Supplementary Material for Harhangi *et al*.

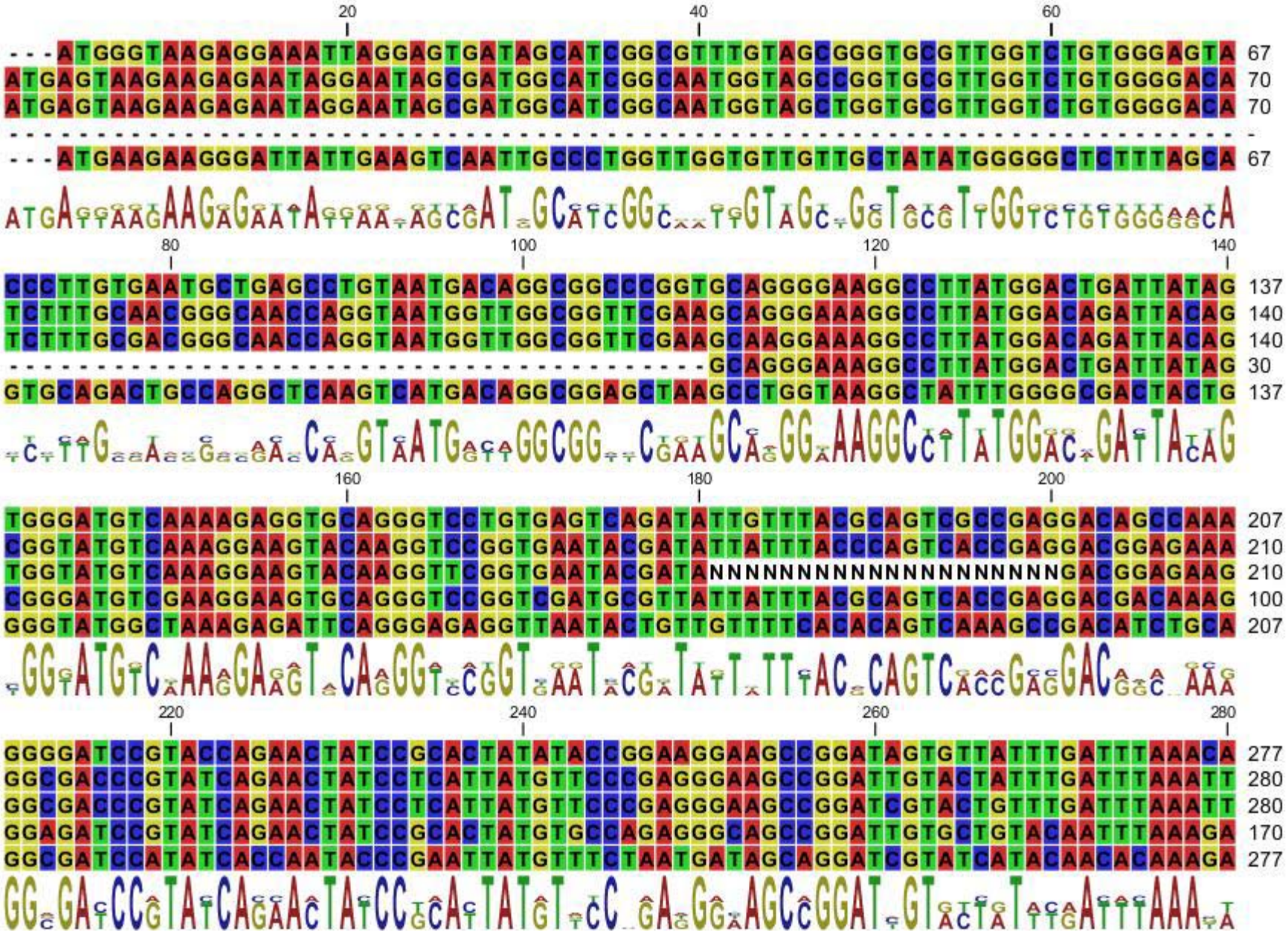
Hydrazine synthase, a unique phylomarker to study the presence and biodiversity of anammox bacteria

