Molecular Phylogeny of Viperidae Family from Different Provinces in Saudi Arabia

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Abstract

Molecular systematic is important in solving the problematic taxonomy of venomous snakes and development of antivenins. The current study aimed to investigate the molecular phylogeny of 4 venomous species in Saudi Arabia using mitochondrial (mt) 16S rRNA gene.

DNA extracted from blood and mt16S rRNA gene amplified by PCR. Sequences submitted to gene bank, examined for similarity with other sequences in the data base using BLAST search, aligned using Clustal W method, and phylogenetic tree was constructed.

E. coloratus clustered as a separate group along with the isolate from Oman and Yemen with insignificant relation. *Cerastes* and *Bitis arietans* groups strongly correlated as sister taxa. The current *B. arietans* sample did not significantly correlate to the previously published sample from the same province (Taif). *Cerastes* groups did not significantly correlate with samples from Egypt or Israel.

This information might reflect the need for multiple gene markers for better molecular systematic.

Keywords: Venomous snakes, Viperidae, Molecular systematic, Phylogeny, Mt16S rRNA gene

1. Introduction

Snake bite affects hundreds of thousands of people and tens of thousands are killed or maimed by snakes annually (Warrell, 2010). Envenomation due to terrestrial snakes is a common and frequently devastating environmental and public health problem in some areas of Saudi Arabia (Malik, 1995; AlHarbi, 1999; Ismail *et al*., 2007). Effective clinical interventions rely on better identification of snake subspecies.

The taxonomy of venomous snakes is complicated because of the complex variable nature of the medically important species (Gillissen *et al.,* 1994). Their classifications have been changed frequently based on new discoveries. Attempting to classify snakes was challenged by identification of novel species and variations in venom composition within subspecies.

The phylogeny of snakes is of considerable interest for the resolution of a problematic taxonomy, which furthermore impinges on the treatment of snakebite. The mitochondrial (mt) genomes of snakes contain a number of characteristics that are unusual among vertebrates. Studies based on complete or near-complete snakes' mt genome sequences demonstrated a peculiar accelerated mt gene evolution (Douglas & Gower, 2010). Higher-level snake relationships are inferred from sequence analyses of four mitochondrial genes [12S rRNA (ribosomal ribonucleic acid), 16S rRNA, ND4 (NADH dehydrogenase subunit 4) and cytochrome b) (Heise *et al*., 1995; Keogh et al., 1998; Lenk *et al*., 2001; Vidal & Hedges 2002a & b). rRNA is the one of the only genes present in all cells (Smit *et al*., 2007). For this reason, genes that encode the rRNA are sequenced to identify an organism's taxonomic group, calculate related groups, and estimate rates of species divergence. Thus many thousands of rRNA sequences are known and stored in specialized databases such as RDP-II (Ribosomal Database Project-II) (Cole *et al*., 2003) and SILVA (a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB soft ware) (Pruesse *et al*., 2007).

Here we present analyses of DNA sequence data bearing on the relationships and biogeography of Viperidae family. We sampled 18 samples of Viperidae (*Cerastes cerastes, Cerastes gasperettii, Echis coloratus, and Bitis arietans*) from different provinces in Saudi Arabia (Hail, Taif, Riyadh, and Medina). Our analyses indicate a presence of genetic diversity of Viperidae in Saudi Arabia and elucidate the urgent need for peculiar antivenin

against those species.

2. Materials and methods

Blood samples were collected from fieldwork collections (table 1). The use of animals for research followed the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education by the New York Academy of Sciences, Ad Hoc Animal Research Committee. Genomic DNA (deoxyribonucleic acid) was extracted using the Axy Prep Blood Genomic DNA Miniprep kit (Axygen Biosciences, USA). Briefly, 200 µl of anti-coagulated whole blood mixed with 500 µl of buffer AP1 (cell lysis buffer) by vortexing at top speed for 10 seconds. Subsequently, 100 µl of buffer AP2 (protein-depleting buffer) were added and mixed by vortexing at top speed for 10 seconds. The mixture was then centrifuged at 12,000×g for 10 minutes at ambient temperature to pellet cellular debris. Binding was performed by applying the clarified supernatant to the Miniprep column and centrifugation at 6,000×g for 1 minute. The bound DNA was washed using 700 µl of buffer W1A (wash buffer). The Miniprep column allowed to stand at room temperature for 2 minutes then centrifuged at 6,000×g for 1 minute. Desalting was performed twice using 800 µl then 500 µl of buffer W2 (desalting buffer) followed by centrifugation at 12,000×g for 1 minute. The Miniprep column was then centrifuged at 12,000×g for 1 minute to get rid from any residual solutions. Finally, DNA was eluted in 200 µl of pre-warmed (at 65°C) TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA). Columns allowed to stand at room temperature for 1 minute then centrifuged at 12,000×g for 1 minute and the eluted DNA stored at -30°C till use.

