# CHAPTER 3

## *Bartonella* spp. detection using nested-PCR of the 16S rRNA and 23S rRNA ITS region and species identification

### 3.1 INTRODUCTION

Classical methods for isolation of *Bartonella* from tissue or blood have shown to be time-consuming procedures. A more rapid and direct method of detection is polymerase chain reaction (PCR) (Johnson *et al*., 2003). PCR has found widespread application for rapid, sensitive, and specific detection of infectious agents. PCR dependability has been evaluated under both research and routine diagnostic conditions for a broad spectrum of pathogens. The principal of PCR allows for the amplification and visualization of large amounts of genetic product in a short space of time (Ritzler and Altwegg, 1996).

There is a heightened focus on the development of new molecular-level diagnostic methodologies for *Bartonella* rapid identification and differentiation (Houpikian and Raoult, 2001; Maggi & Breitschwerdt, 2005). There are various genes that have been targeted, the most common being the citrate synthase gene, the 16S gene (Relman *et al.,* 1990; Maurin *et al*., 1997), the riboflavin synthase gene, the *groEL* gene, the RNA polymerase beta subunit gene, and 16S-23S rRNA ITS region (Maggi & Breitschwerdt, 2005). PCR is the most sensitive and practical of the diagnostic tools used for the detection and species subtyping of *Bartonella* spp. using genus-specific primers based on the ITS region (Birtles *et al*., 2000; Jensen *et al*., 2000; Houpikian & Raoult, 2001; Maggi & Breitschwerdt, 2005). Variation in species-specific amplicon sizes allow for the subtyping (Maggi & Breitschwerdt, 2005). It was reported that PCR of the 16S-23S rRNA ITS region offers 93% positive predictive value for determining *Bartonella* infections in cats (Chang *et al*., 2006). Various studies have looked at the PCR prevalence of *Bartonella* spp. from both human and animal hosts.

DNA hybridization and pulsed-field gel electrophoresis are reportedly the most sensitive methods used for the molecular characterization of *Bartonella*; however, these techniques are not suitable for routine diagnosis as prior cultivation of the organism is required (Houpikian and Raoult, 2001).

The objective of this study was to optimize and run a nested PCR for the detection of *Bartonella* spp. infecting humans, cats, dogs, and rats. The PCR was used to confirm culture isolates as *Bartonella* spp. and sequencing was carried out on the culture isolates for species identification.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Samples

A total of 382 HIV-positive patients, 42 clinically healthy volunteers, 98 cats, 179 dogs, and 124 rats were tested by nested PCR for *Bartonella* prevalence. In addition, 20 culture isolates (Chapter 2) were subjected to a single-round PCR to confirm the isolate as a *Bartonella*.

### 3.2.2 DNA extraction and quantification

All cultures and blood samples were tested by PCR for evidence of *Bartonella* spp. DNA was extracted using the QIAamp DNA mini kit (Qiagen, Germany) according to kit protocol (Appendix 3, Section 3.2.2a). The final elution volume was 100 µl instead of the recommended 200 µl final eluted volume allowing for a more concentrated DNA sample.

The DNA was assessed by running 5 µl of the extracted DNA on 2% (w/v) TAE agarose gel (method: Appendix 3, Sections 3.2.2b and 3.2.2c) against Hyper-ladder 1 (HL1)(Bioline, United Kingdom) to verify sizes of the fragments as described in Section 3.2.3. Control DNA was quantified using NanoDrop ND-1000 (Thermo Scientific) and double stranded-DNA (ds-DNA) fragment purified for sequencing was quantified by BioPhotometer (Eppendorf, Germany).

### 3.2.3 Agarose gel electrophoresis

DNA analysis was performed on 2% (w/v) TAE molecular grade low electro end osmosis (EEO) point agarose (WhiteSci, USA) gels. Gels were supplemented with 0.5 µg/ml ethidium bromide (EtBr) to facilitate visualization. Electrophoresis was carried out at 100 V in 1x TAE (40 mM Tris-HCl; 2 mM EDTA; 20 mM acetic acid; pH 8.5) for 40 min. Gels were visualized by ultraviolet (UV) illumination in a Vacutec gel documentation system under 300 ms exposure. The image was captured by GeneSnap (SynGene) software and analyzed by GeneTools (Syngene).

### 3.2.4 PCR amplification

***Primer selection***

Two sets of genus-specific primers were selected from the published works of Roux & Raoult (1999) and Seki *et al.* (2006) (Table 3.1). The reverse primer QHVE-14 was modified by elongating the 5' end by 3 nucleotides to decrease non-specific amplification. Detection from culture DNA was carried out in a single-step PCR using primers QHVE-1 and QHVE-3, whereas the blood-extracted DNA required a nested PCR using the QHVE-12 and QHVE-14b inner primers. Table 3.2 shows 6 commonly isolated human *Bartonella* spp., binding localities of the primers and product sizes; however, other species within the genus were also detected using these primers. Primers were ordered from Inqaba Biotechnical Industries (Pty) Ltd (South Africa), the stock solutions were prepared to a concentration of 100 µM (100 ρmol/µl), and were diluted to a 10 ρmol/µl working concentration.

**Table 3.1** Primers used for a single-step and/or nested PCR for detection of *Bartonella* genus-specific sequences within the hyper-variable ITS region between 16SrRNA and 23SrRNA genes

| Name: | ←→ | Sequence: | Bp: | Reference: |
|---|---|---|---|---|
| **QHVE-1** | → | 5' – TTC AGA TGA TGA TCC CAA GC – 3' | 20 bp | Roux and Raoult, 1995; La Scola and Raoult, 1999 |
| **QHVE-3** | ← | 5' – AAC ATG TCT GAA TAT ATC TTC – 3' | 21bp | Roux and Raoult, 1995; La Scola and Raoult, 1999 |
| **QHVE-12** | → | 5' – CCG GAG GGC TTG TAG CTC AG – 3' | 20bp | Seki *et al*, 2006 |
| **QHVE-14** | ← | 5' – CAC AAT TTC AAT AGA AC – 3' | 17bp | Seki *et al*, 2006 |
| **QHVE-14b** | ← | 5' – CCT CAC AAT TTC AAT AGA AC – 3' | 20bp | unpublished |

**Table 3.2** Six most often isolated spp. of *Bartonella*, binding positions on the 16S rRNA and 23S rRNA ITS region, and number of base pairs within the region.

| Species: | Outer primers | | | Inner primers | | |
|---|---|---|---|---|---|---|
| | # bp: | QHVE-1 | QHVE-3 | # bp: | QHVE-12 | QHVE-14 |
| *Bartonella henselae* Accession #: **L35101** | **723** | 318 – 337 | 1021 – 1041 | **568** | 448 – 467 | 1000 – 1016 |
| *Bartonella quintana* Accession #: **L35100** | **640** | 353 – 372 | 973 – 993 | **500** | 468 – 487 | 952 – 968 |
| *Bartonella vinsonii* Accession #: **L35102** | **661** | 336 – 355 | 977 – 997 | **481** | 491 – 510 | 956 – 972 |
| *Bartonella elizabethae* Accession #: **L35103** | **788** | 359 – 378 | 1135 - 1147 | **572** | 558 – 577 | 1114 – 1130 |
| *Bartonella clarridgeiae* Accession #: **DQ683194** | **711** | 313 – 332 | 1004 – 1024 | **573** | 425 – 445 | 982 – 998 |
| *Bartonella grahamii* Accession #: **AJ269789** | **736** | 311 - 330 | 1026 - 1046 | **487** | 538 - 557 | 1005 - 1024 |

### *Taguchi square optimization*

Optimization was based on the Taguchi checkerboard principle where the various combinations of PCR reagent concentrations reveal the effects and interactions of each specific reaction component simultaneously (Cobb and Clarkson, 1994).

**Table 3.3** The variables DNA, $MgCl_2$ and primers optimized at three different amounts/concentrations A, B, and C.

| | A | B | C |
|---|---|---|---|
| DNA (ng) | 20 | 40 | 60 |
| $MgCl_2$ (mM) | 1.5 | 2.0 | 2.5 |
| Primers (ρmol) | 5 | 10 | 20 |

| Tube # | DNA | $MgCl_2$ | Primers |
|---|---|---|---|
| **1** | A | A | A |
| **2** | A | B | B |
| **3** | A | C | C |
| **4** | B | A | B |
| **5** | B | B | C |
| **6** | B | C | A |
| **7** | C | A | C |
| **8** | C | B | A |
| **9** | C | C | B |

The total volume for each reaction was 50 µl and contained 1x Buffer II (without $MgCl_2$), 1.5 U *AmpliTaq* DNA polymerase (Applied Biosystems, USA), and 200 µM of each deoxyribonucleotide triphosphate (dNTP) (Thermo Scientific, United Kingdom). The primer, $MgCl_2$ and DNA concentrations were varied as shown in Table 3.3. PCR reactions were performed on a VERITI Thermocycler (Applied Biosystems) under the following conditions: denaturation at 94°C for 6 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s, elongation at 72°C for 1 min, and a final elongation of 72°C for 6 min.

