*Sh*AR2β, a divergent nicotinic acetylcholine receptor subunit from the blood fluke *Schistosoma*

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SUMMARY

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate the fast actions of the neurotransmitter, acetylcholine. Invertebrate nAChRs are of interest as they are targets of widely-selling insecticides and drugs that control nematode parasites. Here, we report the cloning of *Sh*AR2β, a candidate nAChR subunit from the blood fluke, *Schistosoma haematobium*, which is the third trematode nAChR subunit to be characterized. While *Sh*AR2β possesses key structural features common to all nAChRs, its amino acid sequence shares considerably low identity with those of insect, nematode and vertebrate nAChR subunits. In particular, the second transmembrane domain of *Sh*AR2β, which lines the ion channel, bears unusual amino acid residues which will likely give rise to a receptor with distinct functional properties. Phylogenetic analysis shows that *Sh*AR2β is a divergent nAChR subunit that may define a clade of trematode-specific subunits. We discuss our findings in the context of potentially exploiting this receptor as a target for controlling schistosome parasites.

Key words: Schistosoma, nicotinic acetylcholine receptor, cholinergic, ion channel, degenerate PCR.

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are members of a wider 'cys-loop' superfamily of ligand-gated ion channels (LGICs) which also includes serotonin (5-HT3), glycine, γ-amino butyric acid (GABA), as well as invertebrate glutamate and histamine receptors (Sine and Engel, 2006). The nAChR channel is best known for its role in mediating excitatory responses to acetylcholine (ACh) in the neuromuscular junction and neuronal synapses of both vertebrates and invertebrates. The channel itself is a multimeric complex comprising 5 subunits, and the discovery of large subunit families in many species provides a molecular basis for broad functional diversity. In birds and mammals, 17 nAChR subunits have been identified (Millar, 2003) but the largest and most diverse nAChR gene family identified to date is that of the nematode *Caenorhabditis elegans*, whose genome contains the coding regions for at least 27 subunits (Jones and Sattelle, 2004).

The identification and characterization of invertebrate nAChRs has become the subject of intensive research in recent years, much of which has been stimulated by the recognition of the importance of these receptors as targets for insecticides and agents that control nematode parasites (Kohler, 2001; Raymond-Delpech *et al*. 2005; Brown *et al*. 2006). For our own part, we are interested in investigating potential nAChR drug targets in the trematode blood fluke, *Schistosoma*, parasitic worms that live in the bloodstream of vertebrate hosts including man and domestic livestock. Human schistosomiasis currently affects over 200 million people generally in economically disadvantaged areas of the tropics, and is recognized by the World Health Organization as the second most important parasitic disease after malaria (Ribeiro-Dos-Santos *et al*. 2006). Means of control are extremely limited, being reliant on a single effective drug, praziquantel. Extensive use of this drug in the field is leading to evidence of emerging resistance, and there is an acknowledged need for new control measures to be developed. Although these trematode parasites are not sensitive to conventional cholinergic anthelmintics, the fact that human schistosomiasis can be treated using the organophosphate acetylcholinesterase (AChE) inhibitor, metrifonate, demonstrates that the parasite's cholinergic system presents a viable and potent drug target. Our own investigations using cultured schistosomes *ex vivo* have led us to identify a unique regulatory role for nAChRs located on the schistosome surface, which

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together with surface-anchored AChE, form components of an unusual cholinergic regulatory system that functions to modulate the parasitic uptake of essential nutrients, such as glucose, from the host blood in response to ACh circulating in the host bloodstream (Camacho *et al*. 1994, 1995, 1996; Camacho and Agnew, 1995). In order to identify new drug targets in this parasite, we began our characterization of the individual components of the schistosome cholinergic system by cloning and functionally characterizing the schistosome surface AChE (Jones *et al*. 2002; Bentley *et al*. 2003, 2005) as well as cloning 2 nAChR subunits from *Schistosoma haematobium*, the first trematode nAChR subunits to be identified. One of these subunits is of the α type (*Sh*AR1α) and the other is non-α (*Sh*AR1β) (Bentley *et al*. 2004). *Sh*AR1β was localized within the musculature and on discrete cell bodies within the connective parenchyma, where it is likely to have a conventional role in synaptic transmission. On the other hand, *Sh*AR1α was localized exclusively to the surface membranes, suggesting it may contribute to the regulatory receptor that is responsible for the modulation of glucose uptake in the parasite.

