3 of caecilian rod opsin sequences.

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Scolecomorphus vittatus CTCCATAG
Rhinatrema bivittatum TTCTCTAG
Geotrypetes seraphinii TTTCGTAG
Typhlonectes natans TCCCATAG
Siphonops annulatus CTCTGTAG

Table 5.3 Intron sizes in each species of caecilian.

	Intron 1	Intron 3
Boulengerula taitanus	474	106
Geotrypetes seraphinii	408	87
Ichthyophis glutinosus	487	103
Ichthyophis cf. *kohtoaensis*	503	103
Rhinatrema bivittatum	494	88
Scolecomorphus vitattus	323	107
Siphonops annulatus	339	81
Typhlonectes natans	487	87
Uraeotyphlus narayani	484	-

Table 5.4 Size of and number of informative characters for each partition.

Partition	Number of characters	Number of informative characters per partition and (1st, 2nd and 3rd positions)
Exon1 and Exon 2	384	48 (11, 2, 35)
Aligned Intron 1	539	203
Exon 3 and 4	297	40 (9, 3, 28)
Aligned Intron 3	109	19

Different phylogenies result from the deparate analyses of the four data partitions (Fig 5.3). The partitions that include *Ureaotyphlus narayani* (Exon 1 & 2 and Intron 1)

produce trees that differently resolved but mutually compatible and entirely congruent with current understanding of relationships within caecilians as determined using mitochondrial genomes and RAG 1 sequences (San Mauro et al. unpublished) and concatenated nuclear and mitochondrial genes (Roelants et al. 2007).

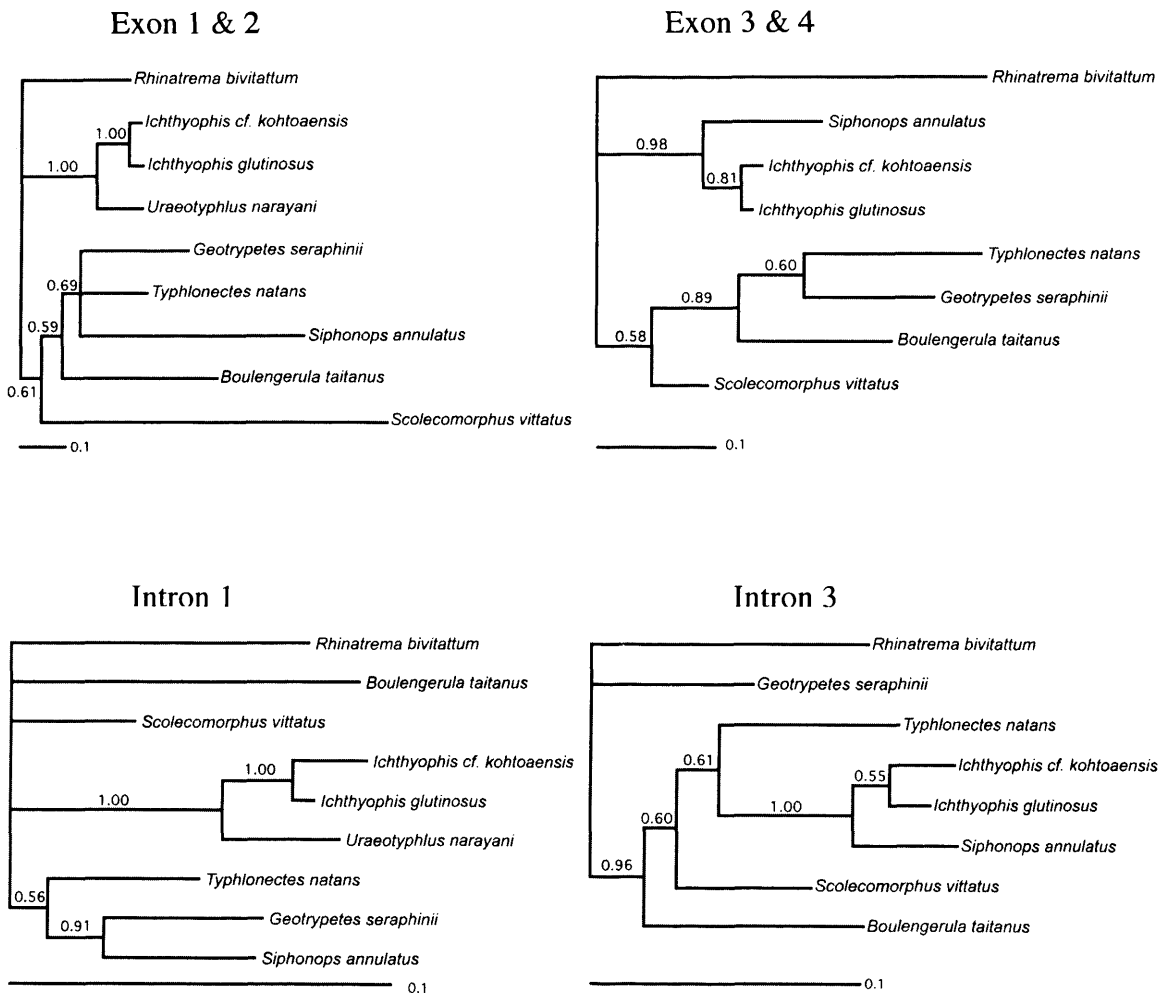

Fig 5.8 Results of separate Bayesian analyses of coding and non-coding caecilian RH1 nucleotide sequences. Numbers indicate posterior probabilities.

Both fragments provide maximal support for monophyly of *Ichthyophis* and for the clade Diatriata (*Ichthyophis* and *Uraeotyphlus*). In addition, Intron 1 provides

good support for the close relationship of the caeciliids *Geotrypetes seraphini* and *Siphonops annulatus* but other relationships are either unresolved or poorly supported.

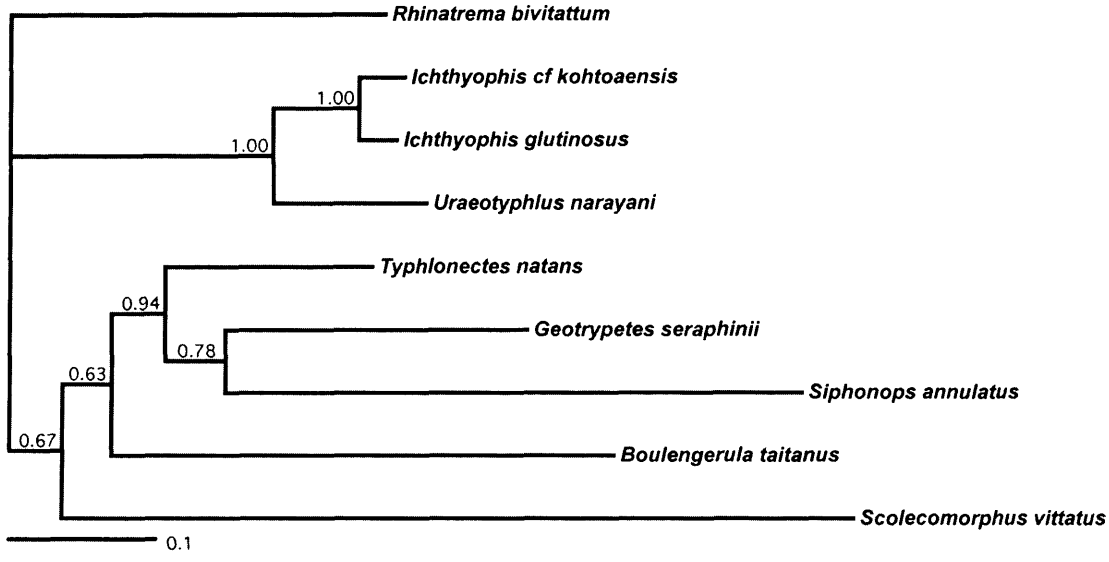

Fig 5.9 Results of combined Bayesian analyses of Exon 1 & Exon 2 and Intron 1 caecilian RH1 nucleotide sequences. Numbers indicate posterior probabilities.