Culture-extracted DNA from *B. henselae* (ATCC 49882) was used for PCR optimization and *B. clarridgeiae* (ATCC 70095), *B. grahamii* (ATCC 700132), *B. vinsonii* subsp. *berkoffii* (ATCC 51672), and *B. elizabethae* (ATCC 49927) were subsequently subjected to this PCR to assess primer annealing. Once the method was found successful in detecting all control strains, the rodent culture isolates were crudely extracted by boiling the pure culture in 200 µl sterile water for 15 min and tested by PCR. Rodent isolates were confirmed positive as *Bartonella* spp. and DNA was thereafter extracted from the original blood samples of confirmed culture isolates for use in natural-infection PCR optimization.

***PCR of DNA extracted directly from blood***

DNA extracted from 13 *Bartonella* culture-positive rodent samples were run in the first-round optimized PCR described above. Reactions were set up as before and amplicons

were electrophoresed as described in 3.2.3. BART 0377 (culture-confirmed sample) DNA was used as template for optimization. The temperature, $MgCl_2$, and primer concentrations were optimized and the surfactant additive Triton-X 100 was used in the first round PCR in order to decrease the appearance non-specific bands. The final reaction volume was 50 µl and contained: 1x Buffer II (without $MgCl_2$), 2 mM $MgCl_2$, 1.5 U *AmpliTaq* DNA polymerase, 20 pmol of each primer (QHVE-1 and QHVE-3), 200 µM of each dNTP, 5 µl of the 1% (v/v) dilution of Triton-X 100, and 5 µl DNA. Reactions were performed under the following conditions: 2 min initial denaturation step at 94°C, followed by 35 cycles of the following steps: denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and elongation at 72°C for 60 s. A final elongation step concluded the amplification at 72°C for 6 min. PCR products were maintained at 4°C until being added to the reaction mixtures of the nested round.

The PCR reaction (50 µl) of the nested round contained: 2 µl of first-round amplicons, 1x Buffer II (without $MgCl_2$), 1.5 mM $MgCl_2$, 30 pmol of each inner primer (QHVE-12 and QHVE-14b), 200 µM of each dNTP, and 1.5 U *AmpliTaq* DNA polymerase. The reactions were amplified as above, with a variation in the annealing temperature (55°C).

### 3.2.5 Nucleotide sequencing

***Purification of amplicons from agarose gels***

Isolated DNA from blood of 15 rats and 5 cats was amplified in duplicate to ensure that sufficient product was available for extraction and purification. Amplicons were run on a 2% (w/v) TAE agarose gel (Section 3.2.3) in order to assess the product quality. Bands were visualized over a UV light box and were individually cut from the gel using disposable pipette-cutters. The excised bands were placed into 1.5 ml safelock tubes, and gel segment weights were calculated (total weight – empty tube weight = gel weight) in order to determine the amount of Buffer QG (*QIAquick* Gel Extraction Kit, Qiagen, Germany) required for extraction and purification of the excised fragment from the gel (Appendix 3, Section 3.2.5a). Gel-purified amplicons were run on an agarose gel as before, the concentrations were determined using a Biophotometer, and concentrations were adjusted to 20 ng/µl.

***Cloning of amplicons into the pGEM® -TEasy vector***

Three rodent isolates were cloned to assess the integrity of the primer binding sites. The pGEM®-T Easy vector (3 Kb) cloning system (Promega, USA) was used for cloning of the fragments. Vectors were supplied at 50 ng/µl concentration (Figure 3.1). Nucleotide concentrations and approximations of base pairs numbers (kilo-base pairs, Kbp) were important in determining the amount of PCR product required for optimal fragment incorporation.

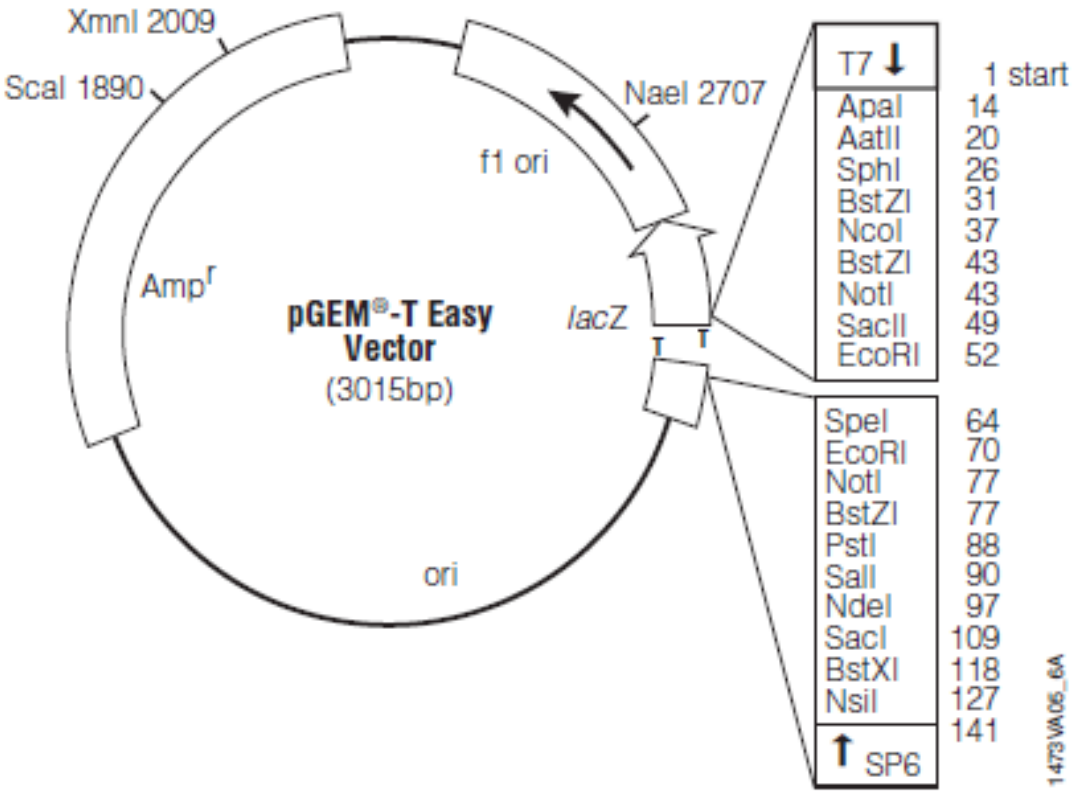



**Figure 3.1** pGEM®-T Easy vector circle map and sequence reference points.

***Ligation of amplicons and plasmid vectors***

Ligation reactions were set up in 0.5 ml low-binding capacity tubes to a final volume of 10 µl as follows: 5 µl of 2x rapid ligation buffer, 50 ng pGEM®-T easy vector; 3 U T4 DNA ligase (Promega, 3 U/µl), approximately 38 ng insert DNA, and deionised water.

The vector-to-insert ratio was 1:3. Reactions were gently mixed and incubated at 4℃ for 16 hrs (overnight) to ensure maximum number of transformants were achieved.

***Transformation of competent cells***

*One shot* competent cells (Invitrogen, USA) were used for transformations. Five microliters (5 µl) of each ligation reaction was pipetted into separate vials of 50 µl ice bath-thawed competent cells. Inoculated vials were incubated for 30 min on ice, followed by a 30 s heat-shock at 42℃ and immediately returned to ice for 2 min. Super optimal broth with catabolite repression (SOC) medium (250 µl) at room temperature was added to transformed cells and mixtures were incubated at 37℃ with shaking at ~170 rpm. Forty microliters (40 µl) of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) was added to 100 µl of each transformation to facilitate blue/white colour selection, and mixtures were plated out onto Luria Burtani (LB)/Ampicillin plates (Appendix 3, Section 3.2.5b). The transformations were evenly spread by a sterile glass spreader and remaining transformation mixture was refrigerated at 4℃. Plates were incubated overnight (16-24 hrs) at 37℃. Competent cells that had been successfully transformed were white, whereas non-transformed cells were blue (Figure 3.2).

***Screening recombinant plasmids by size***

White colonies selected from each plate were inoculated into 5 ml of LB broth supplemented with 50 µg/ml ampicillin. The inoculated broth aliquots were incubated at 37℃ in a shaking incubator (150 rpm) for 24 hrs. Turbid suspension (250 µl) was centrifuged at 10 900 rpm for 15 – 20 s and the supernatant was discarded. Forty microliters of loading dye and 14 µl phenol:chloroform (1:1) were added to the pellet, and vortexed for 10 s to lyse the cells. These were subsequently centrifuged at 10 900 rpm for 3 min. Using the original non-recombinant vector as a reference, 6.5 µl of the solution was loaded onto an 2% (w/v) TAE agarose gel (Section 3.2.3).

***Sequencing and analysis***

Recombinant plasmids were screened by size as described by Beuken *et al.* (1998). The cloned colonies were boiled in 200 µl sterile water for 10 min and run through the single round PCR to check that the cloned fragments were still amplified by PCR primers.