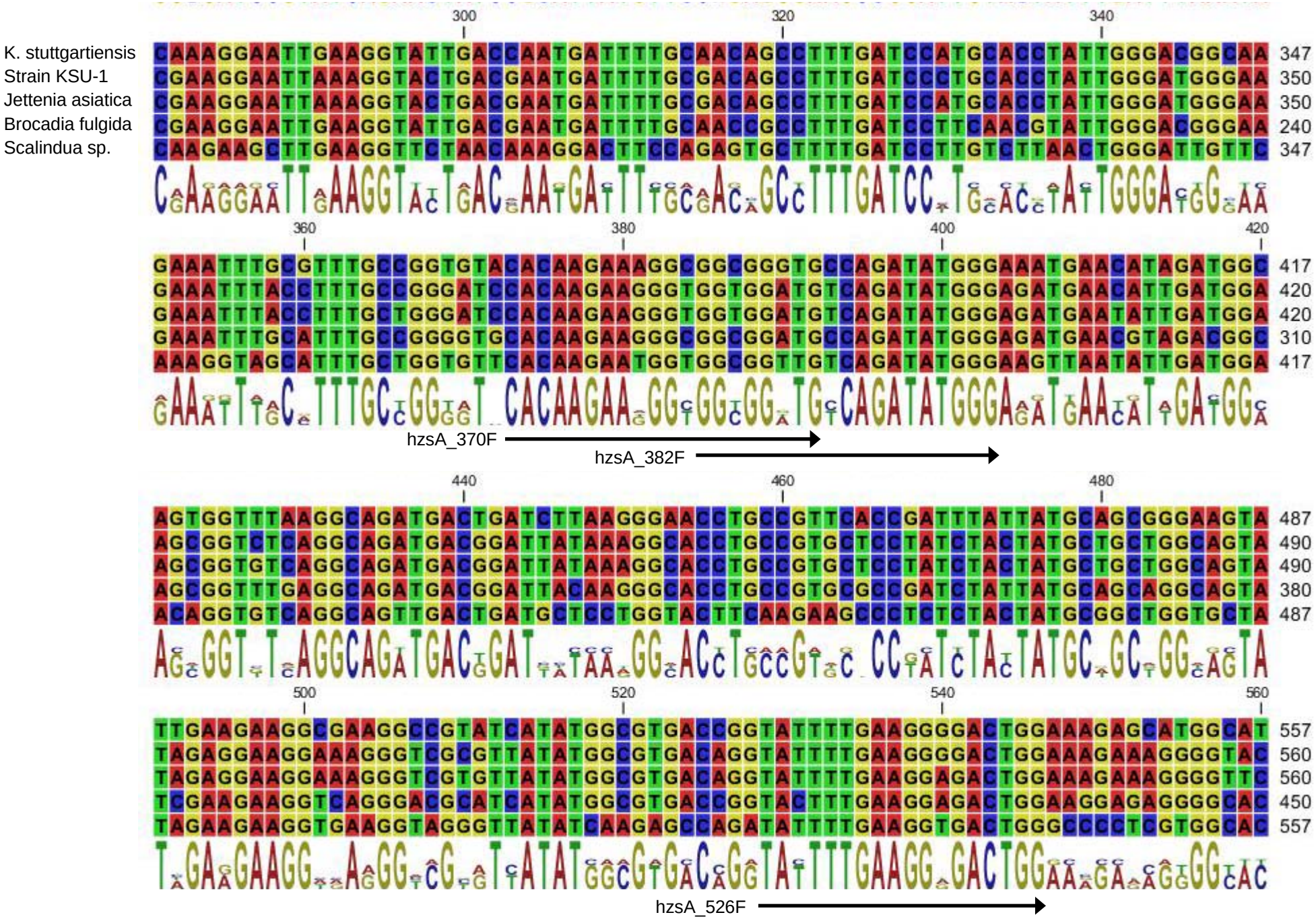
This material includes a table with paiwise sequence identities, a figure with the ClustalW alignment of the *hzs*A genes of five different anammox bacteria (Figure S1), a figure showing agarose gel electrophoresis of the *hzs*A gene PCR products amplified using gDNA from reactor biomass (Figure S2), a figure showing the phylogenetic analysis of cloned *hzs*A gene qPCR products (Figure S3) and a figure with Real time RT-PCR of *hzs*A/*nir*S mRNA expression level for *Kuenenia stuttgartiensis* (Figure S4).