Combined analysis of these data partitions (Fig. 5.9) replicates precisely the relationships recovered by San Mauro et al. (unpublished) and Roelants et al. (2007). Importantly. these opsin sequences provide additional evidence relevent to the major discrepancy between the analyses of these authors and the findings of Frost et al. (2006) which concerns the earliest divergence within the Teresomata. Frost et al. (2006) recovered *Boulengerula* as the sister group of all other teresomatans whereas the opsin sequences add to the bulk of evidence supporting the hypothesis that the Scolecomorphidae occupies this position. This is of significance for taxonomy given that

it is only in the Frost et al. (2006) hypothesis that the Caeciliidae is paraphyletic with respect to the Scolecomorphidae.

In contrast, analyses of the fragments that do not include *Uraeotyphlus narayani* (Exon 3 & 4, and Intron 3) provide results that are incongruent with each other (e.g. over the positions of *Boulengerula*, *Scolecomorphus* and *Geotrypetes*) and, with the sister-grouping of the two species of *Ichthyophis*, are at odds with current understanding of caecilian relationships and are considered unlikely *a priori*. In particular, both fragments recover *Siphonops annulatus* as the sister group of *Ichthyophis*, a relationships for which there is no other evidence from either morphology or molecules.

5.5 Discussion

Analysis of the caecilian opsin Amino Acid sequences provide phylogenetic confirmation that these are RH1 class rod opsins, that they are orthologous with other amphibian sequences and that they provide some additional support for the Batrachia hypothesis. The different topologies observed using different substitution matrices highlights the importance of model choice. No model has been found that is universal for all protein data set (Keane et al 2006). The WAG data matrix is based on substitution rates in globular proteins (Whelan and Goldman 2001) and may not be the most suitable model for analysis of opsin sequences. A substitution matrix developed for use with GPCR's (Hedman et al. 2002) might prove useful in future analyses. This data matrix was not used as it is not one of the models implemented in MrBayes. The Hedman data matrix would have to be implemented into a different phylogenetic program such as P4

(Foster 2004). Due to time constraints was not able to use this matrix for the current analyses.

That the phylogeny produced from Intron 1 and its flanking coding regions in Exon 1 and 2 is consistent with current understanding of caecilian phylogeny suggests that these rod opsin sequences are a good phylogenetic marker for elucidating the relationships of caecilian genera. The different performance of these fragments when analysed separately may reflect their relative utility either for infering divergences at different depths in the tree and/or for divergences that have occured more or less rapidly. Intron sequences are expected to evolve at an elevated rate, due to a lack of functional constraints compared to coding sequences, and thus to be of more use in resolving more recent or rapid divergences (Graur et al. 2000). Thus whereas the coding sequences resolve earlier divergences, the non-coding sequence resolved the more recent and possibly more rapid divergences of *Geotrypetes*, *Typhlonectes* and *Siphonops* (Roelants et al. 2007). The lack of resolution at nodes could also be attributed to the small number of informative characters, and concatenating the partitions appears to have countered this and led to a final tree that took advantage of the strengths of both partitions. In an analysis of the avian family Megapodidae, evolution of the rod opsin first intron was found to occur at a relatively conservative rate compared to mitochondrial genes (Birks et al. 2002) and it can be anticipated that rod opsin sequences would usefully complement mitochondrial and other nuclear markers upon which phylogenetic analyses of caecilians have been based.

In contrast, Exons 3 and 4 and Intron 3 do not appear to provide much phylogenetic information as judged against current understanding of caecilian phylogeny.

Reasons for this are not at all clear at present and merit further investigation. In particular, the strong support they provide for the relationship of *Siphonops* and *Ichthyophis* is problematic. It is noteworthy that no sequences of these markers were obtained for *Uraeotyphlus* which is expected to occupy this position and it is worth considering the possibility of contamination and the need to verify that the *Siphonops* sequences are indeed from this taxon by amplifying overlapping fragments from flanking regions. However, the spurious results could also be attributed to the small number of informative characters in these analyses. Relatively few caecilian sequences have been published so it is imperative that new analyses do not result in misleading phylogenetic relationships caused by contamination. Possible contamination was identified by the comparison of the results with previous analyses. Reproducibility and reliability of results has to be determined before publication. The caecilian samples are from rare and exotic animals. Therefore it is especially important that contamination is not introduced or tissue is not wasted due to errors in the preparation for molecular analysis.

The source of contamination is probably from the PCR stage of analysis. It is unlikely to be a result of the extraction as the results for exon1-2 gave the expected results from recent analyses (Roelants et al 2007). However, this does not exclude the possibility of contamination being introduced. In tackling the contamination a number of laboratory procedures have to be implemented. Caecilian specific primers were used which reduces the possible contamination from the laboratory i.e. microscopic particles of the aerosol coming from open tubes of other specimens containing a number of DNA molecules adequate for enzymatic amplification (Francalacci 1995). Contamination can

be prevented by confining the experimental procedures to an area that can be thoroughly cleaned and irradiated with UV light to destroy any DNA molecules present.

It would be particularly interesting to obtain and analyse a larger caecilian data to determine the utility of these markers at different taxonomic levels and to investigate whether there are any detectable differences in modes of evolution between species that have retained relatively well-developed eyes and those that have independently evolved the most vestigial visual systems. A larger alignment of nucleotide sequences would allow for studies of selection through the comparison of rates of synonymous and non-synonymous substitutions and more extensive comparisons of amino acid sequences might prove of use in detecting substitutions that are causally responsible for the small spectral shift in the absorption of caecilian rod opsins compared to other amphibians.

5.6 Conclusion

Phylogenetic analysis confirms the identity of caecilian rod opsin sequences and provides some support for the Batrachia hypothesis. High support and resolution of caecilian interrelationships is seen when a combination of coding and non-coding sequences of rod opsin are included in caecilian data sequence analyses. The results of these analyses are consistent with recent combined and large molecular analyses. This suggests rod opsin is a useful marker for resolving caecilian relationships at different taxonomic levels. It remains to be seen if rod opsin introns can provide support for caecilian relationships at very low taxonomic levels as might be expected on non-coding sequences.

Chapter 6

Conclusions and Prospects for Future Work.

6.1 Conclusions

In this thesis I have (1) provided the first description of the morphology of the eye of a member of the Rhinatrematidae, the sister group of all other caecilians. (2) elucidated the previously unknown sequence of the rod opsin gene and the physiology, using spectrographic measurements of visual pigments, of the rod opsin gene of caecilians (3) investigated the use of rod opsin sequences as a phylogenetic tool. The obvious reduction in visual system morphology in caecilians is associated with a change in the genes involved in visual function. The rod opsin, identified using direct measurement and phylogenetic analysis, is the only visual pigment detected in caecilians suggesting a loss of colour and diurnal vision, which are probably unnecessary in these predominately burrowing vertebrates with nocturnal habits. There is a change in the maximum absorbance of the rod opsin in caecilians compared to other amphibians examined to date. The structure of the eye, with an organised retina and lens and the functional rod opsin, suggests that the eye has a visual function as least in aquatic and some terrestrial caecilians. A sharp image is unlikely due to the coverings over the eye and the lack of the possibility of extensive accommodation. The rod opsin has been retained in caecilians with very reduced eyes, e.g. *Boulengerula* possibly for a photoperiodic function rather than for vision. Current relationships in caecilian phylogeny are

supported using coding and non-coding nucleotide sequences of the rod opsin. Analysis of full length amino acid sequences give a monophyletic grouping of amphibian rod opsins and some support for the Batrachia hypothesis.