Cloned isolates were plated onto LB agar, incubated overnight at 37 ºC, and sent to Inqaba Biotechnologies for sequencing. Forward and reverse strands were sequenced using T7 (5' - TAA TAC GAC TCA CTA TAG GG - 3') and Sp6 (5 '- ATT TAG GTG ACA CTA TAG - 3') universal primers designed for sequencing cloned pGEM®-T Easy vectors.

Amplicons from 12 rat isolates and 5 cat isolates were gel purified as described previously and were sent to Inqaba Biotechnologies for direct-sequencing. Direct sequencing of these amplicons was done using 2 ρmol of primers QHVE1 or QHVE3 depending on forward or reverse sequencing. Both strands were sequenced.

Sequences were aligned and analysed using BioEdit freeware (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Strands were aligned by pairwise alignment allowing the ends to slide. Sliding ends were completed by viewing the FinchTV chromatograms. Sequences were exported into National Center for Biotechnology Information (NCBI) website's Basic Local Alignment Search Tool (BLAST) database for species identification of the isolates. The phylogenetic tree was drawn using the Neighbor-joining method (Saitou & Nei; 1987) using molecular evolutionary genetics analysis (MEGA4) freeware (http://www.megasoftware.net/) (Tamura *et al.,* 2007).

## 3.3 RESULTS

### 3.3.1 PCR prevalences

***PCR of culture isolates***

Figure 3.2 illustrates the cultured isolates confirmed by a single round PCR. DNA was extracted from a bacterial suspension in 200 µl sterile water. The isolates slightly varied in amplicon size, although all amplicons were between 600 and 800 bp.

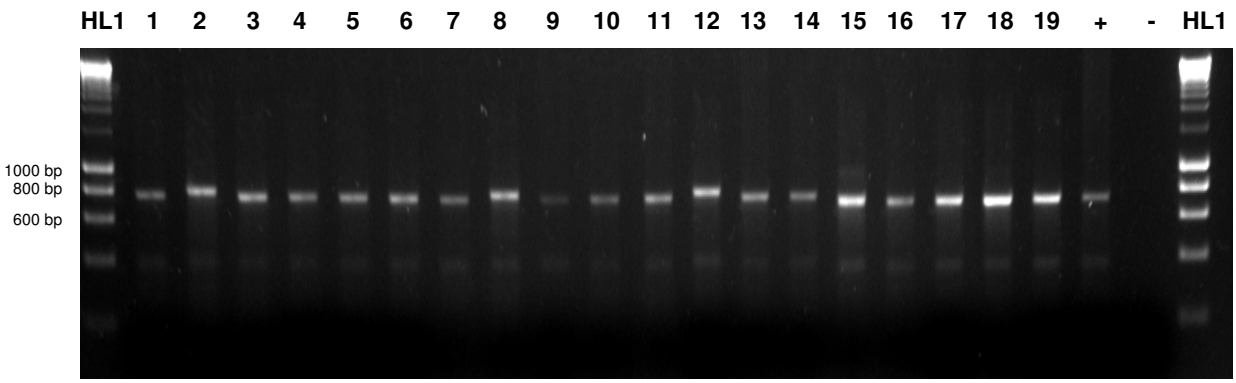



**Figure 3.2** Agarose gel analysis of rodent and cat isolates tested by a single round PCR. There are slight differences in the band sizes for the different isolates. Lanes: HL1, Hyperladder 1; 1, BART 0268; 2, BART 0271; 3, BART 0272; 4, BART0323; 5, BART 0324; 6, BART 0354; 7, BART 0355; 8, BART 0357; 9, BART 0358; 10, BART 0359; 11, BART 0361; 12, BART 0377; 13, BART 0379; 14, BART 0381; 15, BART 0480; 16, BART 0483; 17, BART 0484; 18, BART 0519; 19, BART 0538; +, *B.henselae* (ATCC 49882); -, non-reactive control.

***Population prevalences***

PCR of the HIV-positive population yielded a prevalence of 22.5% (86/382) (95% confidence; 18.5 – 27.1), whereas the clinically healthy group had a prevalence of 9.5% (4/42) (95% confidence; 3.1 - 23.5). This is a significant difference (p-value: 0.05; chi-square statistic: 3.818 with 1 degree of freedom) in the proportion of current infection for the two populations. This difference is unlikely to have occurred through mere chance, although the limited healthy volunteer sample size may not have allowed for an accurate indication of the total population.

The feline bloods tested by PCR indicate 23.5% (23/98) (95% confidence; 15.8 – 33.3) *Bartonella* prevalence. This is significantly different (p-value: 0.0002; chi-square statistic: 13.500 with 1 degree of freedom) to the culture prevalence (5%). Both test techniques test for current infection however, due to the fastidious nature of the bacteria, PCR is the far more efficient method for detection of *Bartonella* spp. as it does not rely on the bacteria being alive in the blood to be detected.

Rat bloods tested by PCR indicate 25% prevalence (31/124) (95% confidence;17.9 – 33.7). There is a significant difference (p-value: 0.0151; chi-square statistic: 5.907 with 1 degree of freedom) between PCR prevalence and culture prevalence (13%). When the

prevalence for rats was compared with that of the felines, it was found that there is no significant difference (p-value 0.7918; chi-square statistic: 0.070 with 1 degree of freedom).

The dog PCR prevalence was found to be 9% (16/179) (95% confidence; 5.4 – 14.4), significantly lower than the prevalences of the felines (p-value: 0.0009; chi-square statistic: 11.053 with 1 degree of freedom) and rodents (p-value: 0.0001; chi-square statistic: 14.419 with 1 degree of freedom).

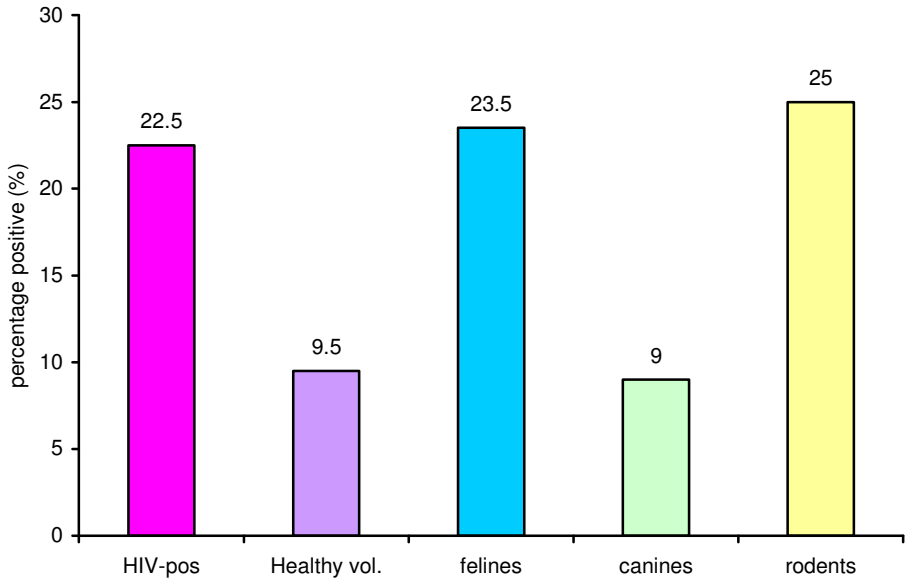



**Figure 3.3** Bar graph comparing the *Bartonella* prevalences of human and animal populations tested by PCR

Figure 3.3 illustrates that the highest infection rates belong to the rats, felines and HIV-positive patients.

### 3.3.2 Nucleotide sequencing

***Purification of amplicons from agarose gels***

Purified amplicons were electrophoresed as described in 3.2.3 to assess whether the band was the correct size and that the DNA had not sheared during gel purification.

Figure 3.4 illustrates the 3 rodent-derived bacterial isolates gel-purified for cloning. The purified amplicons were 700 – 766 bp in size and no shearing was observed. Amplicons were subsequently used for ligations into pGEM®-T Easy vectors which were transformed into competent cells.

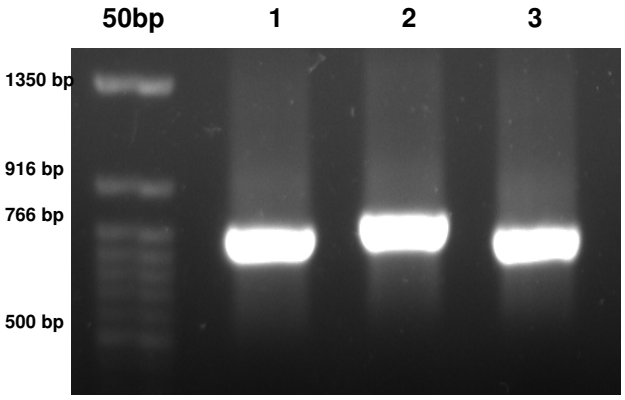



**Figure 3.4** Agarose gel (2% (w/v) TAE) analysis of the PCR products of the 3 rodent isolates that were cloned and sequenced. Lanes: 50bp, MWM (New England BioLabs); #1, BART 0357; #2, BART 0377; #3, BART0381.

***Transformation of competent cells and screening recombinant plasmids by size***

Blue/white screen illustrated that the insert DNA had successfully been incorporated into the plasmids. Figure 3.5 illustrates transformed competent *E. coli* cells in the presence of X-GAL. The white colonies were selected for sequencing as the insert DNA fragments were successfully ligated.