We now describe the identification of a third *Schistosoma* putative nAChR subunit, a structurally unique non-α polypeptide which does not appear to be closely related to any nAChR subunit sequences reported to date.

MATERIALS AND METHODS

cDNA synthesis

Adult (20 week) *S. haematobium* (Kenyan strain) were obtained from hamsters by portal vein perfusion. *S. haematobium* mRNA was purified using the Qiagen Oligotex System from total RNA extracted in phenol-chloroform from frozen ground tissue. The cDNA library was synthesized using a modification of the Clontech SMART system (BD Biosciences) which selected for full length cDNAs and introduced unique 5′ and 3′ flanking sequences to facilitate RACE-type PCR without the need to package the cDNA.

Cloning and sequencing

S. haematobium cDNA was screened by PCR using a pair of degenerate primers derived from an EST sequence identified in *Schistosoma japonicum* which appears in dbEST (dbEST Id: 14234012, GenBank Acc: BU769415). This sequence encodes a 190 amino acid polypeptide which has close homology to the region of invertebrate and vertebrate nAChR subunit sequences extending from loop C of the ligand binding domain to the third transmembrane domain (TM3). The degenerate primers encoded the ten amino acids at the N-terminal side of TM2 (forward primer KMMLGINIFV; aar atg atg ctn ggn ath aay ath tty gt) and the nine residues at the C-terminal end of TM3 (reverse primer FYKGNDGQP; gg ytg ncc stc rtt ncc ktt rta raa) where n = a/c/t/g, y = c/t, r = a/g, k = g/t, h = a/c/t, w = a/t. The following PCR conditions were used: 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. A PCR product of approximately 200 bp was generated and sequence analysis confirmed that this product represented the *S. haematobium* homologue of the *S. japonicum* EST. Non-degenerate forward and primers were derived from this sequence, and these were used in conjunction with primers directed towards the library's unique 5′ and 3′ flanking sequences in order to obtain overlapping 5′ and 3′ RACE fragments which together represented the complete cDNA along with flanking non-coding sequence. Initially, PCRs were carried out using Taq DNA polymerase (Helena Biosciences), but then reactions were repeated using a proof reading polymerase mix (Clontech HF-2, BD Biosciences) in order to obtain high fidelity template DNA for automated sequencing. Analysis of the complete cDNA sequence suggested that it encoded a nAChR polypeptide of the non-α type; in keeping with the nomenclature employed for previous *Schistosoma* nAChR subunits, we named this *Sh*AR2β.

Sequence analysis

The multiple protein sequence alignment was constructed with ClustalX 1.8 (Thompson *et al*. 1997) using the slow-accurate mode with a gap opening penalty of 10 and a gap extension penalty of 0·1 as well as applying the Gonnet 250 protein weight matrix (Benner *et al*. 1994). The protein alignment was viewed using the GeneDoc program (http:\\www.psc.edu\biomed\genedoc) which was also used to calculate identity and similarity values between subunit sequences. The tree was constructed using ClustalX (Thompson *et al*. 1997) with the neighbour-joining method (Saitou and Nei, 1987) and displayed using TreeView (Page, 1996). Numbers at each node signify bootstrap values with 100 replicates (Felsenstein, 1985) and the scale bar represents the number of substitutions per site. Signal peptide cleavage sites were predicted using the SignalP 3.0 server (Dyrlov Bendtsen *et al*. 2004) and membrane-spanning regions were predicted using the TMpred program (available at http://www.ch.embnet.org/software/TMPRED_form.html). The PROSITE database (Hulo *et al*. 2006) was used to identify potential phosphorylation sites.