Mitochondrial small subunit of 16S ribosomal RNA (rRNA) gene fragments were amplified by polymerase chain reaction (PCR) using 2X DreamTaq™Green PCR master mix (Fermentas life sciences) (DreamTaq™ DNA polymerase supplied in 2X DreamTaq™ Green buffer, dNTPs, 4 mM MgCl2, a density reagent, blue and yellow dyes) and sequence specific primers (5`-CGCCTGTTTATCAAAAACAT-3`& 5`-CCGGTCTGAACTCAGATCACGT-3`) (Pook *et al*., 2009). Template DNA (0.3µg) was added to 50 pmole of each primer, and 25 µl of 2X DreamTaq™Green PCR master mix in a reaction volume of 50 µl. The amplification conditions were: denature at 95°C for 3 minutes, then 35 cycles of 95°C denature for 30 seconds (s), 43°C anneal for 45 s, extension 72°C for 1 minute, followed by one final extension step of 72°C for 5 minutes (Pook *et al*., 2009). The amplified products were separated on 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet (UV) light.

DNA sequencing was carried out at King Faisal Specialist Hospital & Research Centre (KFSHRC) (Saudi Arabia, Riyadh). Samples were processed on 3730xl ABI Sequencer with POP7 and 50cm Cap Array-plate number AB6516.

The resulting sequences were submitted to the gene bank, compared to that present in the data base using BLAST analysis (Altschul *et al*., 1990 & 2009). Multiple sequence alignment was carried out using Clustal W method (Chenna *et al*., 2003, Thompson *et al*., 1994). Phylogenetic and bootstrapping analyses (Efron *et al*., 1996) were carried out using DNASTAR Laser gene 8.1MegAlign program (DNASTAR, Inc).

3. Results

3.1 Sequence analysis

The PCR produced fragments > 300nt (ranged from 514 to 866nt) of 16S rRNA (Figure 1) which used for final alignment. All sequences showed quite typical mitochondrial nucleotide composition (Table 1); A = 33.2%, C = 24.4%, G = 22.8%, T = 18.7% (Average value). BLAST (Basic Local Alignment Search Tool) search revealed high similarity (92-99%) with 24 isolates (Table 2). Similarities with other isolates which are not known to be present in KSA were excluded from the analysis.

3.2 Phylogenetic analysis

Since the rRNA genes are transcribed, but not translated, they fall in the category of non-coding genes. Therefore, no indels or stop codons were tested and the phylogenetic tree was constructed using DNA sequences. *E. coloratus* from Saudi Arabia clustered as monophyletic group along with the isolates from Oman (Thumrait) and Yemen (Ghoyal Ba-Wazir, and Bir Ali) with insignificant relation (Figure 2) and low divergence (Figure 3). No relation was detected between *E. Coloratus* and *Cerastes* groups. Instead a significant support for a sister-group relationship was detected between *B. arietans* and *Cerastes* groups. The current *B. arietans* sample (sequence identity: 96.6%, Figure 4) did not significantly correlate to the previously published sample (Pook *et al*., 2009) from the same province (Al-Taif). *Cerastes* groups did not significantly correlate with neither samples from Egypt or Israel (Figure 3) although the sequence identity is high (Figure 3 & 4). There was cross clustering between *Cerastes cerastes* and *Cerastes gasperettii* with no significant correlation (Figure 3).

4. Discussion

Our results urge for development of specific antivenin against the Viperidae family present in Saudi Arabia provinces. The current results did not support any significant sister-group relationship between *Echis* and *Cerastes* which was previously reported by Pook *et al*. (Pook *et al*., 2009). In our analysis this relation was strongly negative. However, our results were in accordance with their study regarding the clustering of *E. coloratus*. Different algorithms, data size and data quality might account for such incongruent data (Pruesse *et al*., 2007, Spinks *et al*., 2009). On the other hand, single relationship always achieved for species and genus monophyly (Spinks *et al*., 2009) which might explain the congruent part.