Some features in the *Rhinatrema bivittatum* eye suggest visual functionality, i.e. the crystalline lens, the positioning of the lens using zonule fibres, the presence of six extrinsic muscles, and the retinal organization. However the lack of iris muscles and the covering of the eye suggest that a sharp image is not formed. Good resolving power is important in diurnal vision whereas sensitivity to low light levels is the primary concern in nocturnal conditions. Adaptations that enhance visual acuity, whether optical or anatomical, generally improve sensitivity. A nocturnal eye may therefore be characterised as having poor resolving power, a large nearly spherical lens with substantial amounts of spherical aberration, a cornea with relatively large radius of curvature and a virtually absent accommodative mechanism. (Sivak 1980). In agreement, in even the most well developed eyes of caecilians certain elements have degenerated giving an indication of their nocturnal and burrowing lifestyle of the animal. The skin over the eye is cloudy and zonule fibres have almost disappeared, the lens has an outer cellular layer. These are all expected to decrease resolving power. There are also features that are expected to enhance the detection of light, for example the rod based retina and the dark pigment epithelium. The dark epithelium reduces interference from light bouncing off the inside of the eye.

6.2 Further studies of rudimentation and caecilian vision

6.2 .1 Further morphological studies

Transmission electron microscopy could reveal finer details of the caecilian eye. Examination of sections of eyes from caecilian species already described and used in phylogenetic analysis show that a comprehensive re-examination of the Wake (1993) and Wilkinson (1997) data are needed. The results indicate that the apparently global morphological regression of the visual system conceals a selective progression of structures related to chemoreception and possible photoperiodic functions. The evolution of an atrophied eye and reduced visual system is an adaptively advantageous response to the subterranean environment. Factors that may favour regression include mechanical aspects, metabolic constraints, and competition between sensory systems. The primary advantage of sensory atrophy is hypothesised to be the metabolic economy gained by the reduction of visual structures that do not contribute significantly to the animal's fitness (Nevo 1999). The senses of subterranean animals display impressive structural and functional convergence that could be studied in detail to investigate generalities.

6.2.2 Developmental stages

The molecular, physiological and morphological data has been studied in most detail from adult caecilians. (Himstedt, 1995;Wake 1985) Given that gene expression and morphology could change over the lifespan of an individual, completing the study within this order would require looking at the different life history stages in different species of Gymnophiona. Visual pigment has been shown to express differently and changes in morphology can occur at later life history stages in fish. (Shand et al. 2002; Carleton 2001; Hoke 2006; Cheng 2006)

Further, distinct metamorphic life stages are observed in caecilians especially in oviparous forms with an aquatic juvenile and terrestrial adult forms. In particular, there is evidence of different developmental timing depending on whether the species have free-living larvae or are livebearers. The eye of the viviparous *Dermophis mexicanus* continues to grow after birth (Wake 1985). In contrast eyes of the oviparous *Ichthyophis* remain unchanged from hatching to adulthood and the lens and retinal cell number does not alter as observed in other amphibians (Himstedt 1995). It is possible that the developmental genes and pathways could differ between caecilians that show distinct metamorphosis, e.g. *Ichthyophis*, compared to viviparous species, e.g. *Dermophis mexicanus*. These caecilians with contrasting life histories would be worthy of comparative analysis using gene expression molecular techniques.

Previous work on cave-dwelling fish (Jeffrey 2001) and on degenerate conditions in humans would suggest suitable candidate genes for investigation of eye development would be Prox1, SOX2, SHH, OTX2, Pax 6 (Yamamoto et al 2001; Van Heyningen et al 2002) to compare the caecilian species.

6.2.3 Other Species

Vertebrates that live in dark conditions exhibit similar morphological and physiological changes as seen in the caecilian visual system. Subspecies of the cave salamander *Proteus anguinus* show a similar morphological diversity and rudimentation of eyes (Kos et al 2001). One population of *P. anguinus* in particular appears to have lost cone opsins but retained the rod opsin for scotopic vision. Similarly, most species of deep-sea fish have lost cone opsin and photoreceptors (Douglas et al 1998). Morphological convergent

evolution of the visual system of caecilians and burrowing reptiles, for example snakes and amphaesbanians, has been inferred (Caprette et al. 2002). Also a few anuran frog species (e.g. *Leptodactylus pentadactylus*) have been shown to display negative phototaxis (Kicliter and Goytia 1995) as demonstrated in the caecilian *Ichthyophis* cf. *kohtoaensis* (Himstedt 1995). There is some similarity between the life histories of *L. pentadactylus* and *Ichthyophis* inasmuch as they form burrows to lay eggs (Kokubum et al. 2005). Reptile and amphibian species showing similar morphological or behavioural traits have not been directly investigated with regards to molecular evolution.

Further research could investigate the new mechanistic basis causing the rod opsin spectral shift, to detect alterations within the phototransduction pathway, and during caecilian development of the visual system compared to other amphibians. Further understanding of rudimentation of the visual system would be provided by study of other ecological analogues within reptilian and amphibian clades. Morphological convergent evolution of the visual system of caecilians and burrowing reptiles, for example snakes and amphaesbanians could be examined for similar loss or changes in opsin maximum absorbance.

6.2.4 Spectral Shift

A spectral shift has been discovered from the MSP data. The absorbance values of three caecilian species mentioned in Chapter 4 are 489nm, 488nm and 487nm respectively. Usually the absorption of rod opsin is closer to 500nm, as found in all other amphibians studied to date (Fyhrquist et al. 1999). Other blue shifted rod opsins are found in deep-sea fish (Hope et al. 1997). This degree of shift in caecilians has not been observed in any

other terrestrial animal. As all caecilian species measured have a similar value, it can be inferred that the causative mutation in the nucleotide sequence occurred early in caecilian evolution. There are four amino acid changes conserved within two complete caecilian rod opsin sequences compared to other amphibians. None occur within the retinal-binding pocket as would be expected for a single mutation to cause a significant shift. Therefore a new mechanism is being used. It is possible that these four amino acids together or one or two amino acid changes cause the spectral shift but exist outside the retinal binding pocket (Yokoyama 2000). Site directed mutagenesis could be used to find the specific causative mutations.