RESULTS

Degenerate primers derived from an *S. japonicum* EST sequence (dbEST Id: 14234012), which showed similarity in BLAST searches to neuronal-type

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nAChR subunits, were used to screen an adult *S. haematobium* cDNA library by PCR. A single PCR product of 200 bp was obtained, the sequence of which was used in the design of non-degenerate forward and reverse primers. These in turn were used in combination with primers directed to unique cDNA library 5′ and 3′ flanking sequences in order to generate 2 RACE PCR products which represented the overlapping 5′ and 3′ fragments of a single cDNA. Sequence analysis indicated that this cDNA was 2306 bp long and incorporated a single open reading frame encoding a protein of 545 amino acids (Fig. 1). This polypeptide possesses features typical of nAChR subunits including: an N-terminal signal leader peptide; an extracellular N-terminal domain with many of the aromatic and acidic residues conserved in the 6 distinct regions (loops A–F) which are involved in ACh binding (Corringer *et al*. 2000); the cys-loop which consists of 2 disulphide bond-forming cysteines separated by 13 amino acid residues; 4 transmembrane domains (TM1–TM4); and a highly variable intracellular domain between TM3 and TM4. The absence of 2 adjacent cysteines within loop C signifies that it is a non-α subunit (Corringer *et al*. 2000). This is the second non-α *Schistosoma* sequence to be described and accordingly we named it *Sh*AR2β.

As is common in nAChR subunits, *Sh*AR2β has potential N-glycosylation sites within the extracellular N-terminal domain (Fig. 1), which may play a role in various aspects of ion channel function such as desensitisation and conductance (Nishizaki, 2003). The first glycolsylation site (N33) is located in a region which is frequently glycosylated in other nAChR subunits and the second site, located between loops D and A, is highly conserved in neuronal nAChR subunits at a position equivalent to N68 in the human α7 nAChR subunit. The third glycosylation site is unusual and is located immediately proximal to TM1. Also, as is typical of nAChR subunits, *Sh*AR2β possesses potential sites for phosphorylation in its cytoplasmic TM3–TM4 domain (Fig. 1), 3 of which are protein kinase C sites (S373, S416 and S466), 1 is a casein kinase II site (S386) and another is a tyrosine kinase site (Y447). Although there is generally very little conservation within the cytoplasmic TM3–TM4 domain, phosphorylation mediates a number of important functions that have been ascribed to this region including the regulation of nAChR expression and localization, channel desensitization and ACh sensitivity (Swope *et al*. 1999; Williams *et al*. 1999).

Comparison of the deduced sequence of the mature *Sh*AR2β polypeptide with those of the previously identified *Schistosoma* nAChR subunits *Sh*AR1α and *Sh*AR1β reveals that *Sh*AR2β is not as closely related to these subunits as *Sh*AR1α and *Sh*AR1β are to each other. One notable structural distinction is in the form of the putative intracellular loop linking TM3 and TM4. In both *Sh*AR1α and *Sh*AR1β this region is exceptionally large, at 346 and 353 amino acids respectively, whereas in *Sh*AR2β it comprises 149 residues (Fig. 1). Excluding this region from sequence comparisons, the mature polypeptide of *Sh*AR2β shares 25% identity/51% similarity with *Sh*AR1β and 27% identity/49% similarity with *Sh*AR1α whilst in the same comparison, *Sh*AR1α and *Sh*AR1β share 54/73% identity/similarity with each other. When compared with vertebrate nAChR subunits, the mature *Sh*AR2β polypeptide most closely resembles the neuronal-type α2–α6 and β2–β4 with values of overall identity/similarity ranging from 21–23/41–46%. Values for similarity/identity between *Sh*AR2β and α7–α10 are only slightly lower at 20–21/37–41%. *Sh*AR2β appears to be least similar to muscle-type nAChRs, but again the distinctions are marginal e.g. 20/41% identity/similarity for α1, 20/39% for β1, 19/38% for δ and 17/37% for γ subunits of human. Amongst invertebrate species, *Sh*AR2β is most similar to *Drosophila* Dα6 (23/41%) and out of the *C. elegans* nAChR subunits *Sh*AR2β most closely resembles UNC-29 (20/43%). *Sh*AR2β bears less resemblance to other member of the cys-loop LGIC superfamily, showing, for example, 17/36% identity/similarity to the rat 5-HT3$_a$ subunit, 13/31% to the *C. elegans* GABA receptor UNC-49, 10/25% to the *C. elegans* glutamate receptor GLC-1, and 12/28% to the *Drosophila* histamine receptor HisCl1.