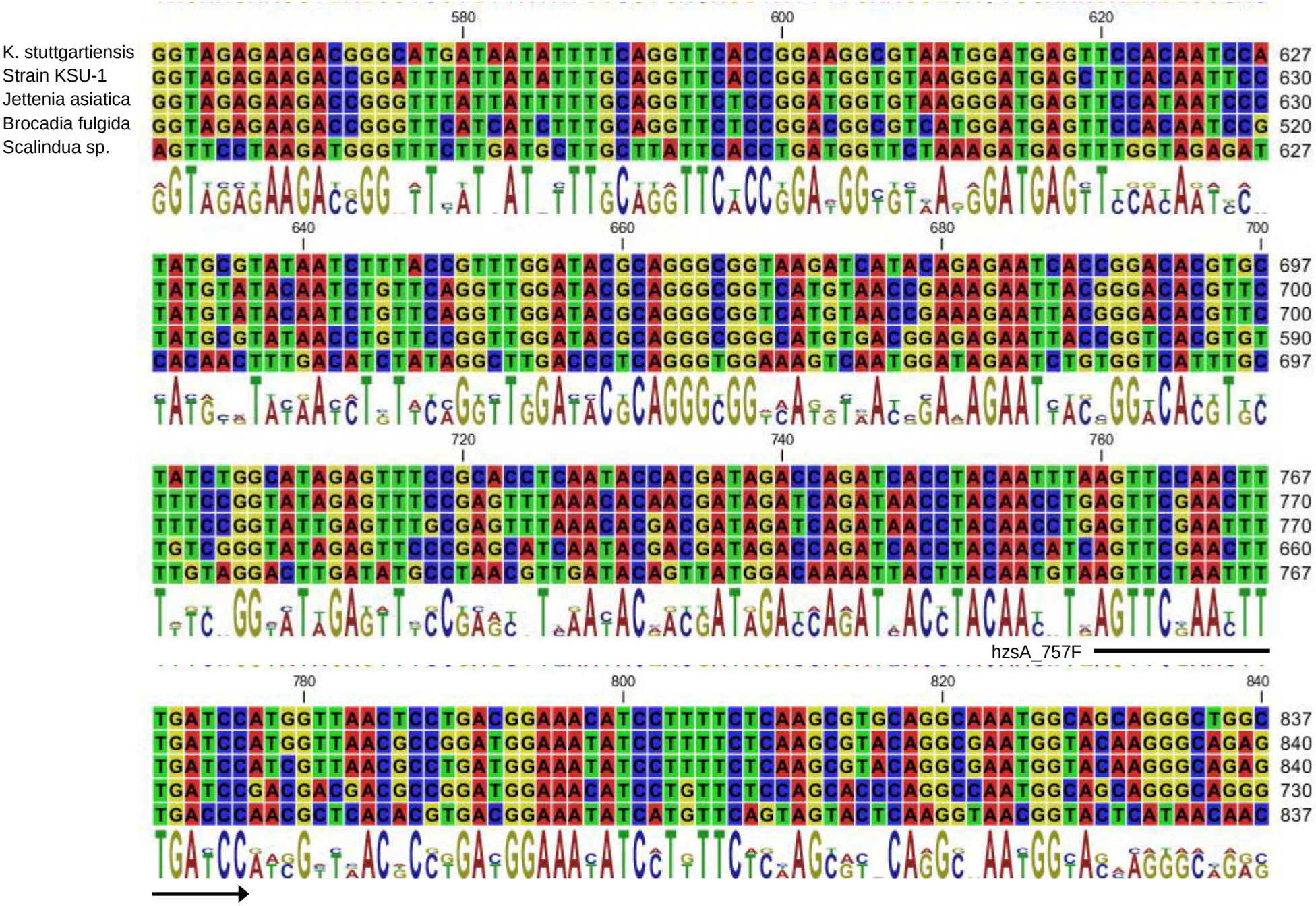
Supplementary Table S1 Pairwise sequence identities (%) for the *hzs*A genes and derived protein sequences of the anammox bacteria used to design primers (see also Supplementary Figure S1).

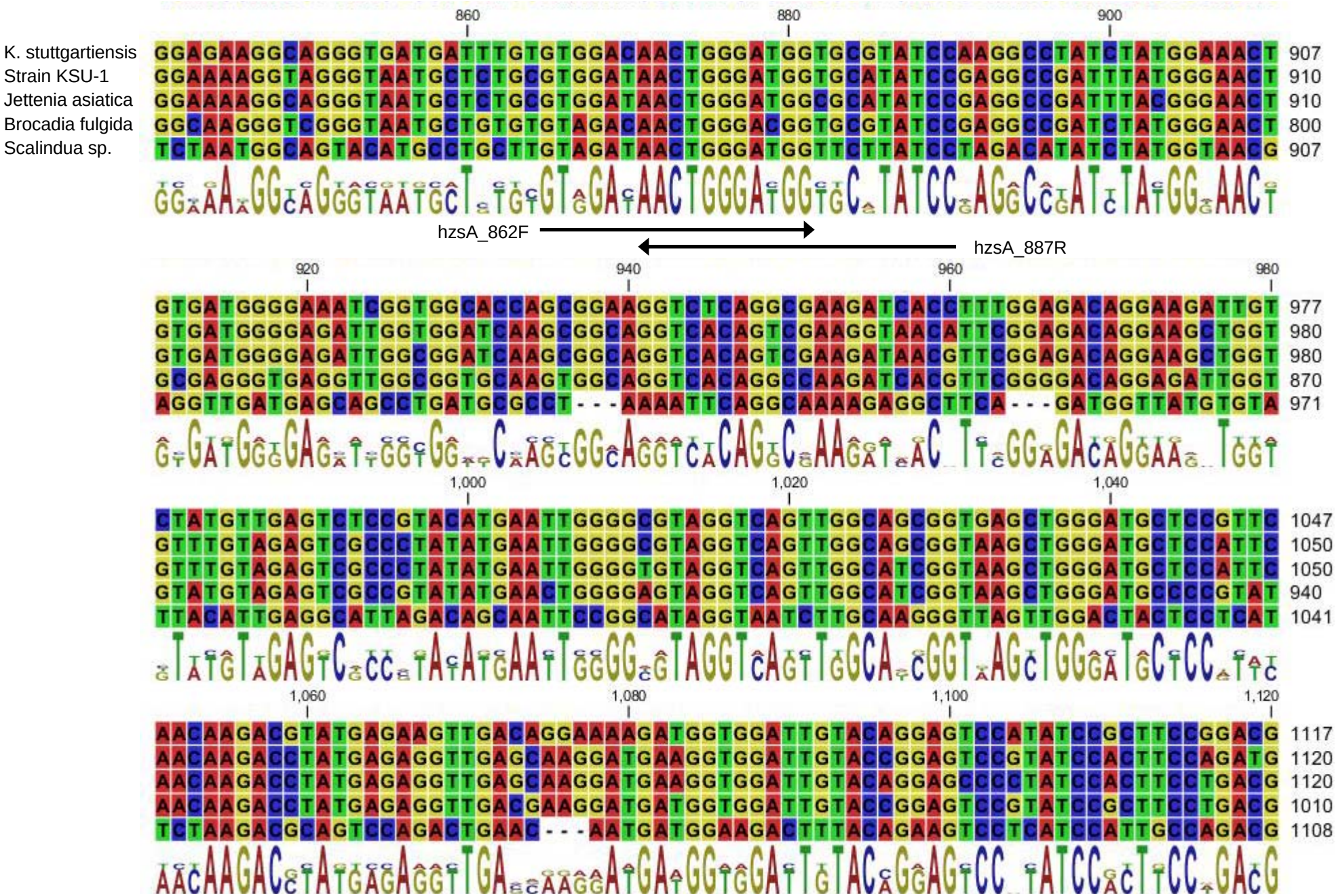
DNA	1	2	3	4	5		Protein	1	2	3	4	5
1							1					
2	77.5						2	81.8				
3	75.5	92.8					3	82.0	97.0			
4	78.3	82.0	79.1				4	83.5	89.4	88.7		
5	64.0	63.6	61.7	62.0			5	61.5	61.8	62.3	64.0	