**Figure 3.5** Blue/White screen of transformed competent *E.coli* cells in the presence of X-GAL. White colonies illustrate that the DNA fragment was successfully ligated into the competent cells and were therefore the selected colonies. The blue colonies had not successfully taken up the DNA fragment and were thus not used.

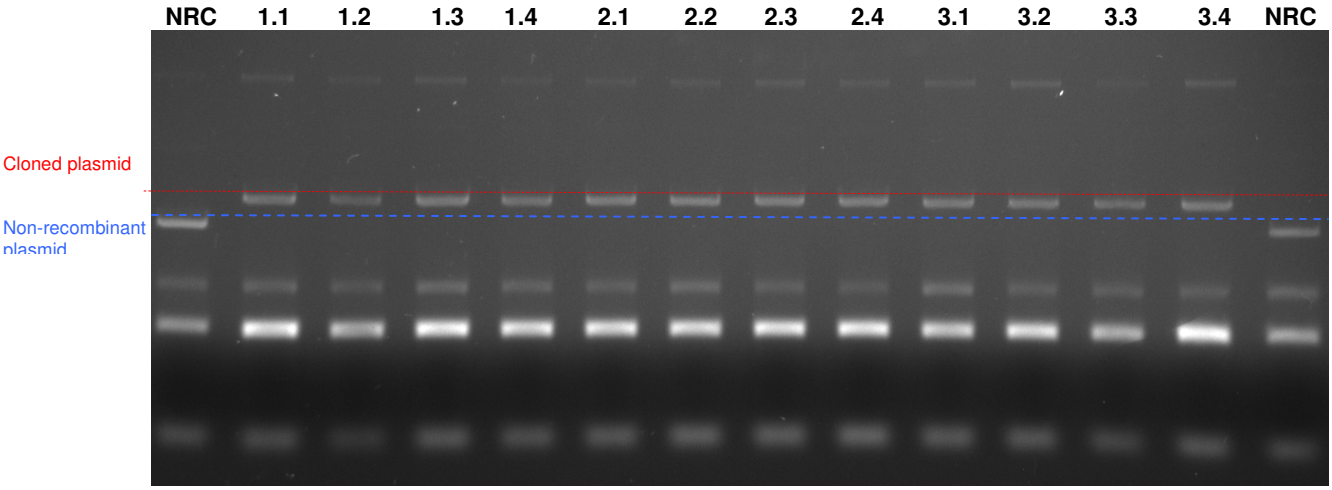



**Figure 3.6** Cloned vector plasmids run on a 1.2% (w/v) TAE agarose gel at 100V for 40 min. The non-recombinant plasmid of the negative control was used as a reference to illustrate the difference between it and the plasmid with the cloned DNA fragment. Lanes: NRC, Non-recombinant clone; 1.1, 1.2, & 1.3, BART 0357; 2.1, 2.2, & 2.3, BART 0377; 3.1, 3.2, & 3.3, BART 0381.

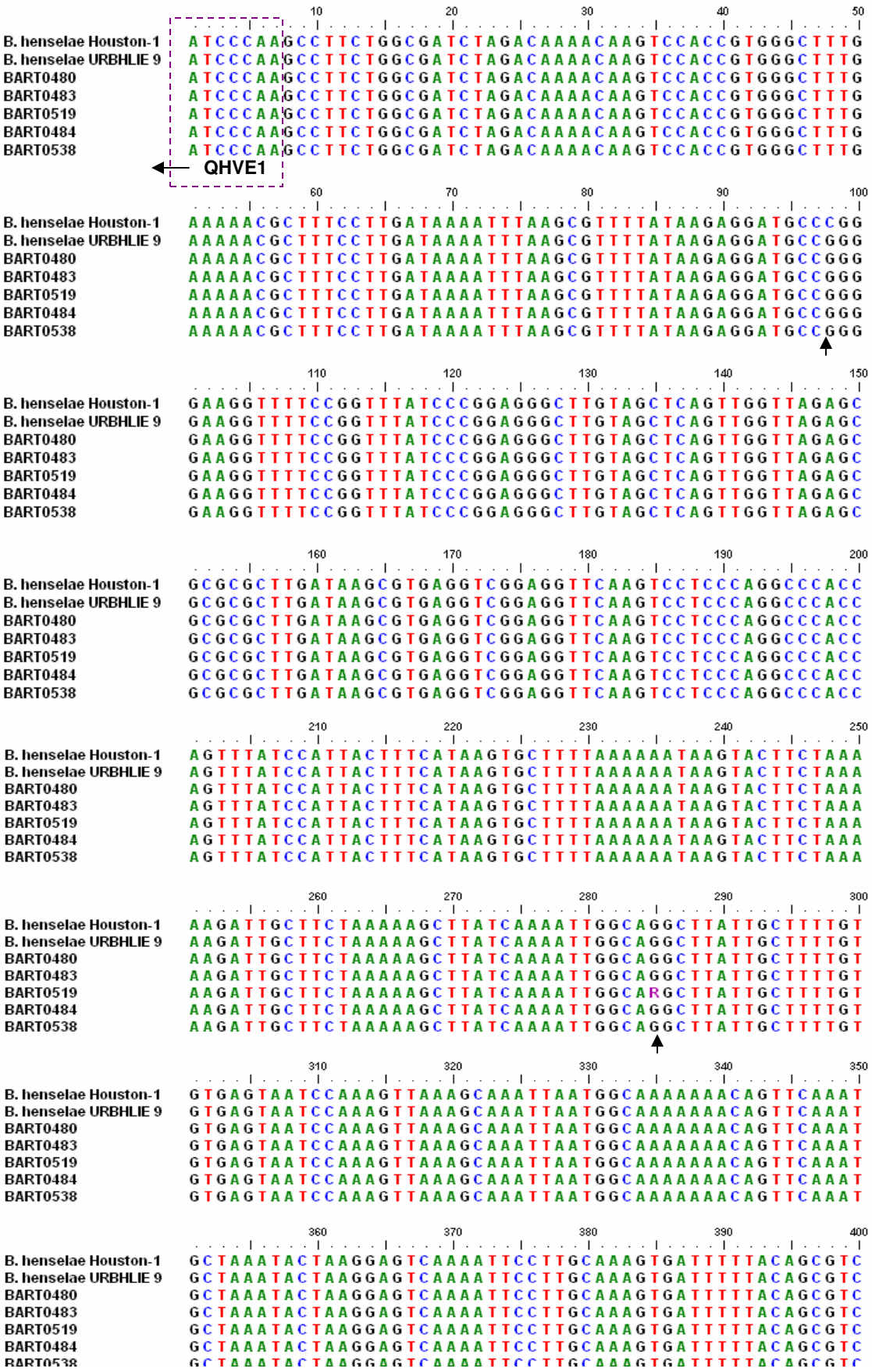
The recombinant clones from which DNA was extracted was electrophoresed as described in 3.2.3 to assess the plasmid sizes. DNA from a non-recombinant was also run on the gel as a visual reference. Recombinant clones were higher up the gel illustrating that the plasmids were larger after having successfully ligated with the inserted fragment.

### *Sequencing and analysis of isolates*

Once sequences were resolved, a ClustalW sequence alignment was run. RN24BJ, RN28BJ, and URBHLIE9 sequences were also aligned with the rat and feline isolates respectively obtained from this study.

**Table 3.4** Sequenced rodent and feline *Bartonella* isolates BLASTed on the NCBI GeneBank website.

| BART #: | # base pairs: | Similarity: | Percentage similarity (%): |
|---|---|---|---|
| Isolates similar to: RN24BJ | | | |
| 0268 | 736 | 728 | 98 |
| 0272 | 736 | 728 | 98 |
| 0312 | 736 | 728 | 98 |
| 0323 | 736 | 728 | 98 |
| 0324 | 736 | 728 | 98 |
| 0354 | 736 | 728 | 98 |
| 0357 | 736 | 728 | 98 |
| 0358 | 736 | 728 | 98 |
| 0359 | 736 | 728 | 98 |
| 0361 | 736 | 728 | 98 |
| 0379 | 736 | 728 | 98 |
| 0381 | 736 | 728 | 98 |
| Isolates similar to: RN28BJ | | | |
| 0271 | 797 | 765 | 98 |
| 0355 | 779 | 774 | 99 |
| 0377 | 779 | 774 | 99 |
| Isolates similar to: *B. henselae* (isolate URBHLIE 9) | | | |
| 0480 | 702 | 702 | 100 |
| 0483 | 702 | 702 | 100 |
| 0484 | 702 | 701 | 99 |
| 0519 | 702 | 701 | 99 |
| 0538 | 702 | 701 | 99 |


QHVE1
B. henselae Houston-1
B. henselae URBHLIE 9
BART0480
BART0483
BART0519
BART0484
BART0538