Conservation between *Sh*AR2β, the 2 other *Sh*AR subunits and across species is greatest within the putative transmembrane domains TM1 to TM3, reflecting the importance of these regions in defining the structural and functional properties of the nAChRs. For example, comparing *Sh*AR2β with *Sh*AR1α, the degree of identity/similarity is; 64/82% (TM1), 35/65% (TM2), and 35/60% (TM3). Comparing *Sh*AR2β with human α7 the equivalent values are 50/82% (TM1), 41/71% (TM2) and 30/70% (TM3). Within TM2, 8 residues have been shown by labelling experiments to be exposed to the channel lumen (Corringer *et al*. 2000), 5 of which, T245, L248, V252, L255 and E259 (human α7 numbering), are known to be crucial for defining the intrinsic conductance and ionic selectivity of the channel. In *Sh*AR2β the first 4 of these are either conserved or conservatively substituted (N285, V288, V292 and L295 respectively) (Fig. 1). Significantly, α7E259, which is located near the extracellular rim of the pore, is substituted by the positively charged K299 in *Sh*AR2β. This substitution may affect the ion channel properties of *Sh*AR2β since mutagenesis of the equivalent residue in the *Torpedo californica* α subunit, αE262, to a lysine decreased conductance for both inward and outward currents (Imoto *et al*. 1988). The narrowest portion of the channel is defined by the 'loop component' residues α7D235

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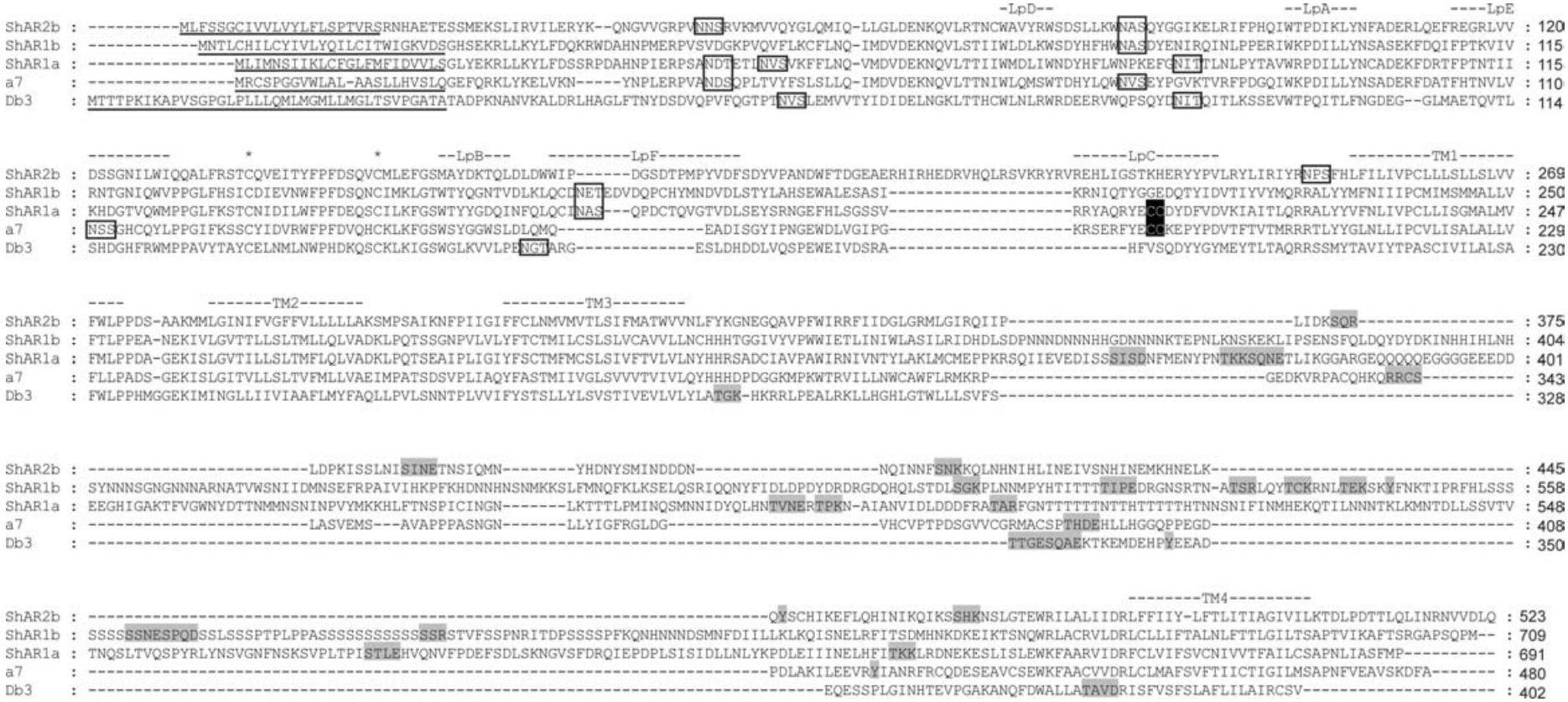