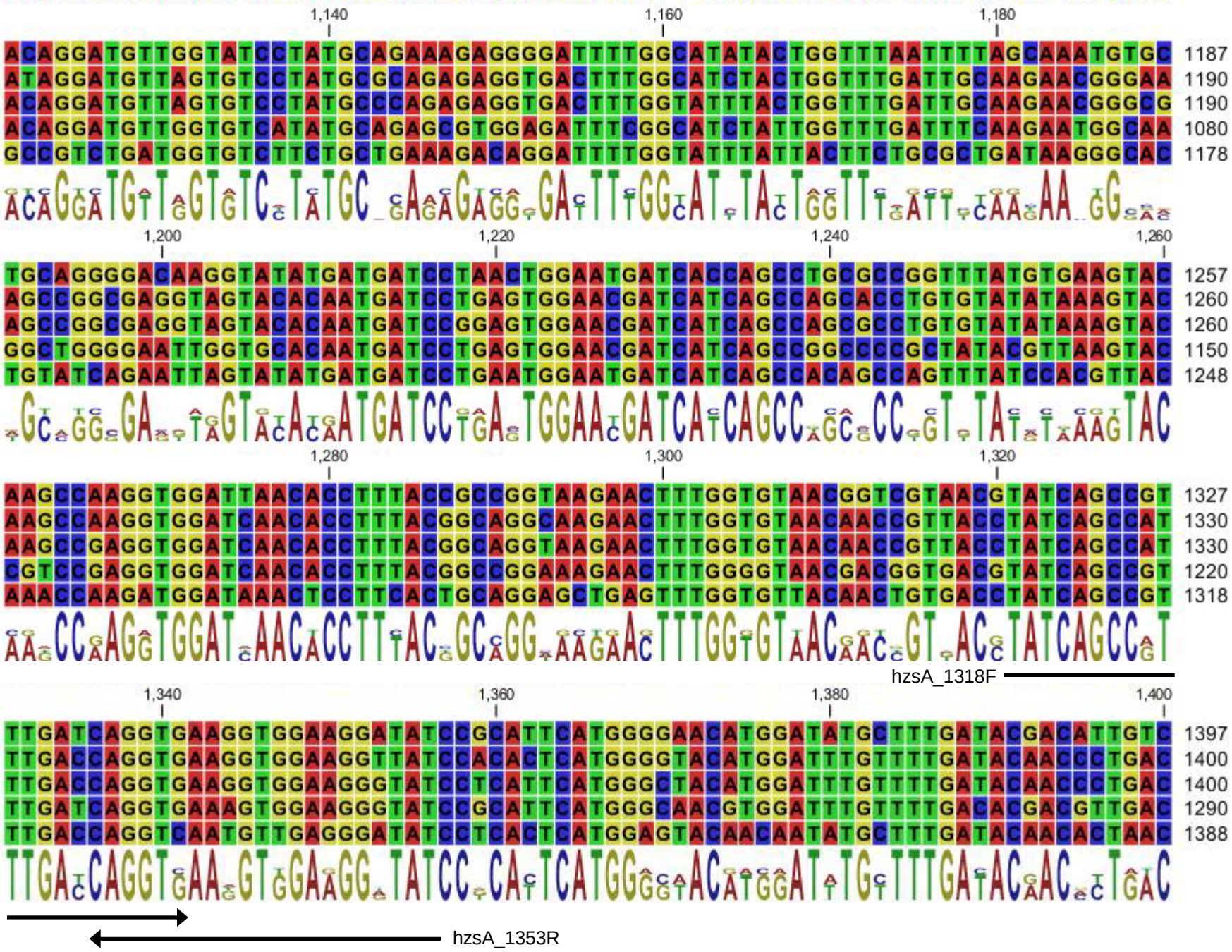
1 = *Kuenenia stuttgartiensis*
2 = Strain KSU-1
3 = *Jettenia asiatica*
4 = *Brocadia fulgida*
5 = *Scalindua* sp.