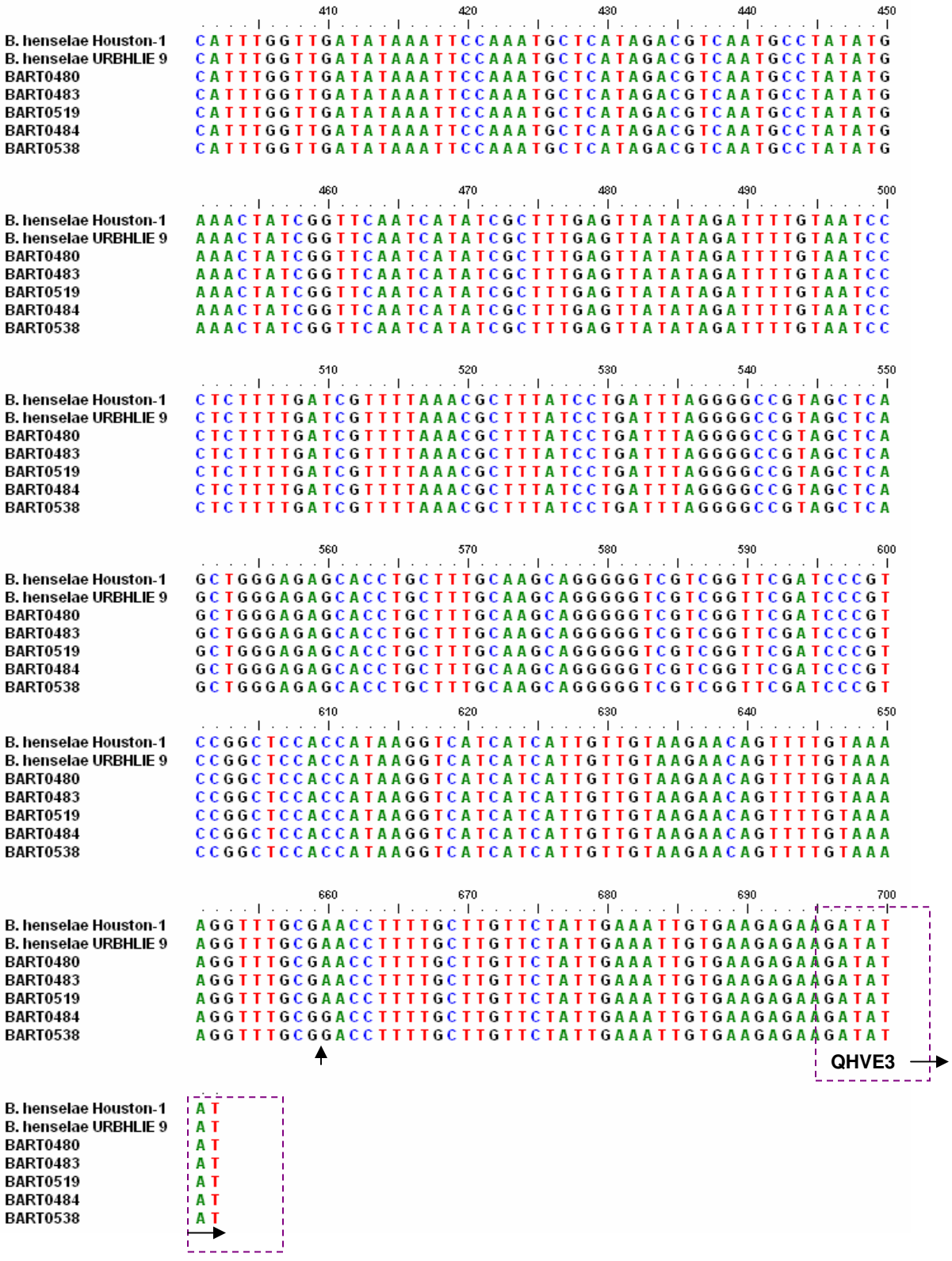



**Figure 3.7** Alignment of 16S-23S rRNA ITS region amplicons derived from 5 feline *Bartonella* culture isolates and 2 published *B. henselae* strains: *B. henselae* Houston-1 (accession #: L35101) and *B. henselae* URBHLIE 9 (accession #: AF312496 (Houpikian and Raoult, 2001).

Table 3.4 shows that the 5 feline isolates were 99 - 100 % similar to *B. henselae* URBHLIE9 (accession number: AF312496.1). Primers (QHVE1 & QHVE3) amplified a region consisting of 687 bp (excluding primers) for all the feline isolates. BART0480 and BART0483 were 100% identical to the *B. henselae* URBHLIE 9 strain, and had only 1 nucleotide difference from *B. henselae* Houston-1 (accession number: L35101) strain at position 98 (Figure 3.7). BART0519 was 99% similar to URBHLIE 9 with a heterogenous nucleotide at position 285 (i.e. nucleotide adenosine (A) or guanine (G) equally expressed). BART0519 and BART0484 were identical to each other and 99% similar to URBHLIE 9. One nucleotide difference was observed at position 660.

The 15 rodent culture isolates sequenced by Inqaba Biotechnologies and BLASTed on GeneBank (NCBI website) were found to be 1 of 2 *Bartonella* spp.: RN28BJ (accession number: EF213776.1) or the recently named novel species candidatus "*B. thailandensis*" (RN24BJ; accession number: EF190333.1) first described in Beijing, China (Saisongkorh *et al.,* 2009). Isolates ranged in percentage similarity from 97 – 99% to either RN24BJ or RN28BJ (Table 3.4).

The rodent isolates were slightly more variable and a phylogenetic tree (Figure 3.8) contingent from the ITS data using parsimony and distance methods illustrated 2 well-supported (more than 90% bootstrap values) clusters within the isolates. The first cluster places RN24BJ with 12 of the isolates (BART0272, BART0323, BART 0268, BART0354, BART0379, BART0381, BART0312, BART0324, BART0359, BART0361, BART0357, and BART0358) and the second group clusters RN28BJ with the remaining 3 isolates (BART0271, BART 0355, and BART 0377). *B. elizabethae* (GeneBank accession number: L35103) and *B. grahamii* (GeneBank accession number: AJ269785) were used as sources of comparison. *B. elizabethae* was found to be most similar to the rodent isolates from this study.

Alignments (Figure 3.9) further illustrated the differences between the cluster similar to RN24BJ and the cluster similar to RN28BJ. BART0271, BART 0355, and BART 0377 are hereafter referred to as the 'RN28BJ' cluster, and BART0272, BART0323, BART 0268, BART0354, BART0379, BART0381, BART0312, BART0324, BART0359, BART0361, BART0357, and BART0358 as the 'RN24BJ' cluster.