The weak relation achieved for the current *B. arietans* sample and that previously published appear to be partially due to variability in the compared fragment lengths (517 versus 691nt; respectively). Although >300nt was considered sufficient to include sequences in alignments (Pruesse *et al*., 2007), Spinks *et al*., reported insufficient recovery of well-supported relationships among many genera or species using ~6Kb of nuclear sequence but ~1 kb of mtDNA yielded similar support levels (Spinks *et al*., 2009). Thus longer mt sequences might be needed in the future studies.

Bad resolution of *Cerastes gasperettii* and *Cerastes cerastes* might be resolved in the future using multiple gene markers instead of a single gene marker. More sequence populations with higher quality and combining nuDNA and mtDNA markers might be used in the future studies to resolve the problematic molecular systematic.

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Table 1. Base composition of the new 16S rRNA DNA sequences

Accession number (Province)	Species	Full Length (nt)	Nucleotide composition (%)				
			A	C	G	T	Ambiguous
HQ267789 (Nakbeen, Hail)	*E. coloratus*	517	33.08	23.79	18.57	24.37	0.19
HQ267790 (Saha, Hail)	*E. coloratus*	517	33.08	23.79	18.18	23.79	1.16
HQ267791 (Saha, Hail)	*E. coloratus*	518	33.20	23.94	18.53	24.13	0.19
HQ267792 (Al-Taif)	*B. arietans*	517	34.62	22.24	18.76	24.18	0.19
HQ267798 (Faid, Hail)	*C. cerastes*	515	33.01	23.5	18.83	24.27	0.39
HQ267799 (Al-Fatkha, Hail)	*C. cerastes*	637	33.12	21.66	18.68	24.33	2.2
HQ267800 (Al-Fatkha, Hail)	*C. gasperettii*	634	31.07	21.92	18.3	23.97	4.73
HQ267801 (Baqaa, Hail)	*C. cerastes*	640	31.25	21.09	19.38	25.31	2.97
HQ267808 (Al-Taif)	*C. cerastes*	514	33.07	22.96	19.07	24.71	0.19
HQ267809 (Baqaa, Hail)	*C. gasperettii*	516	33.33	23.26	19.19	24.03	0.19
HQ267810 (Faid, Hail)	*C. gasperettii*	744	34.41	22.18	19.35	23.79	0.27
HQ267811 (Al-Owaid, Hail)	*C. cerastes*	740	34.19	22.43	19.32	23.78	0.27
HQ267812 (Al-Fatkha, Hial)	*C. cerastes*	866	32.10	21.94	17.55	25.98	2.42
HQ267815 (AL-Medina)	*C. cerastes*	679	33.28	22.53	18.56	24.59	1.03
HQ267816 (Riyadh)	*C. cerastes*	514	33.27	22.96	18.87	24.71	0.19
HQ658449 (Al-Taif)	*E. coloratus*	526	33.65	23.38	18.25	24.52	0.19
HQ658451 (Baqaa, Hail)	*C. gasperettii*	530	34.15	23.02	18.30	24.53	0.00
HQ658452 (Al-Owaid, Hail)	*E. coloratus*	529	33.08	23.44	18.53	24.57	0.38

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Table 2. Similarity with other sequences in the data base, representative values