The sensitivity hypothesis states that visual pigments will best detect contrast if their spectral sensitivity matches the background space-light while in others 'offset' visual pigments will be better (Lythgoe 1979). Caecilians are found in wet tropical environments in agricultural, swamp, open bush and forested habitats (Jones et al. 2006, Gower et al 2004). Within these habitat types, animals have been found to seasonally inhabit relatively loose, shallow soil and epigeic microhabitats such as under leaf litter, rotting wood and rotting vegetation (Burger et al 2007). Most species burrow in soils and are considered nocturnal (Burger et al 2007, Kupfer et al 2004, Himstedt 1995). Therefore it could be hypothesized that the wavelength of visual pigments may either be similar to that of the forest, subterranean or night environment. Tropical forests are heterogenous in ambient light colour (Endler 1993). The forest environment can be divided into four different habitat types depending on sun sky and vegetation (Endler 1993). The habitats are forest shade, woodland shade, small gaps, and large gaps. Forests are groups of trees in which most of the crowns overlap, forming a nearly

continuous canopy with small holes or gaps. Woodlands are groups of trees in which most of the crowns are separated leaving large as well as small gaps in the canopy. The light will vary from white in large gaps to yellowish-red in small gaps. In forest shade essentially all of the light has been transmitted through or reflected from leaves which is between 470-490nm. As a result the greenish leaf spectrum should dominate the irradiance spectrum (Endler 1993). In woodland shade the bluish sky radiance should dominate the irradiance spectrum in woodland shade. The visual pigment of caecilians does match the spacelight of a shaded forested environment and it is possible that the visual pigments evolved.

At night the wavelengths of light are long-wave shifted so again the caecilian visual pigment does not match. However the light at twilight light is predominately 480nm similar to the λmax of caecilians. After the sun sinks below the horizon the loss of middle wavelengths (570nm-630nm) becomes pronounced; the light becomes purplish until colour vision is no longer possible (Endler 1993). Species signalling in early/late conditions should use blues, reds or purple for maximum brightness and with a small amount of yellow, yellow – green or green for maximum contrast (Endler 1993). It is possible that the main role of the visual pigment is to detect twilight. In many fish species the twilight period is often a critical period during which diurnal and nocturnal species are changing behaviour patterns and positions and when prey detection and predator avoidance tasks become highly significant (Munz and Mcfarland, 1977).

Following a suggestion made by de Vries (1949), has proposed that the shift in maximum sensitivity of the eye toward the blue end of the spectrum at low levels of illumination (Purkinje shift) may have evolved as a means of obtaining a more favourable

signal-to-noise ratio, thereby greatly increasing sensitivity of the eye to light. Such a shift in the spectral absorption of a visual pigment should be accompanied by a decreased thermal instability of the photochemical, producing the more favourable ratio. The noise hypothesis is based on the fact that visual pigments produce a 'dark' noise signal due to thermally induced isomerizations. These random events will set a lower limit for photon sensitivity below which it is impossible to distinguish the 'light' signal from the 'dark' noise. It is predicted that visual pigments absorbing at shorter wavelengths are less prone to thermal noise and that this could form the basis for increasing sensitivity in low light levels and at higher temperatures. Thermally induced noise events decreasing with a shift to shorter wavelengths have been demonstrated with porphyropsins also being noisier than rhodopsins (Donner et al 1990). This lowering of the visual threshold should be advantageous to a fish living in a low light-intensity environment. (Munz 1958) and suggests an alternative explanation for the evolution of the blue-absorbing rod opsin of deep-sea Lake Baikal fishes (Bowmaker et al 1994). The study on Lake Baikal fishes show the sensitivity of rods shifts towards shorter wavelengths at increasing depths. Much of the surface water of Lake Baikal is yellow and the water remains blue-green to significant depths, implying that the shift goes too far. The authors suggest that the shift in spectral sensitivity with depth is not simply a product of the sensitivity hypothesis, but also may be due to the need to use short-wave absorbing visual pigments with more stability in environments where light levels are low and thermal noise may be more significant. Thus the caecilian visual pigment may be blue-shifted as a match to the twilight environment and/or for the reduction of noise at low light levels.

The loss of cone opsin and therefore loss of colour and diurnal vision in caecilians need to be confirmed. Using degenerate primers in PCRs with *Rhinatrema bivittatum* cDNA would be useful in determining if cone opsin is present. Other methods of determining loss would be to redo the Southern blot and in-situ hybridization techniques of eye sections using amphibian cone opsins sequences for probes. In cetaceans, the African giant rats *Cricetomys gambianus* and *C. emini*, *Spalax ehlenbergi* (Peichl et al 2004), and the owl monkey a loss occurs of the UVS/VS opsin gene due to an accumulation of mutations (Hunt et al. 2001, Levenson and Dizon 2003). If the same mechanism occurred in caecilians then given the long time of divergence then pseudogenes are unlikely to be found.

6.2.5 Other visual genes

Creation of cDNA libraries from different species of caecilian, other amphibians and reptiles, to produce expressed sequence tags would generate the most amount of sequence for comparative analysis of phototransduction and developmental genes. Other photransduction genes. Excitation of the visual pigments initiates the enzymatic phototransduction cascade. Phototransduction is seen as the archetype of a sensory transduction module and the retinoid cycle, in which the retinal chromophore is reisomerized in a long series of reactions (Palczewski et al. 2006). These proteins, transducin, phosphodiesterase, cyclic nucleotide-gated channels, opsin kinase, arrestin, guanylate cyclase, S-modulin/recoverin and GC-activating protein within the cascade have been studied in a variety of vertebrates including some frogs (Hisatomi and Tokunaga 1999). Further investigation of sequences of such genes would clarify how

rudimentation of the caecilian visual system extends to these other proteins and the nature of their interaction with rod opsin. In situ experiments could determine how the above mentioned developmental and phototransduction genes are expressed in tissues. The expression of the developing system of the subjects would be also quantified by Real Time PCR (RT-PCR) methods.

6.2.6 Photoperiodicity

Caecilians have a hypertrophied Harderian gland compared to other amphibians (Avivi et al. 2004). Similar hypertrophy in also observed in the mole rat *Spalax ehlerenbergi*. which has a rod-based eye but also has a significant amount of cone photoreceptors in its retina (Peichl et al. 2004). Unlike *Spalax* however, it is thought that the Harderian gland function in caecilians to lubricate the tentacle rather than the eye (Walls 1942). Together with the eye and pineal gland, the Harderian gland participates in photoperiodic perception (Avivi et al. 2004). *Spalax* is considered to be visually blind, as it does not use its eyes for vision. Specific circadian rhythm genes were found to be associated with the Harderian gland Pers and MOP3 (Avivi et al. 2001) and the authors suggested that this was a unique adaptation to life underground. Avivi et al. (2004) found CRY genes that act as photoreceptors and a negative feedback mechanism of the biological clock. CRY have been found to be expressed in the Harderian gland as well as in other tissues such as liver, heart, eye and brain (Avivi et al 2001). These genes are expressed out of phase compared to what is found in diurnal animal (e.g. mice). Melatonin receptors have also been found in the Harderian gland which also plays a part in photoperiodism. To set the biological clock *Spalax* has a brief exposure to light to adapt to the 24-hour light/dark

cycle. As well as light the clocks of animals can be set by changes in temperature (Zhu and Green 2001). Since *Spalax* shows a similar adaptation in life history, i.e. it is a burrower and shows a similar rudimentation of the eye it would be interesting to see if genes involved in photoperiodicity occurred with a similar increase in expression in caecilians.

Since some genes, such as CRY are expressed, throughout the animal it may be possible to isolate these genes from the cDNA library of *Rhinatrema* being produced at the moment and to compare the sequences and possibly expression patterns by localization studies with other species. Other opsins of interest that may have a photoperiodic function can be found extraretinally as well as in the retina. These include pinopsin found in the pineal in the anterior preoptic nucleus, melanopsin found in retinal pigment epithelium, melanophores in *Xenopus*, vertebrate ancient found in the brain and peropsin found in the retinal pigment epithelium (Kojima and Fukada, 1999). The relationships between these opsins has been recently been studied and a paper that I co-authored Pisani et al. 2006 (in Appendix).