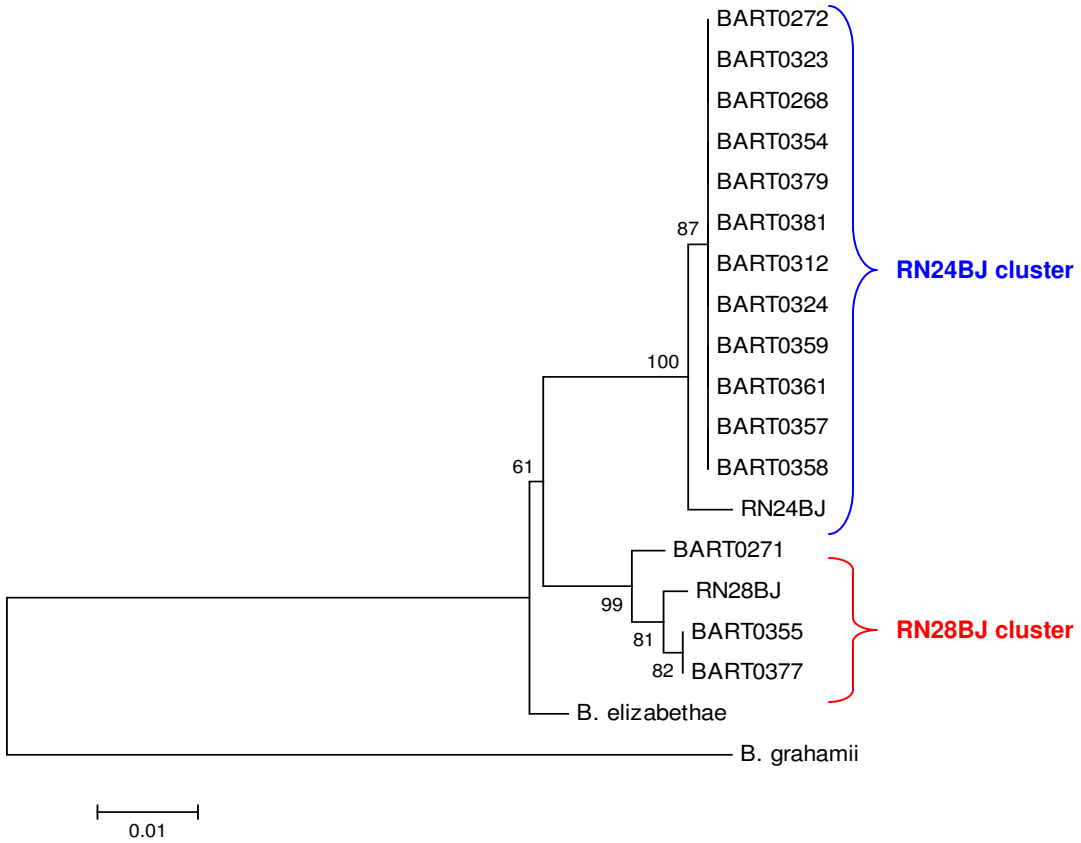
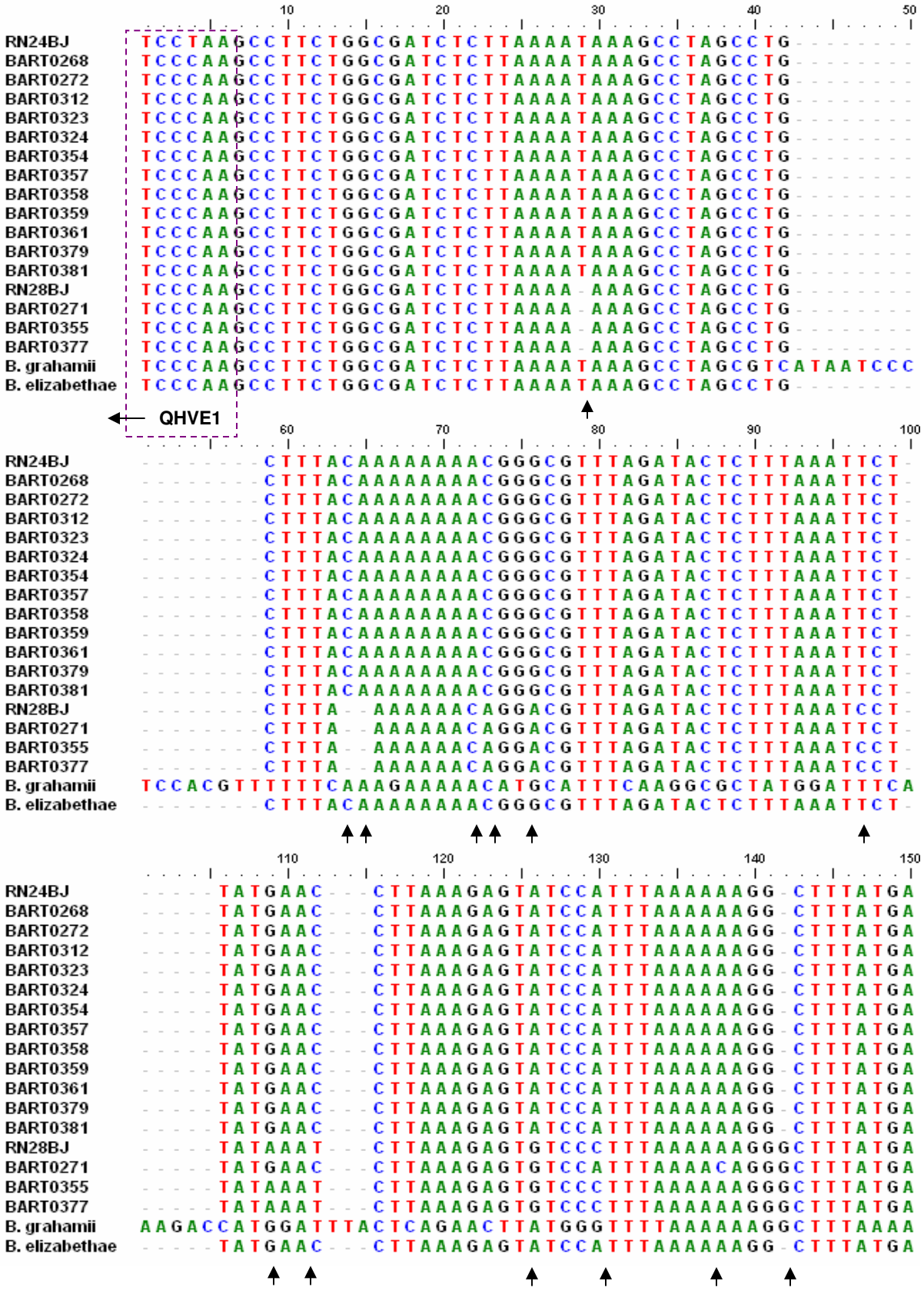



**Figure 3.8** Evolutionary relationships of 19 rodent isolates including *B. grahamii* and *B. elizabethae.* Evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated isolates clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 662 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