Accession Description	Max score	Total score	Query coverage (%)	E value	Max identity (%)
GQ359726.1 *Echis coloratus* isolate 2029 16S ribosomal RNA gene, partial sequence; mitochondrial	878	878	94	0.0	98
GQ359710.1 *Echis coloratus* isolate 597 16S ribosomal RNA gene, partial sequence; mitochondrial	867	867	91	0.0	98
GQ359713.1 *Echis coloratus* isolate 1626 16S ribosomal RNA gene, partial sequence; mitochondrial	869	869	94	0.0	98
GQ359712.1 *Echis coloratus* isolate 1625 16S ribosomal RNA gene, partial sequence; mitochondrial	869	869	94	0.0	98
GQ359711.1 *Echis coloratus* isolate 598 16S ribosomal RNA gene, partial sequence; mitochondrial	869	869	94	0.0	98
EU852321.1 *Echis coloratus* 16S ribosomal RNA gene, partial sequence; mitochondrial	869	869	94	0.0	98
GQ359714.1 *Echis coloratus* isolate 1692 16S ribosomal RNA gene, partial sequence; mitochondrial	848	848	91	0.0	98
GQ359727.1 *Echis coloratus* isolate 2030 16S ribosomal RNA gene, partial sequence; mitochondrial	835	835	89	0.0	99
EU624290.1 *Echis coloratus* isolate 597 16S ribosomal RNA gene, partial sequence; mitochondrial	732	732	79	0.0	98
AJ275760.1 *Echis coloratus* mitochondrial partial 16S rRNA gene (Egypt)	688	688	75	0.0	98
EU852322.1 *Bitis arietans* 16S ribosomal RNA gene, partial sequence; mitochondrial	701	701	94	0.0	92
AF057232.1 *Bitis arietans* 16S large subunit ribosomal RNA gene, mitochondrial gene for mitochondrial RNA, partial sequence	699	699	96	0.0	92
GQ359737.1 *Bitis arietans* isolate 1671 16S ribosomal RNA gene, partial sequence; mitochondrial	691	691	94	0.0	92
GQ359738.1 *Bitis arietans* isolate 1696 16S ribosomal RNA gene, partial sequence; mitochondrial	693	693	91	0.0	92
GQ359739.1 *Bitis arietans* isolate 1567 16S ribosomal RNA gene, partial sequence; mitochondrial	676	676	91	0.0	92
GQ359740.1 *Bitis arietans* isolate 1410 16S ribosomal RNA gene, partial sequence; mitochondrial	785	785	86	0.0	98
EU624280.1 *Bitis arietans* isolate 1571 16S ribosomal RNA gene, partial sequence; mitochondrial	721	721	79	0.0	98
AJ275742.1 *Bitis arietans* mitochondrial partial 16S rRNA gene (Rwanda)	680	680	75	0.0	98
Z46498.1 *B.arietans* mitochondrial gene for 16S ribosomal RNA	678	678	78	0.0	96
AJ275743.1 *Bitis arietans* mitochondrial partial 16S rRNA gene (Morocco)	675	675	75	0.0	97
AJ275741.1 *Bitis arietans* mitochondrial partial 16S rRNA gene (South Africa)	675	675	75	0.0	97
GQ359736.1 *Bitis arietans* isolate 1577 16S ribosomal RNA gene, partial sequence; mitochondrial	697	697	90	0.0	93
AJ275756.1 *Cerastes gasperettii* mitochondrial partial 16S rRNA gene (Israel)	702	702	75	0.0	99
EU852323.1 *Cerastes cerastes* 16S ribosomal RNA gene, partial sequence; mitochondrial	695	695	89	0.0	93

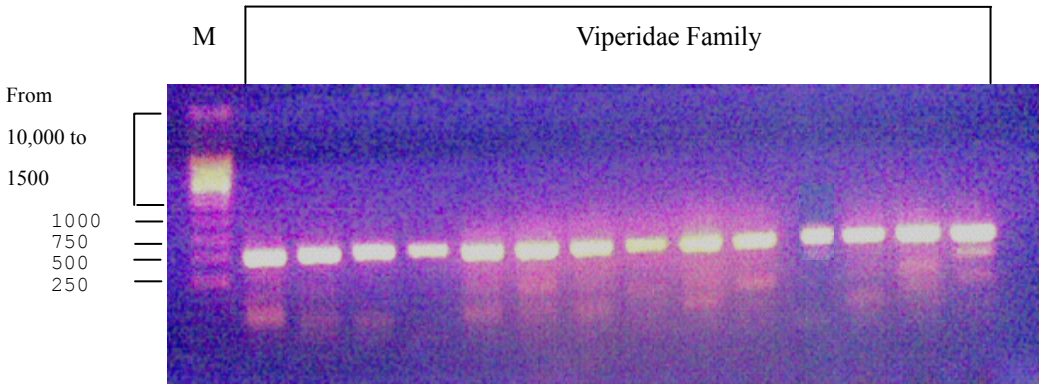

Figure 1. Representative samples of amplified PCR mt16S rRNA gene products. M: O' Gene Ruler™ 1Kb DNA ladder ready-to-use (MBI Fermentas; *Germany*). Ladder bands are indicated