6.2.7 Comparison with other Amphibian sensory systems

A correlation of increase in other sensory systems with a reduction in eye morphology could be examined by comparison with other amphibians.
A general trend for simplification of the sensory systems has been observed in Gymnophiona. In caecilian species, with aquatic to semiaquatic larvae and terrestrial adults, epidermal neuromasts and ampullary organs are lost at metamorphosis (Roth et al 1992). Viviparous species with aquatic adults never develop neuromasts but retain

ampullary organs throughout life. Strictly terrestrial viviparous species develop neither neuromasts nor ampullary organs. Species with direct development and terrestrial adults have both epidermal neuromasts and ampullary organs (Roth et al. 1992). With the auditory system a simplified periphery occurs in that they lack an external ear (tympanum) and a middle ear cavity. The middle ear bones are also reduced. It has also been suggested that eyes and ears are evolutionarily related (Piatigorsky, 2003) due to developmental pathways being shared and the physical association of statocysts (ear precursor) and ocelli (eye precursor) in jellyfish. This association is further reflected also by the occasional linkage between genetic disorders of the eye and ear such as Usher syndrome, which results in deafness and retinal degeneration in humans, and microphthalmia where the reduction in size and function of the eye is associated with defects in external ear morphology (Treisman, 2004).

A similar simplification is observed in the auditory system of some Amphibia compared to other gnathastomes, with members of the salamander family showing reduction compared to Anurans, the lateral line system lack ampullary organs compared to other vertebrates that have both neuromasts and ampullary organs (Roth et al 1992). Most frogs and salamanders even though they depend more heavily on vision have reduced vision compared to other vertebrates. The density of photoreceptor cells, the number of morphologically different neurons in the retina and the number of retinofugal visual projection areas all are decreased in Amphibia. The dorsal nucleus situated within the medulla oblongata and receiving the lateral line afferents is smaller in fossorial species (Roth et al 1992)

The reduction in the visual system is seen with the increase in other compensatory

sensory systems i.e. other sensory systems need to be examined in as much detail similar to the studies on *Astyanyx* whereby the same genes that affect the degeneration of eyes also affect the taste buds to increase them in size and number (Yamamoto et al. 2003). Other sensory systems in caecilian should therefore be investigated.

6.2.8 Development genes.

To understand the evolution of eye degeneration in caecilians it is necessary to determine the molecular and cellular mechanisms of the degenerative process, and whether the same or different genes and mechanisms are involved in the loss of vision. The genes involved in rudimentation have been studied in the cavefish *Astyanyx* in comparison with closely related surface dwelling fish. Lens transplantation by Yamamoto and Jeffery (2000) studies between closely related surface dwelling fish with the cavefish affected the development of the eye. When a cavefish lens was transplanted into a surface fish optic cup it died on schedule. Likewise, when a surface fish lens was transplanted into a cavefish optic cup it continued to grow and differentiate, as it would have in the surface fish host. These results suggest that the cavefish lens is autonomously fated for apoptosis by time of transplantation. After obtaining a surface fish lens, the cornea and iris appeared, which are normally missing in cavefish, and the retina enlarged and became more organised. Further growth resulted in the presence in the presence of a highly developed eye containing all of the expected eye tissues, including the cornea, iris and photoreceptor cells. The cornea and iris are derived in part from optic neural crest cells, indicating that cavefish neural crest cells are present and located in the proper positions to be induced by the lens. Changes in the expression of specific developmental

genes have been observed when comparing the surface dwelling fish with cave-dwelling fish. Pax6 appears to be down regulated in cave-dwelling fish. Pax6 expression appears to be affected by hedgehog gene expression affects midline signalling and is hyperactive in cave dwelling fish (Jeffery, 2001; Strickler, et al. 2001). The developmental and gene expression studies have revealed a negative relationship between midline signalling, which could promote enhancement of other sensory organs that may be advantageous to the survival of blind cavefish. In cavefish the taste buds appear to increase in number due to the increase in SHH expression (Jeffery, 2001). A scenario is proposed for the loss of vision in these cavefish. The developing lens normally produces a factor that is responsible for inducing differentiation of the anterior eye segment (e.g. cornea and iris) (Yamamoto and Jeffery 2000) and sustaining retinal growth by suppression of apoptosis. Although generation of new cells in the retina is not prevented, cell death triggered by the absence of lens signal prohibits net growth, degeneration begins, and the cavefish eye is overwhelmed by the growth of the body. This scenario is not followed by *Ichthyophis* which appears to reach a stage of development at hatching then does not degenerate when considering number of retinal cells which would suggest again possible functionality of the eye (Himstedt 1995). It would be difficult to test this scenario in caecilians due to the number of animals required for lens transplantation between more basal and derived caecilians. However possible ways to study developmental genes is to obtain gene sequences from a cDNA library constructed from caecilian eyes and perform some in-situ hybridisation studies to see the expression of the developmental genes. It would be also informative which genes affects tentacle development, due to the evolutionary history of the tentacle, and to see if similar

developmental genes are involved in eye degeneration in the Gymnophiona.

Theories for the degeneration of the eyes in caecilians other than simplification due to size of the genome include adaptive and neutral mutation theories. The difficulty in explaining eye regression by natural selection resulted in the popularity of the neutral theory hypothesis, where loss of function mutations would be expected to accumulate randomly over time. However, the neutral mutation or adaptation hypotheses are not mutually exclusive (Jeffery et al. 2003). Eye degeneration could have occurred in two steps, the first mediated by natural selection and the second by neutral mutation. Natural selection could have initiated the eye degeneration as a trade-off between forming complete eyes and enhancing taste buds and other cranial sensory organs. The trade-off may be controlled by pleiotropic developmental genes such as the midline signalling gene; sonic hedgehog. Subsequently neutral mutations could have accumulated in different eye genes as eye regression continued under relaxed selection in the cave environment (Jeffery et al. 2003). The accumulation of mutations and gene duplications identified by comparative analysis reflect adaptive responses of various species to different environments (Yokoyama and Yokoyama 1996). Adaptive mutations in genes could be studied by examining caecilian sequences in comparative analyses as it has been done in crayfish and in marine mammals (Crandall and Hillis, 1997, Levenson and Dizon, 2002).

6.2.9 Further Phylogenetic study

Phylogenetic analysis confirms the identity of caecilian rod opsin sequences and provides some support for the Batrachia hypothesis. High support and resolution of caecilian

interrelationships is seen from a combination of coding and non-coding sequences of rod opsin. The results of the analyses included in this thesis are consistent with recent combined and large molecular analyses. This suggests rod opsin is a useful marker for resolving caecilian relationships at different taxonomic levels. It remains to be seen if rod opsin introns can provide support for caecilian relationships at very low taxonomic levels as might be expected on non-coding sequences. Further test of the rod opsin utility can as a phylogenetic marker should be made by comparing it to other nuclear genes such as RAG1 that are widely used in vertebrate phylogenetics.

6.3 Final conclusion and prospectus

Complexity, variation and growing knowledge of the genes involved in development and signal transduction in visual pathways provide many methods to study evolution of caecilians and their vision. Further, the observed morphological diversity within the Gymnophiona order would suggest the group to be a model system in understanding rudimentation and identifying the essential components of a functioning visual system.