Fig. 1. Amino acid sequence alignment of *Sh*AR2β (AY876013) with *Sh*AR1α (AY392150), *Sh*AR1β (AY392151), human α7 (NP_000737) and the divergent *Drosophila* nAChR subunit Dβ3 (CAC48166). Putative N-terminal signal leader peptides are underlined and numbering of amino acids in each mature polypeptide is shown on the right of each line. The loops implicated in ACh-binding (LpA–F) as well as the four transmembrane domains (TM1–4) are indicated. The 2 cysteines forming the cys-loop are indicated by asterisks and the vicinal cysteines definitive of α subunits are highlighted in black shading. Potential N-glycosylation sites are boxed and grey shading highlights putative phosphorylation sites.

to S241, which are located at the cytoplasmic border of TM2. Within this region residues α7D235, E238, K239 and S241 have been shown to be particularly important for contributing to the cation selectivity filter of the channel (Corringer *et al*. 2000). Only 2 of these 4 residues are conserved in *Sh*AR2β (D275 and K279), the glutamate and serine being replaced by the hydrophobic residues alanine (A278) and methionine (M281) respectively. Whilst it is usual amongst nAChR subunits for the polar residue in the position analogous to α7S241 to be conserved, the non-conservative substitution by a hydrophobic residue does occasionally occur, for example in *Sh*AR1β (V262), vertebrate β1(G255) and certain *C. elegans* α subunits including ACR-7, ACR-10 and ACR-15. On the other hand, a charged residue corresponding to α7E238 is very highly conserved in nAChR subunits. The E238A mutation in α7 subunits gives rise to channels which are no longer permeable to divalent cations, although they do retain permeability to monovalent cations (Bertrand *et al*. 1993). It is therefore likely that the presence of an alanine in the equivalent position in *Sh*AR2β, combined with the E259K substitution described above, may have profound consequences on the cation selectivity and conductance of nAChR channels containing *Sh*AR2β subunits.

It is interesting to note that in common with *Sh*AR1α and *Sh*AR1β there is an extended loop F domain in *Sh*AR2β (Fig. 1). Smaller extensions also occur within the same region in some insect subunits (Jones *et al*. 2005). Also, *Sh*AR2β possesses an unusual insertion preceding loop C. A study has demonstrated that the extended loop F as well as the loop B–C interval of the *Drosophila* Dα2 subunit may account in part for the selectivity of the neonicotinoid insecticides for insect rather than mammalian nAChRs (Shimomura *et al*. 2004). The existence of structurally distinct regions within the ligand-binding domain of *Schistosoma* nAChR subunits raises the possibility that these regions might be usefully exploited in the future design and selection of parasite-specific cholinergic agents.

A phylogenetic tree was constructed in order to examine in more detail the relationship between the 3 *Sh*ARs, vertebrate, insect and nematode nAChR subunits as well as other members of the cys-loop LGIC superfamily (Fig. 2). As previously noted, *Sh*AR1α and *Sh*AR1β are highly similar to the vertebrate α7 subunit (Bentley *et al*. 2004). Other invertebrates, such as *Drosophila* with Dα5, Dα6, and Dα7 and *C. elegans* with its ACR-16 group, also possess nAChR subunits closely resembling α7, indicating an ancient lineage for this receptor subtype. Other close relationships are observed where the *C. elegans* UNC-63, ACR-8 and UNC-29 nAChR subunit groups cluster with several *Drosophila* subunits, in particular UNC-29 with Dβ1. In contrast, as might be expected from the relatively low values of sequence identity between *Sh*AR2β and other nAChR subunits, the tree suggests that *Sh*AR2β is a divergent subunit with no close evolutionary relationship between this subunit and those of other organisms.