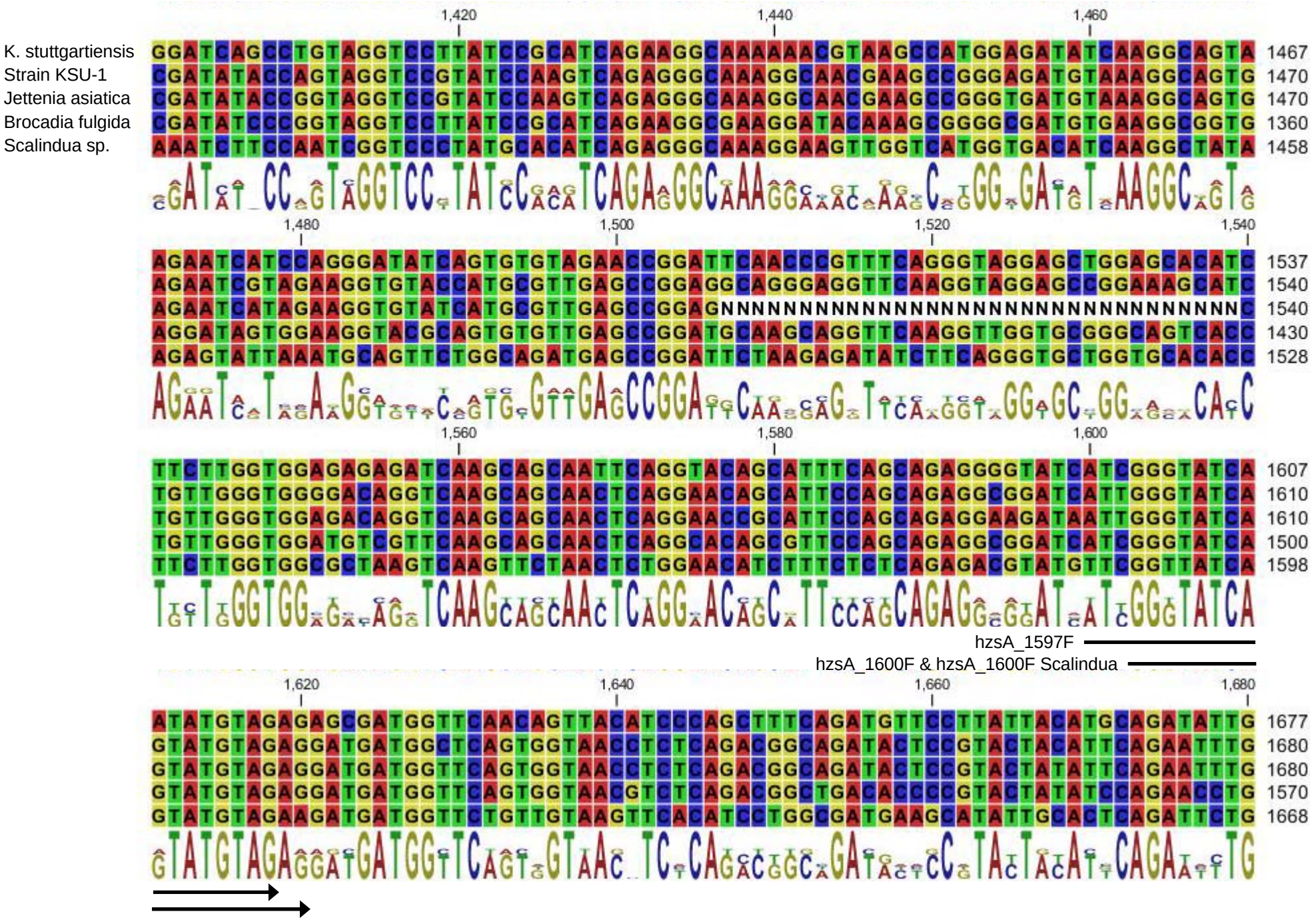

K. stuttgartiensis
Strain KSU-1
Jettenia asiatica
Brocadia fulgida
Scalindua sp.
20
40
60
80
100
120
140
160
180
200
220
240
260
280