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Appendix

Accession numbers of sequences downloaded from NCBI

Rh1: *Rhinatrema bivittatum* DQ283805.1; *Herpele squalostoma* DQ283980.1; *Homo sapiens* NP_000530; *Bos taurus* 1F88_B; *Mus musculus* NP_663358; *Sminthopsis crassicaudata* Q8HY69; *Gallus gallus* P22328; *Alligator mississippiensis* P52202; *Danio rerio* NP_571287; *Latimeria chalumnae* AAD30519; *Danio rerio* BAC21668; *Geotria australis* AAR14682; *Petromyzon marinus* Q98980; *Astyanax mexicanus* P41590; *Homo sapiens* NP_00530.1; *Ornithorhynchus anatinus* ABN43074.1; *Spalax ehrenbergi* AAG25707.1; *Ambystoma tigrinum* Q90245; *Cynops pyrrhogaster* BAB55452.1; *Bufo bufo* P56514; *Rhinella marina* NP_0010900803.1; *Xenopus laevis* AAC42232.1; *Silurana tropacalis* NP_001090803.1; *Lithobates catesbeianus* P51470; *Rana pipiens* P31355; *Rana temporaria* AAB93706.1; *Anolis carolinensis* P41591.1

Rh2: *Takifugu rubripes* AAF44648; *Danio rerio* Q9W6A6; *Gallus gallus* P28683; *Columba livia* AAD32242; *Anolis carolinensis* AAB35062; *Latimeria chalumnae* AAD30520; *Geotria australis* AAR14683; *Astyanax mexicanus* AAB32221

SWS1: *Danio rerio* Q9W6A9; *Carassius auratus* Q90309; *Mus musculus* P51491; *Homo sapiens* P03999; *Phelsuma madagascariensis* AAD45183; *Gallus gallus* P28684; *Xenopus laevis* P51473; *Geotria australis* AAR14684.9; *Cynops pyrrhogaster* BAB79499.1; Gekko gekko P35357; *Phelsuma madagascarensis* AAD25918.1; *Lithobates catesbeianus* BAA96828.1; *Sminthopsis crassicaudata* AAR14685.1; *Bos Taurus* NP_776992.1;

SWS2: *Xenopus laevis* AAO38746; *Geotria australis* AAR14681; *Gallus gallus*

P28682; *Astyanax mexicanus* AAB28911.1; *Ornithorhynchus anatinus* ABN43075.1; *Ambystoma tigrinum* AAC96069.1; *Danio rerio* BAC24133.1; *Lithobates catesbeianus* BAA76864.1; *Cynops pyrrhogaster* BAB39378;

LWS: *Gallus gallus* P22329; *Anolis carolinensis* P41592; *Xenopus laevis* O12948; *Geotria australis* AAR14680; *Mus musculus* O35599; *Homo sapiens* P04001; *Homo sapiens* LWSG NP_00504.1; *Astyanax mexicanus* P22332; *Astyanax mexicanus* LWSG P22331; *Ornithorhynchus anatinus* ABN43076.1; *Ambystoma tigrinum* AAC96070.1; *Phelsuma madagascarensis* AAD25917.1; *Gekko gekko* P35358; *Bos taurus* AAG49893; *Cynops pyrrhogaster* BAB55453.1; *Silurana tropacalis* NP_001084114.1; *Danio rerio* AAD24754.1

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Molecular evidence for dim-light vision in the last common ancestor of the vertebrates

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Supplemental data: Molecular evidence for dim-light vision in the last common ancestor of the vertebrates

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Experimental Procedures

A data set of 47 amino acid sequences, representing all the known vertebrate visual opsins for a wide range of taxa, was downloaded from the NCBI website (see below for accession numbers). These sequences were aligned using ClustalW [S1], default alignment options, but correcting for multiple substitutions when building the guide tree. A second alignment was generated using Muscle [S2] default options. The two alignments, although extremely similar, were not identical, differing slightly in the N- and C- terminal regions. Both the ClustalW and the Muscle alignment were subjected to two GBlocks analyses [S3] to remove poorly aligned sites. The first GBlocks analysis used the default options, but allowing gapped sites. The second GBlocks analysis was more stringent with (1) the minimum length of a block, to be retained increased from 10 to 20 positions, and (2) the maximum number of contiguous non-conserved positions allowed halved from eight to four. The alignments can be downloaded at: http://bioinf.nuim.ie/davide/index.html/.

Phylogenetic analyses were performed on all the four generated datasets, and also excluding all gapped sites. The results of these analyses were always very similar in terms of both recovered trees and nodal support. Accordingly, only the results of the ClustalW generated data set, as cleaned under the less stringent (first) GBlocks analysis are presented (see main text). This alignment was 346 amino acid positions long with very few gaps.

The χ^2 test, as implemented in Tree-Puzzle 5.2 [S4] was used to test the alignment for sequence heterogneity in amino acid composition which can result in spurious phylogenetic results [S5]. No significant heterogeneity was found.

Maximum Likelihood (ML) analyses were performed using both quartet puzzling (Tree-Puzzle5.2 default puzzling options) and standard ML. Standard ML analyses were performed using the software PHYML [S6], PHYML-SPR [S7] and SPR [S7]. Support for the nodes in the ML tree were estimated using the bootstrap (100 replicates) as implemented in PHYML. The Bayesian analysis was performed using the parallel version of MrBayes 3.1.1 [S8]. For the Bayesian analysis an initial value of two million generations was run, sampling each 1000 generations. As suggested in the MrBayes 3.1.1 manual, two independent runs (and four chains for each run) were simultaneously performed, and convergence was tested comparing the average standard deviation of the split frequencies for the two independent runs. The resulting Bayesian tree was derived as the majority rule consensus of the trees sampled from both runs after convergence was reached (c. 300,000 generations).

All likelihood calculations were performed under the best fitting substitution model (WAG + G + I) selected using the Akaike Information Criterion as implemented in MultiPhyl [S9]. In the Bayesian analysis the values of G and I were estimated during the tree search.

Maximum likelihood distances among the 47 sequences were calculated using Tree-Puzzle5.2 and used to build a Minimum Evolution (ME) Tree using PAUP* [S10] (heuristic search). PAUP* was also used to perform a Maximum Parsimony (MP) analysis and a Weighted MP (WMP) analysis of the data. In both cases a heuristic search was performed (100 random addition sequences), and support for the recovered trees was estimated using the bootstrap (1000 replicates, with a single random addition sequence). For the WMP analysis the GPCR specific amino acid substitution matrix of [S11] (see also [S12]) was used.

Potential Long Branch Attraction (LBA) artefacts were investigated using the method of Pisani [8] using the C software Boildown (written and is distributed by SRH). The protocol proposed in [8] was strictly followed and all the sites with LeQuesne Probabilities (LQP; [S13]) ≥ 0.1 were sequentially removed. New phylogenetic analyses were preformed and the results obtained were then compared with those of the analysis of the 346 position data set.

The standard ML tree was compared with the quartet-puzzling tree and a tree displaying the Collin et al. [3] arrangement of the Rh sequences (Figure S1). CODEML, which is part of the PAML package [S14], was used to obtain site-specific likelihood values for each of the three topologies of Figure S1. These values were fed to the software CONSEL [S15] to compare the three alternative topologies using the Approximately Unbiased (AU) test [9].