DISCUSSION

We report here a member of the cys-loop LGIC superfamily from the blood fluke *S. haematobium* which bears closest resemblance to nAChR subunits. This subunit, *Sh*AR2β, is the third trematode candidate nAChR subunit to be described. Unlike the 2 previously characterized *Schistosoma* nAChR subunits, *Sh*AR1α and *Sh*AR1β (Bentley *et al*. 2004), the primary structure of *Sh*AR2β does not appear to be closely related with any known nAChR subunit (Fig. 2). Insects possess at least 1 divergent nAChR subunit which may represent species-specific receptor subtypes to target for future rational insecticide design (Lansdell and Millar, 2002; Jones *et al*. 2005, 2006) whereas members of the DEG-3 group make up a nematode-specific subfamily of nAChR subunits (Jones and Sattelle, 2004). It will be of interest to see whether *Sh*AR2β represents a schistosome specific subunit or is part of a group of nAChR subunits particular to trematodes.

The targeting of nAChRs in other invertebrate species has proven to be a particularly fruitful strategy which has led to the development of a number of classes of highly effective compounds for the control of insect and nematode pests. The high level of species selectivity and potency that is key to the effectiveness of these agents is, in the large part, a consequence of pharmacological specificity at the level of the nAChR complex. As the repertoire of nAChR subunits for an ever increasing number of invertebrates species becomes elucidated, so the molecular determinants of species sensitivity (and insensitivity) to pesticides are beginning to be explored (Sattelle *et al*. 2005; Tomizawa *et al*. 2005). In terms of *Schistosoma*, although this parasite is insensitive to existing anthelmintic nAChR ligands such as levamisole (Pancera *et al*. 1997), we do know that the parasite's surface cholinergic system is likely to present a viable drug target, and there may be additional opportunities to target essential nAChR complexes within the body of the worm.

Information derived from nAChR polypeptide primary sequences combined with recent advances in the modelling of the N-terminal ACh binding domain (Sine and Engel, 2006) is likely to greatly assist in the selection and development of new, improved cholinergic agents with applications to a broader range of invertebrate pests. Functional expression in heterologous systems such as *Xenopus laevis* oocytes has proved useful in characterising pharmacological and functional properties

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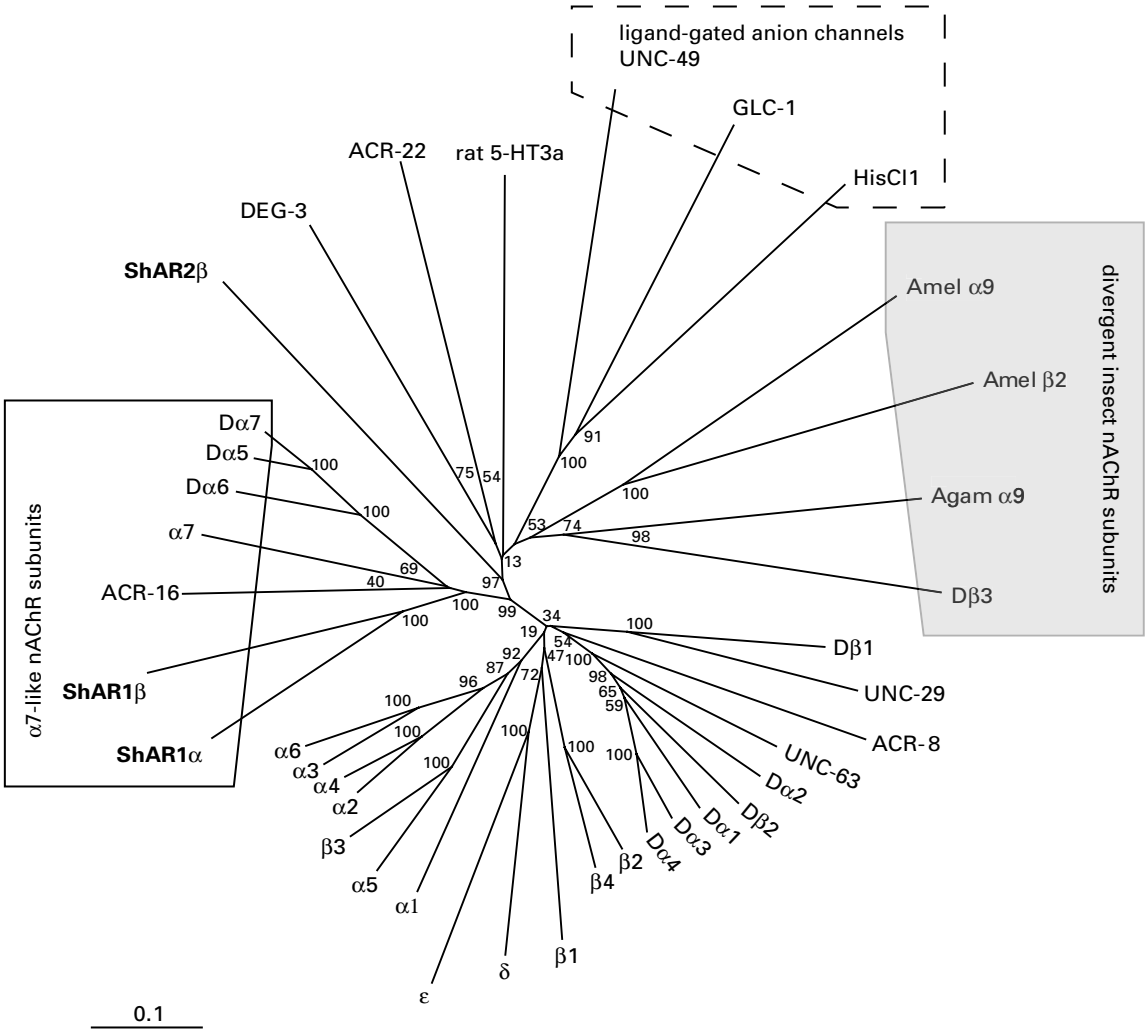