All sequence differences are indicated with the arrow or boxed in area in Figure 3.9. Cluster RN28BJ has at least 3 large nucleotide insertions at positions: 348 – 356 (8 nucleotide insertion); 429 – 442 (10 nucleotide insertion); and 516 – 540 (24 nucleotide insertion).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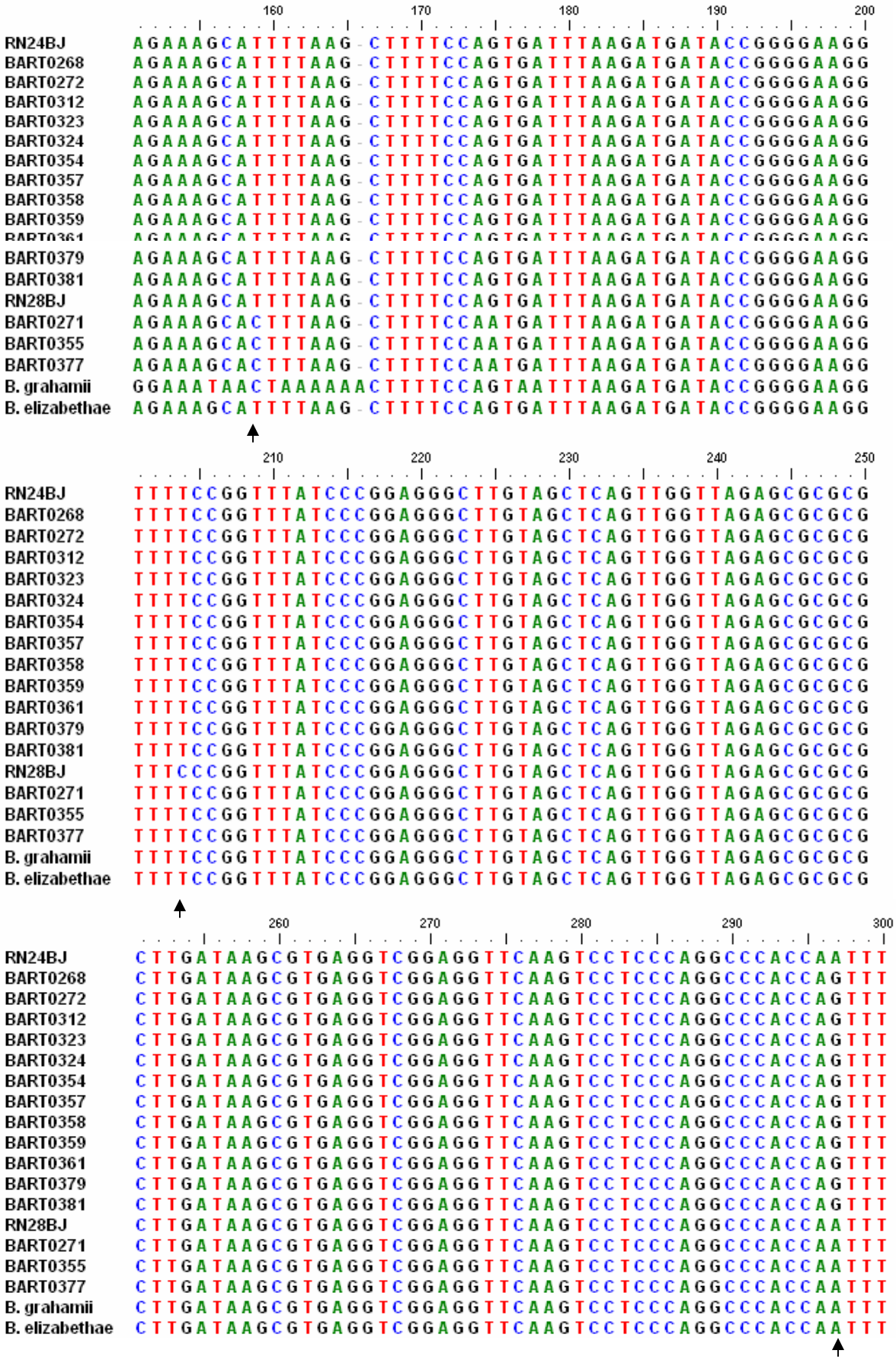
B. grahamii
B. elizabethae
QHVE1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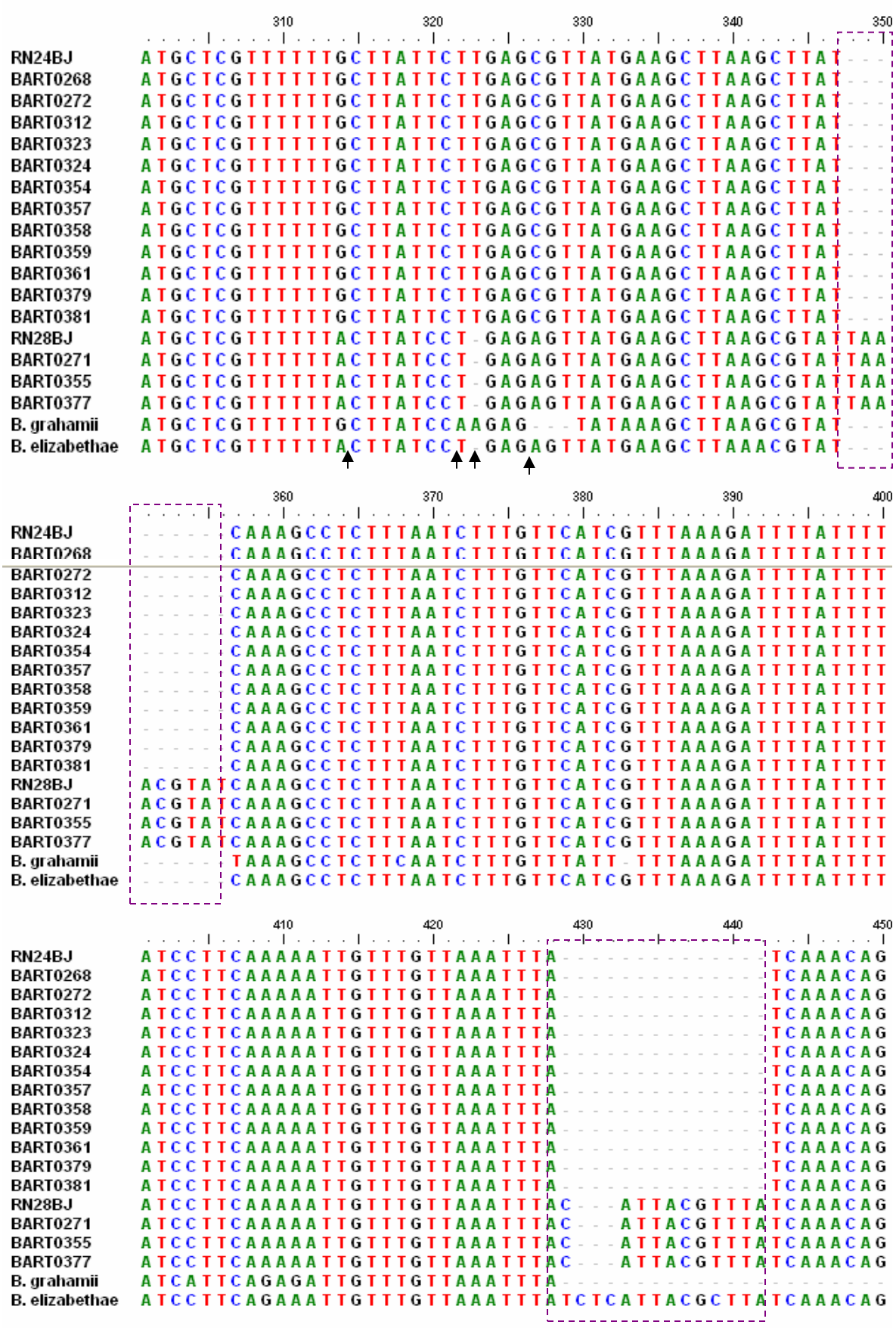
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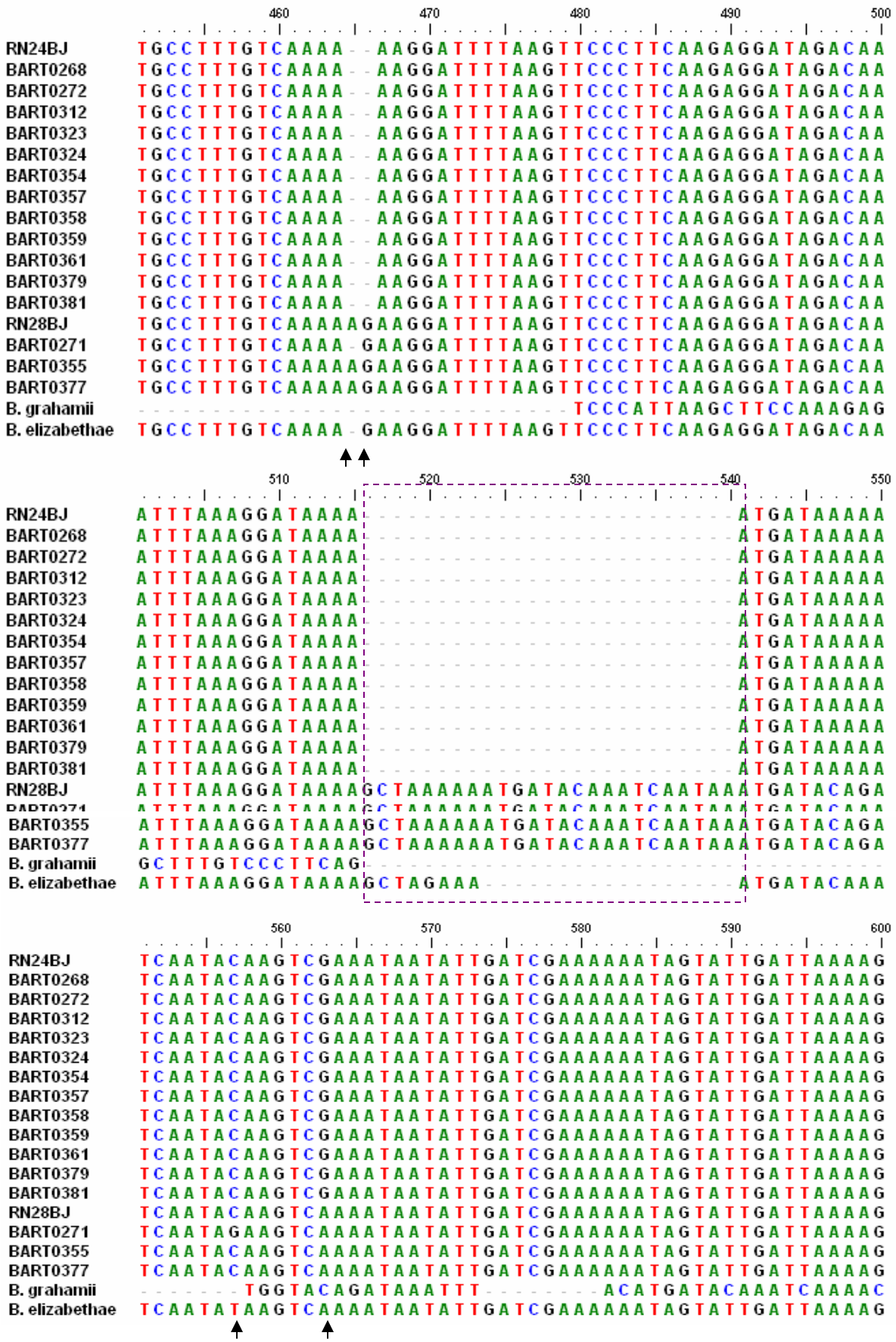
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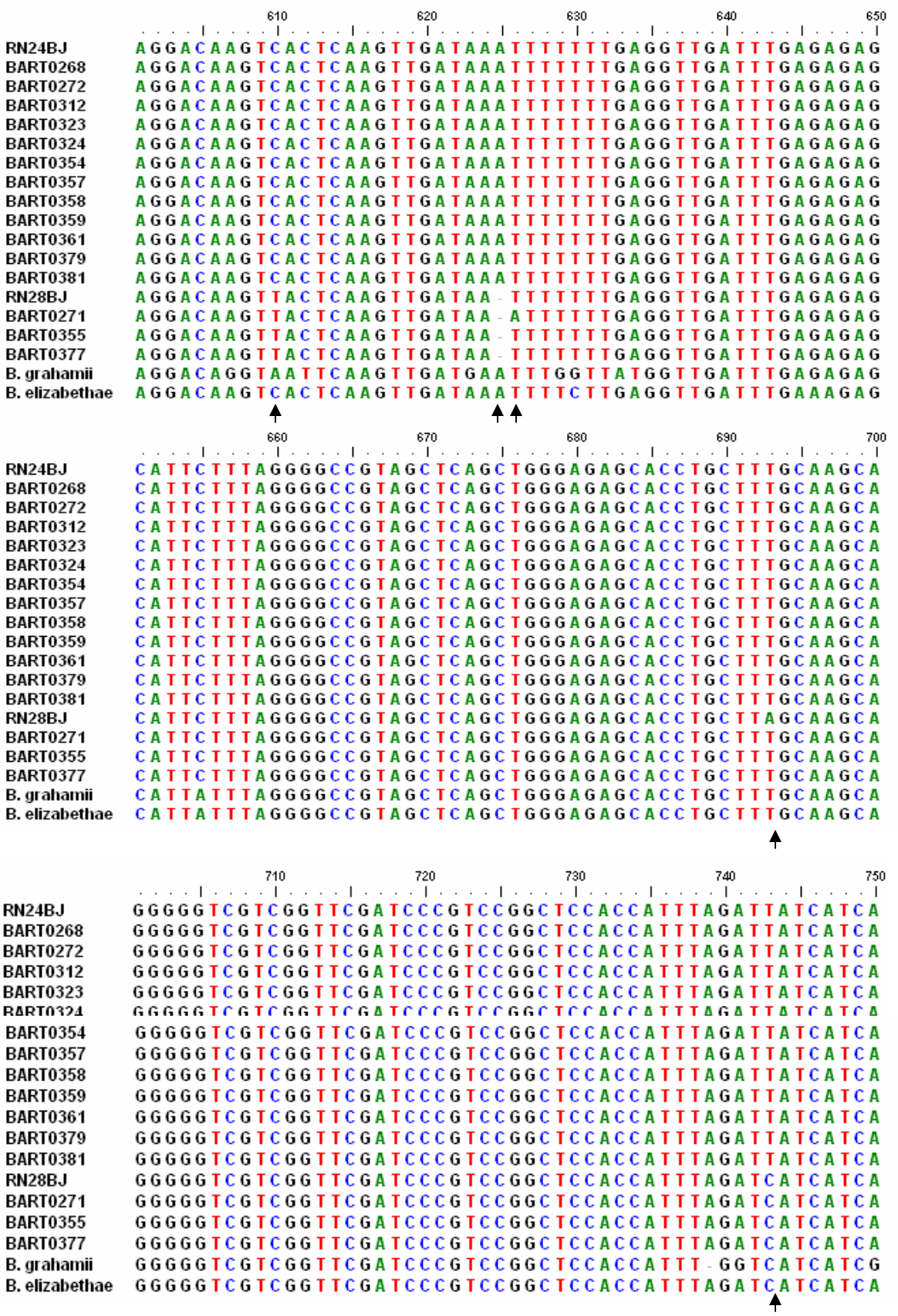
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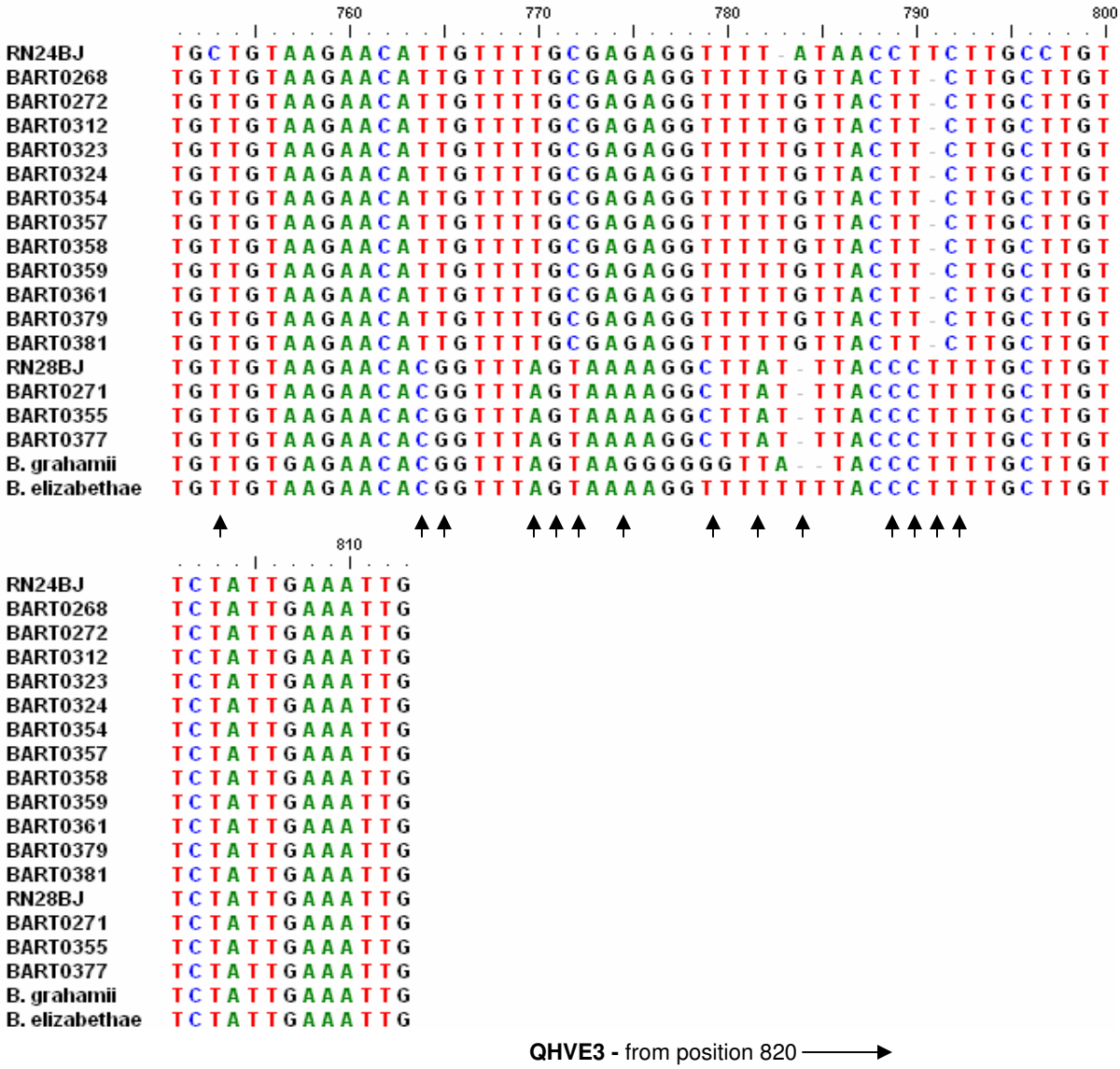