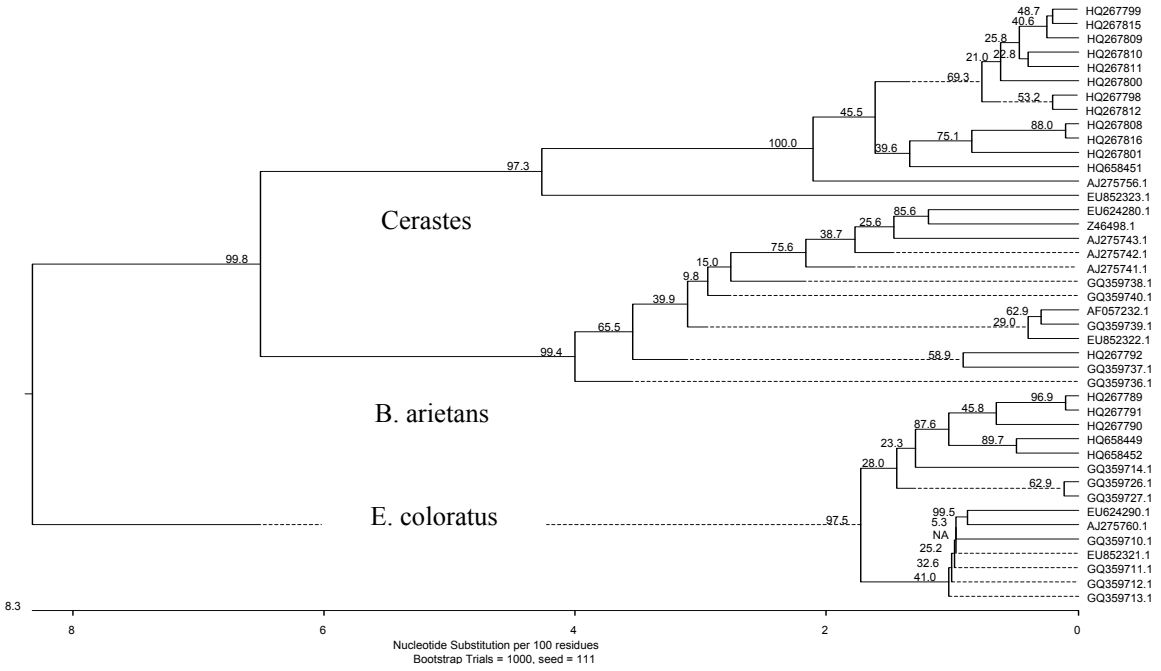

Figure 2. Phylogenetic tree of Viperidae family with bootstrapping Values. Strong nodes with bootstrapping values ≥70 of relevant sequences was considered significant