Supplemental Results and Discussion

Figure 1A and B (main text) summarise the results of the quartet-puzzling, ML (PHYML, PHYML-SPR, SPR), Bayesian, minimum evolution, and equally and differentially weighted parsimony analyses. Complete results of the quartet-puzzling and PHYML analyses are reported in Figure S2. Results of the Bayesian analysis are reported in Figure S3. Because PHYML, PHYML-SPR and SPR all returned the same tree Figure S2 only reports the results of the PHYML analysis. All these analyses agree in providing strong support for the clustering of RhA within the Rh1 group but disagree in the position of Rh2. Very strong support for the clustering of RhA within the Rh1 group was also obtained also using MP (bootstrap support = 95%) and WMP (bootstrap support = 99%) whereas MP provided only 19% and WMP 9% bootstrap support for RhB as an Rh2. We did not perform a bootstrap ME analysis, but the ME tree was consistent with the quartet puzzling ML analysis in supporting RhA as an Rh1 and RhB as an Rh2. No analysis supported a monophyletic RhA plus RhB. Relationships within some putatively orthologous clusters, notably within the LWS cluster are not as expected given the species interrelationships and we suspect this is due to either paralogy or noise within this cluster.

We conjecture the correct classification of RhB is probably as an Rh2, and suggest the position of RhB in the standard ML (non quartet-puzzling), MP, WMP and Bayesian analyses is suggestive of a phylogenetic artefact [S5]. The clustering of RhB as an Rh2 implies a simpler pattern of gene duplication and losses, than its more basal position in the standard ML, Bayesian and parsimony trees. One single gene duplication in the ancestral Rh gene (predating the

agnathans-gnathostomians split) and resulting in the Rh1 and Rh2 genes is needed to explain RhB as an Rh2 (and RhA as an Rh1). Differently, if RhB is assumed to be basal to a ((Rh1,RhA), Rh2) group, then two duplications predating the agnathans-gnathostomians split, followed by two independent gene losses (of RhB within gnathostomians and Rh2 within agnathans) are needed to explain the observed pattern. Whatever the correct position of RhB is, it is clear that accurately placing this sequence proved difficult even using ML-based and Bayesian methods and may have hampered previous analyses.

We must await the isolation and sequencing of further agnathan RhB/Rh2 sequences for this gene to be conclusively and correctly classified. However, this is inconsequential for our primary conclusions that RhA is a member of the Rh1 group. Pisani's method [8] found only 22 potentially fast evolving sites and their sequential removal did not cause RhA to change its position. Also the bootstrap for the RhA plus Rh1 group was unchanged further strengthening our conclusions that RhAs are Rh1, and hence that true (Rh1 mediated) dim-light vision predated the Agnatha Gnathostomata split.

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S15. Shimodaira, H., Hasegawa, M. (2001). CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics. 17, 1246-1247.

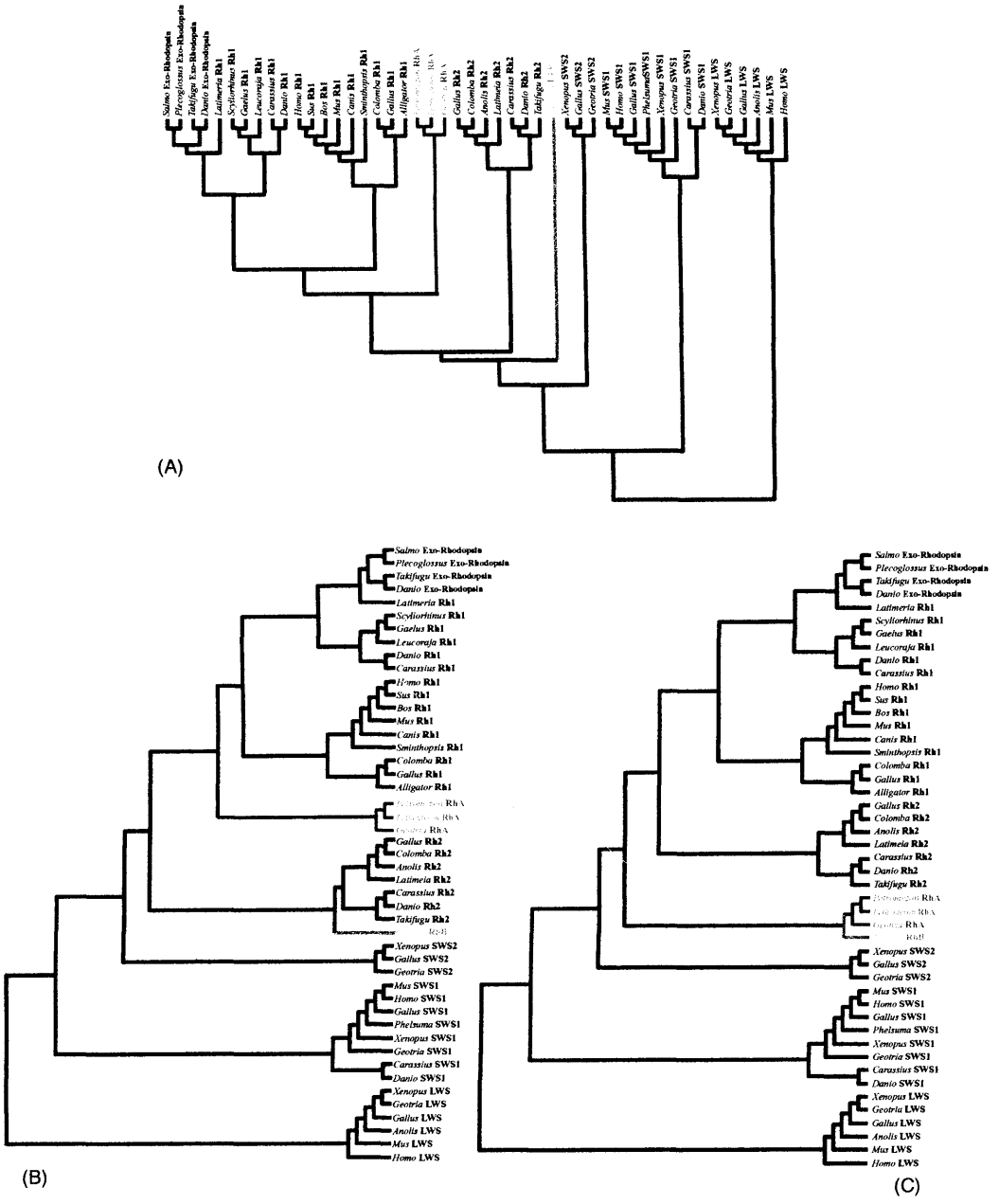

Figure S1. (A) The standard ML (PHYML, SPR-PHYML & SPR) tree (displaying Yokoyama [2] hypothesis). (B) A modification of the standard ML tree illustrating the relationships among the Rh sequences as inferred using quartet-puzzling ML. Note that also this tree display Yokoyama [2] Hypothesis. (C) A modification of the optimal standard ML tree illustrating the relationships among the Rh

sequences as inferred by Collin et al. [3]. These trees has been used to compare the two alternative hypotheses [2 and 3] using the AU test. In Blue: RhA. In Green: RhB. See main text and materials and methods for abbreviations.