**Figure 3.9** Sequence alignments for 15 rodent isolates from Gauteng, aligned with RN24BJ and RN28BJ to which sequences were found most similar. *B. grahamii* and *B. elizabethae*, also rodent species, were aligned with the isolates of this study.

## 3.4 DISCUSSION

*B. henselae* was first isolated from the bloodstream of an AIDS patient (Regnery *et al.*, 1992a). Severely immunocompromised people with bacillary angiomatosis remain bacteremic for a number of weeks (Koehler & Tappero, 1993) and it is this group that is most at risk of contracting a *Bartonella* infection (Boulouis *et al.*, 2005). HIV-infected

patients with CD4+ cell counts of less than 50 /mm$^3$ are more likely to develop BA lesions (Koehler & Tappero, 1993; Boulouis *et al*., 2005). A study conducted in the San Francisco Bay area hospitals reported 3% (12/382) HIV-positive patients PCR-positive for *Bartonella* infection (Koehler *et al*., 2003). HIV-positive outpatients from Johannesburg hospitals were reported to have a 10% prevalence rate of *B. henselae* (Frean *et* al., 2002). This study has shown an even higher prevalence (22.5%) than previously reported. In immunocompromised individuals *B. henselae* infections are usually associated with exposure to cats and cat fleas (Koehler & Tappero, 1993; Boulouis *et al*., 2005).

The highest prevalences found for this study were for the cats (23.5%) and the rats (25%). These prevalences were not as high as some of the other reports published on the prevalence of bartonellae in animals. *Bartonella* prevalence in apparently healthy cats varies from 4 to 70%, depending on the geographical location and the studied population (feral or pet) of cats (Rolain *et al*., 2004b). In Korea, blood collected from 54 dogs and 151 cats was analyzed for the presence of *Bartonella* by nested PCR. *B. henselae* was detected from blood of feral cats (41.8%), pet cats (33.3%), and pet dogs (16.6%). *B. clarridgeiae* was isolated from 9 dog blood samples and 2 dogs were co-infected with *B. henselae* and *B. clarridgeiae* (Kim *et al*., 2009). An interesting finding for this study was the isolation of *B. henselae* URBHLIE9 from all 5 culture-positive cat isolates. This strain was previously isolated from the blood of a patient presenting with endocarditis and implies a strong link between humans and cats as reservoirs for bartonellae (Houpikian and Raoult; 2001).

Other studies showed the following *Bartonella* prevalences: 22% (n=113) from impounded cats in the Netherlands (Bergmans *et al*., 1997); 13% (n=100) from pet cats in Germany (Sander *et al*., 1997); 0.5% (n=198) from sick dogs in Brazil (Diniz *et al*., 2007); 4% (n=50) from dogs in Greece, and 12% (n=60) from dogs in Italy (Diniz *et al*., 2009).

Studies carried out on various rodent populations have shown 29% (n=87) *Bartonella* prevalence in mice and 20% (n=10) in rats from south western Spain (MarQuez *et al*., 2008); 13.9% (n=389) *Bartonella* prevalence from small wild rodents in Korea (Kim *et al*., 2005); 6.2% (n=210) in rodent population sampled in the Greater Jakarta area, Indonesia (Winoto *et al*., 2005); 8.7% (n=195) *Bartonella* prevalence was found in

rodents from northern Thailand (Castle *et al.*, 2004); and 24% (n=79) in rats from Israel (Morick *et al.*, 2009).

PCR results indicate that there a high prevalence of bartonellae in human and animal populations. More work is required to fully understand the extent of disease resulting from these *Bartonella* infections.