Fig. 2. Phylogenetic tree showing the relationship of protein sequences of *Sh*AR2ß (AY876013) with the following nAChR subunits: *Sh*AR1*α* (AY392150); ShAR1ß (AY392151); *D. melanogaster* D*α*1 (P09478), D*α*2 (P17644), D*α*3 (CAA75688), D*α*4 (CAB77445), D*α*5 (AAK67256), D*α*6 (CAD86935), D*α*7 (CAD86936), D*β*1 (P04755), D*β*2 (P25162) and D*β*3 (CAC48166); *Anopheles gambiae* Agam *α*9 (AAU12513); *Apis mellifera* Amel *α*9 (DQ026037) and Amel *β*2 (DQ026039); human *α*1 (X02502), *β*1 (X14830), *δ* (X55019) *ε* (X66403), *α*2 (U62431), *α*3 (M37981), *α*4 (L35901), *α*5 (M83712), *α*6 (U62435) *α*7 (NP_000737); *β*2 (X53179), *β*3 (X67513) and *β*4 (X68275); representatives of the 5 major nAChR groups of *C. elegans*, ACR-16 (P48180), UNC-63 (AAK83056), ACR-8 (NP_509745), UNC-29 (P48181) and DEG-3 (P54244) as well as ACR-22 (AAR89633) which represents the 'orphan' subunit group (Jones and Sattelle, 2004). The following non-nAChR members of the cys-loop LGIC superfamily were also included; the cation permeable rat 5-HT3$_a$ (NP_077370), and the anion-permeable *C. elegans* UNC-49 (AAD42386), GLC-1 (NP_507090) and *D. melanogaster* HisCl1 (AAL74413). The scale bar represents the number of substitutions per site and numbers at each node signify bootstrap values with 100 replicates (Felsenstein, 1985).

of nAChRs, particular those of vertebrates. For invertebrates, however, functional reconstitution of nAChRs has proven more difficult to achieve; a small number of *C. elegans* nAChRs has been expressed while the robust functional expression of receptors consisting entirely of insect nAChR subunits has yet to be reported. *Sh*AR1*α* and *Sh*AR1*β* do not form functional channels in *Xenopus* oocytes (Bentley *et al.* 2004) although it is possible that co-expression with as yet unidentified schistosome subunits is required for the reconstitution of functional nAChRs.

Whilst the molecular characterisation of the complete schistosome nAChR subunit family is in its early stages (several potential *Schistosoma* nAChR subunit sequences are listed (Ribeiro *et al.* 2005)), we have already identified several unusual structural features within the 3 subunits we have cloned so far which suggest that *Schistosoma* nAChRs are likely to exhibit a number of distinctive physiological and pharmacological profiles which could be exploited in the design of parasite-specific drugs.

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