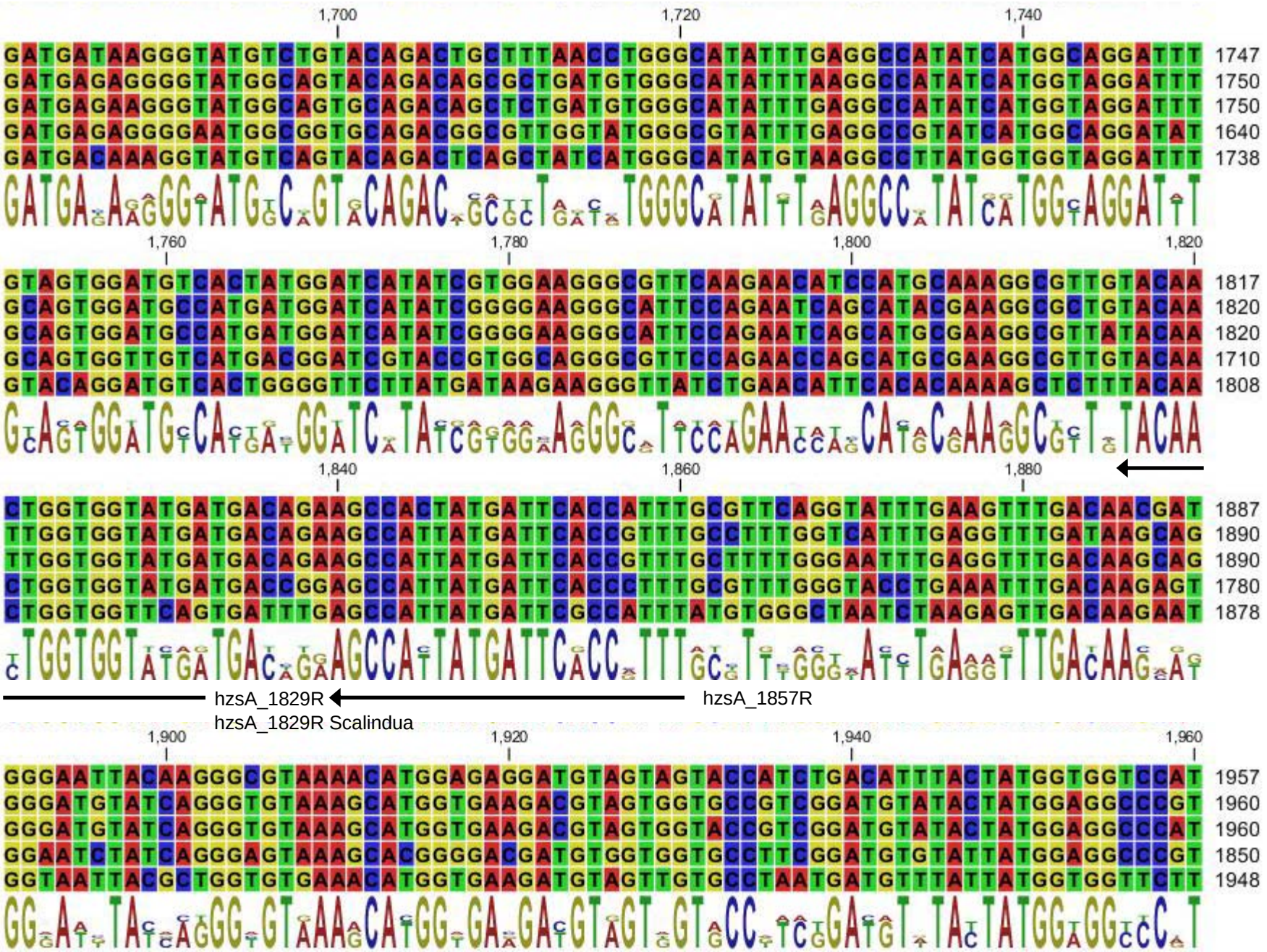

K. stuttgartiensis
Strain KSU-1
Jettenia asiatica
Brocadia fulgida
Scalindua sp.
300
320
340
347
350
350
240
347
360
380
400
420
417
420
420
310
417
hzsA_370F
hzsA_382F
440
460
480
487
490
490
380
487
500
520
540
560
557
560
560
450
557
hzsA_526F