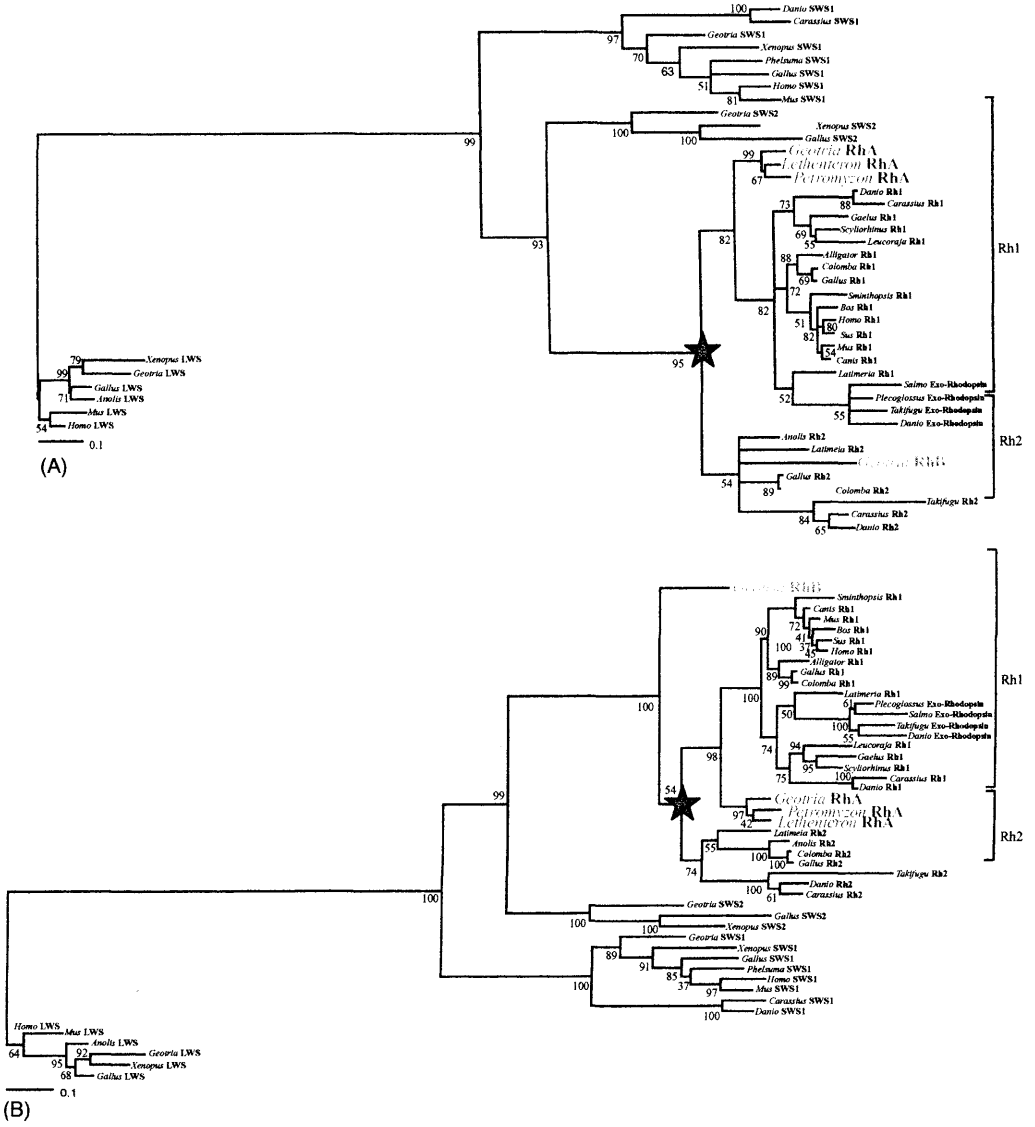

Figure S2. Results of the Maximum Likelihood Analyses. (A) Quartet Puzzling (B) PHYM (see also supplemental results and discussion and main text). Numbers at the nodes represent, respectively, quartet puzzling support values and bootstrap proportions. The star represents the Gene duplication resulting in

the origin of Rh1. In Green: The Jawless Vertebrates RhB sequences, in Blue the Jawless Vertebrates RhA sequences.

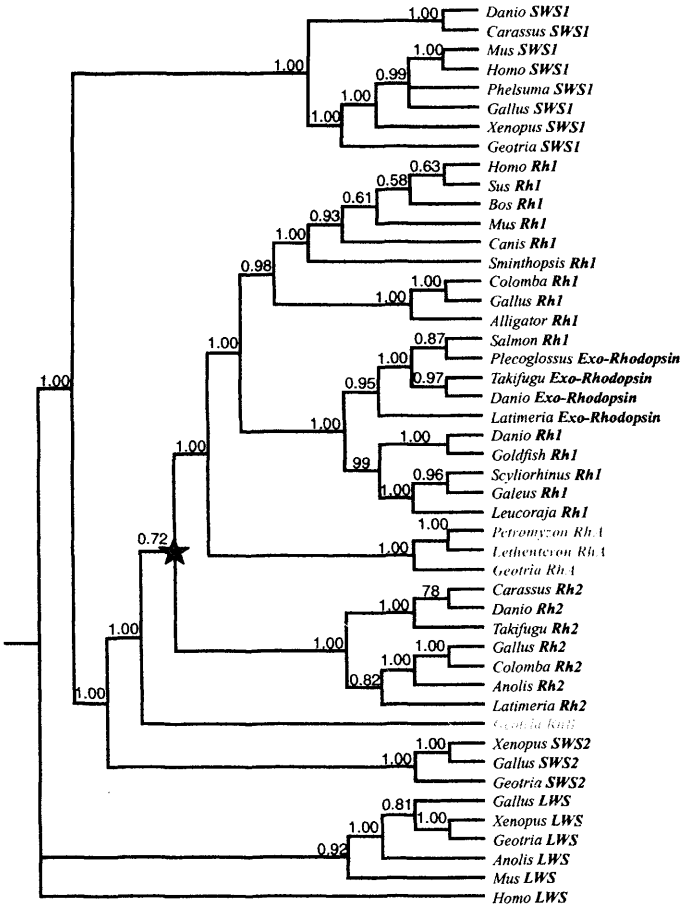

Figure S3. Results of the Bayesian Analysis. The star represents the gene duplication resulting in the origin of Rh1. Number at the nodes represent Posterior Probabilities. In Green the Jawless Vertebrates RhB, in Blue the Jawless Vertebrates RhA.

Supplemental Information (Accession Numbers & Alignments)

SWS1: Danio rerio Q9W6A9; Carassius auratus Q90309; Mus musculus P51491; Homo sapiens P03999; Phelsuma madagascariensis AAD45183; Gallus gallus P28684; Xenopus laevis P51473; Geotria australis AAR14684.

Rh1: Homo sapiens NP_000530; Sus scrofa O18766; Bos Taurus 1F88_B; Mus musculus NP_663358; Canis familiaris P32308; Sminthopsis crassicaudata Q8HY69; Columba livia AAD32241; Gallus gallus P22328; Alligator mississippiensis P52202; Salmo salar AAF44619; Plecoglossus altivelis BAC56700; Takifugu rubripes AAF44622; Danio rerio NP_571287; Latimeria chalumnae AAD30519; Danio rerio BAC21668; Carassius auratus P32309; Scyliorhinus canicula O93459; Galeus melastomus O93441; Leucoraja erinacea P79863; Lethenteron japonicum P22671; Geotria australis AAR14682; Petromyzon marinus Q98980.

Rh2: Carassius auratus P32311; Takifugu rubripes AAF44648; Danio rerio Q9W6A6; Gallus gallus P28683; Columba livia AAD32242; Anolis carolinensis AAB35062; Latimeria chalumnae AAD30520; Geotria australis AAR14683.

SWS2: Xenopus laevis AAO38746; Geotria australis AAR14681; Gallus gallus P28682.

LWS: Gallus gallus P22329; Anolis carolinensis P41592; Xenopus laevis O12948; Geotria australis AAR14680; Mus musculus O35599; Homo sapiens P04001.

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