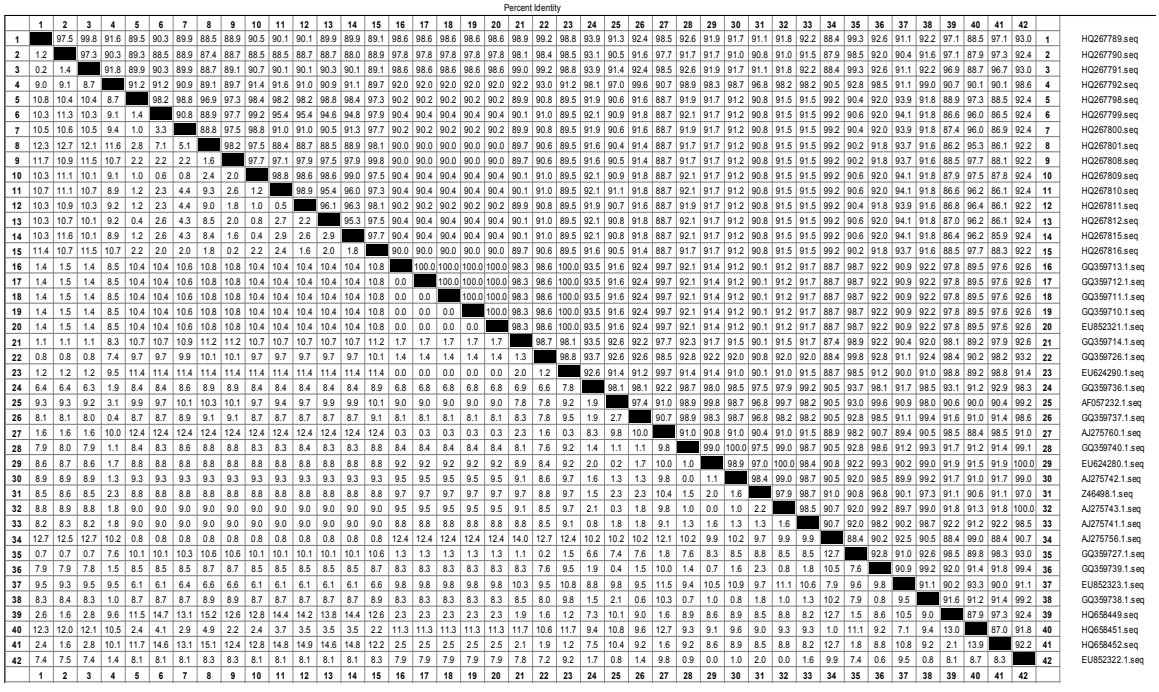

Figure 3. Sequence distance of Viperidae family

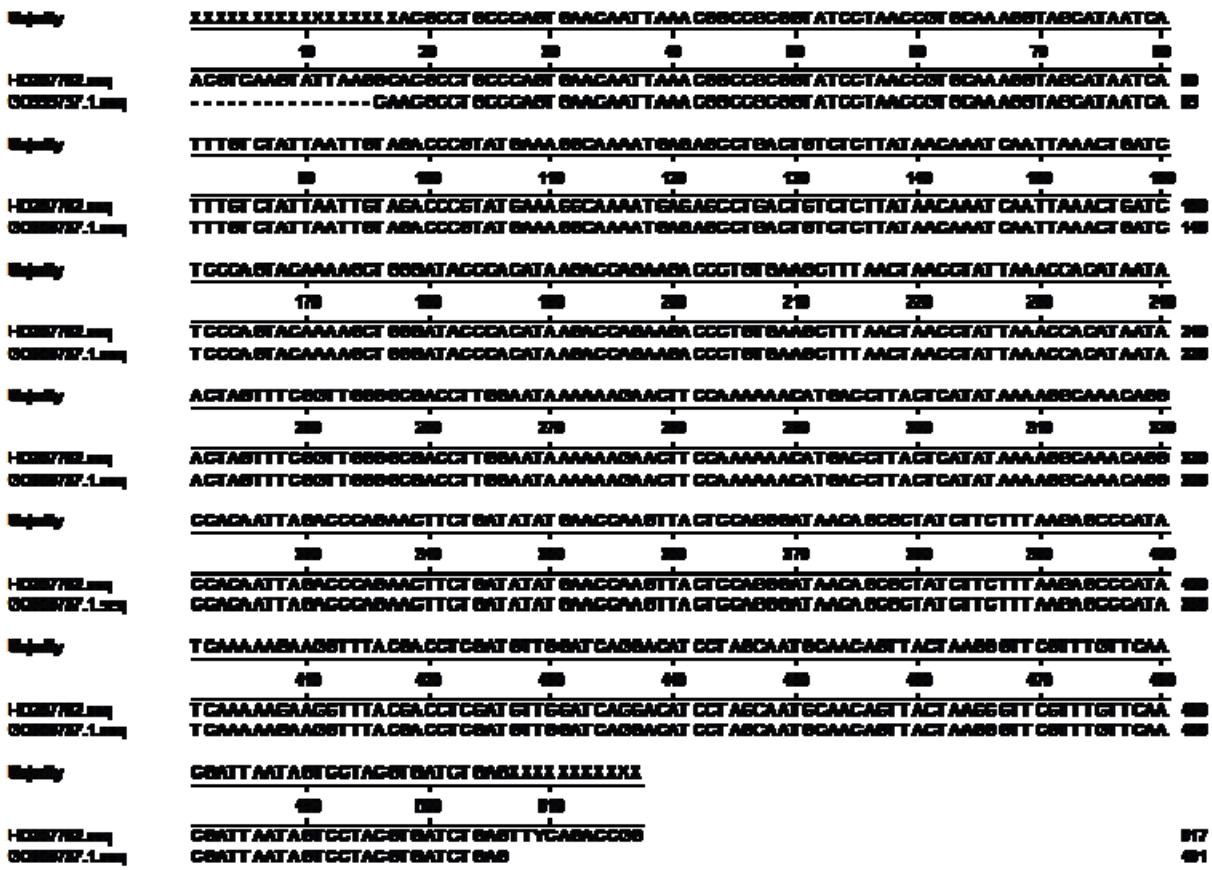

Figure 4. Sequence alignment of two *B. arietans* samples from Al-Taif