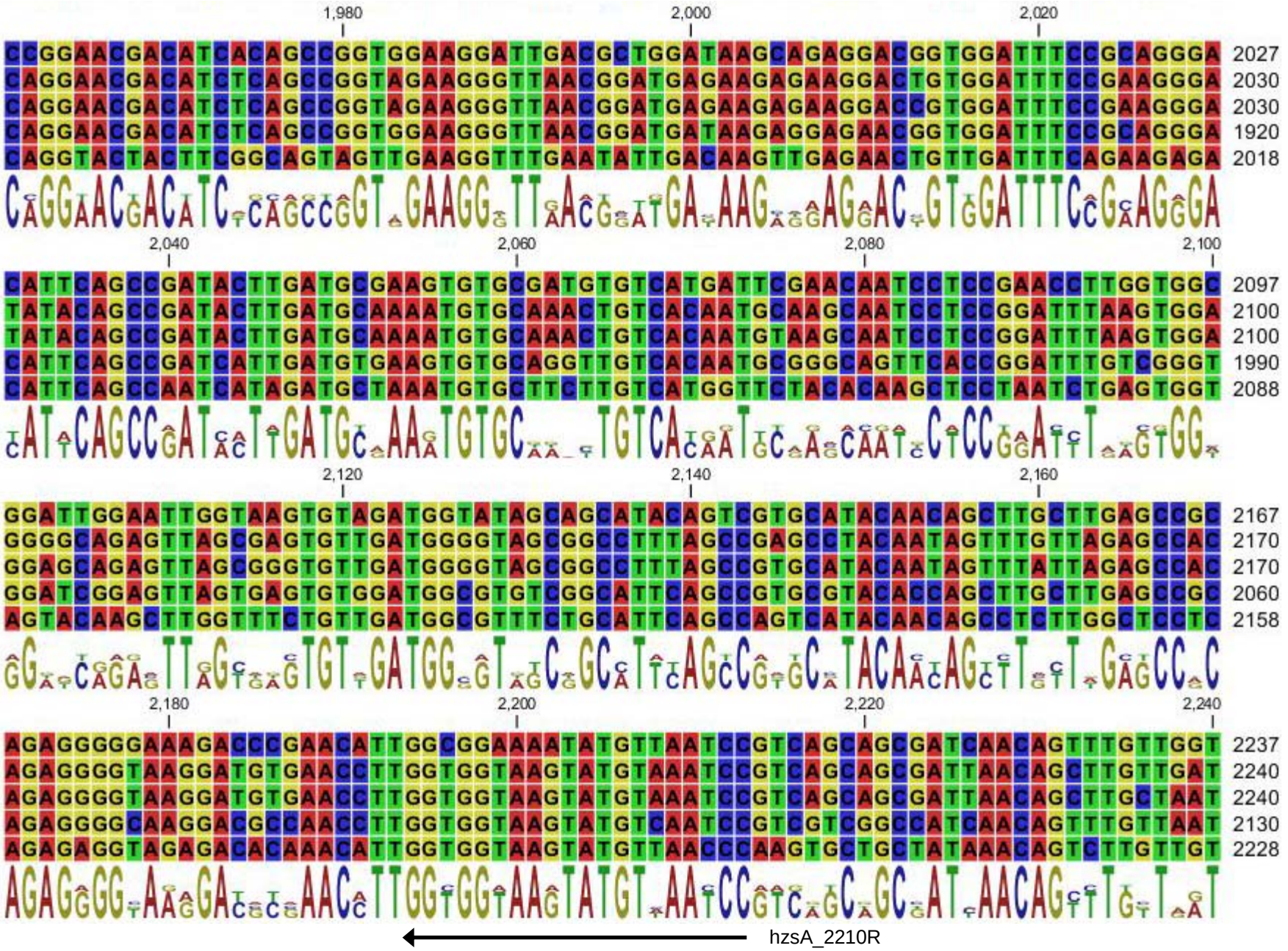

K. stuttgartiensis
Strain KSU-1
Jettenia asiatica
Brocadia fulgida
Scalindua sp.
580
600
620
627
630
630
520
627
640
660
680
700
697
700
700
590
697
720
740
760
767
770
770
660
767
hzsA_757F
780
800
820
840
837
840
840
730
837

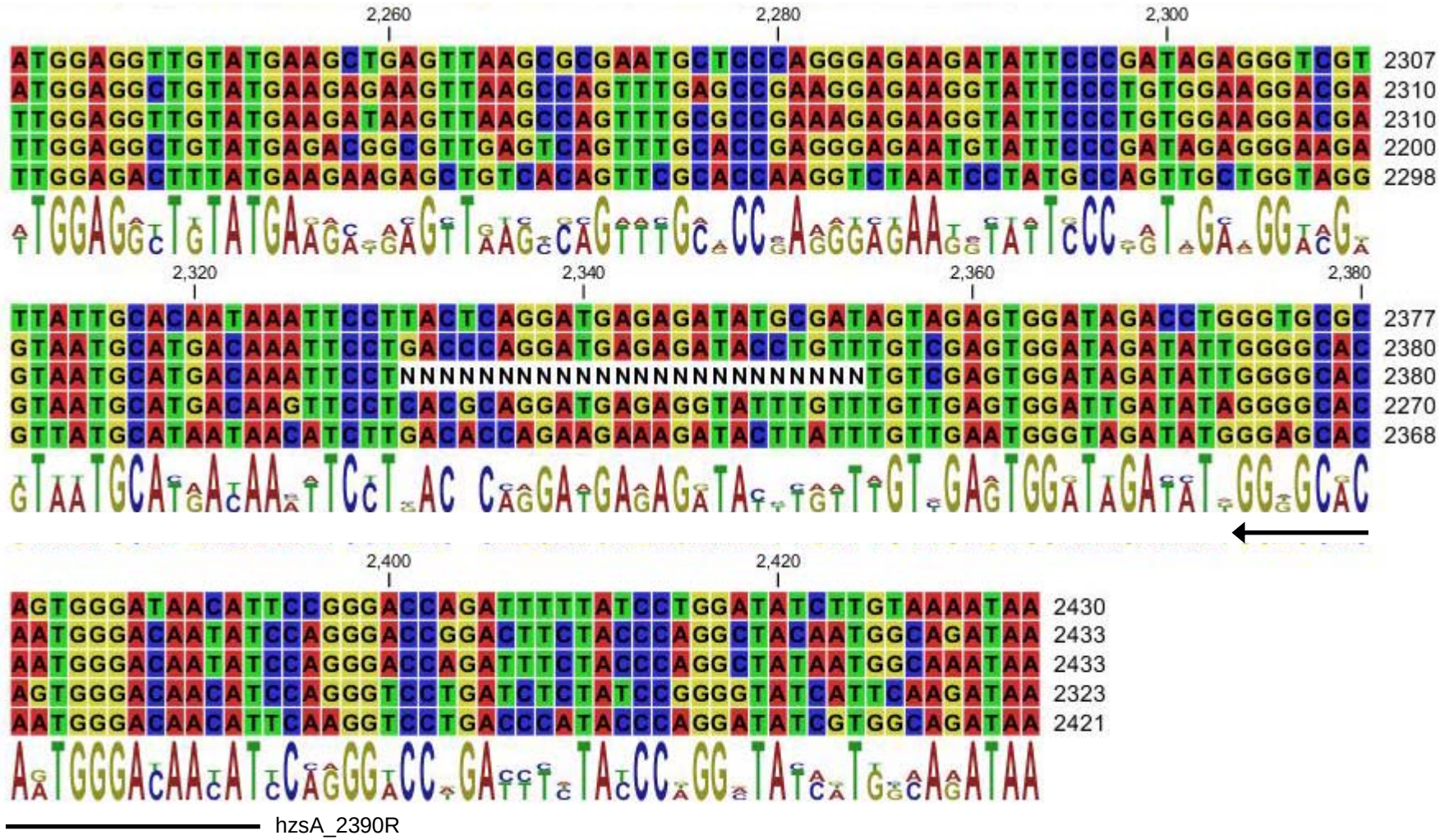

K. stuttgartiensis
Strain KSU-1
Jettenia asiatica
Brocadia fulgida
Scalindua sp.
860
880
900
907
910
910
800
907
hzsA_862F
hzsA_887R
920
940
960
980
977
980
980
870
971
1,000
1,020
1,040
1047
1050
1050
940
1041
1,060
1,080
1,100
1,120
1117
1120
1120
1010
1108


K. stuttgartiensis
Strain KSU-1
Jettenia asiatica
Brocadia fulgida
Scalindua sp.
1,140
1,160
1,180
1187
1190
1190
1080
1178
1,200
1,220
1,240
1,260
1257
1260
1260
1150
1248
1,280
1,300
1,320
1327
1330
1330
1220
1318
hzsA_1318F
1,340
1,360
1,380
1,400
1397
1400
1400
1290
1388
hzsA_1353R

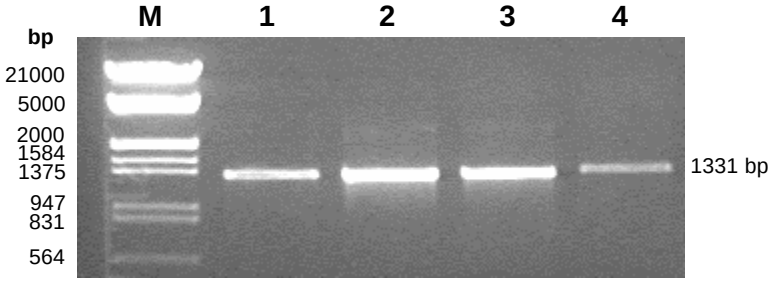

K. stuttgartiensis
Strain KSU-1
Jettenia asiatica
Brocadia fulgida
Scalindua sp.
1,420
1,440
1,460
1,480
1,500
1,520
1,540
1,560
1,580
1,600
1,620
1,640
1,660
1,680
1467
1470
1470
1360
1458
1537
1540
1540
1430
1528
1607
1610
1610
1500
1598
1677
1680
1680
1570
1668
hzsA_1597F
hzsA_1600F & hzsA_1600F Scalindua


1,700
1,720
1,740
K. stuttgartiensis
Strain KSU-1
Jettenia asiatica
Brocadia fulgida
Scalindua sp.
1747
1750
1750
1640
1738
1,760
1,780
1,800
1,820
1817
1820
1820
1710
1808
1,840
1,860
1,880
1887
1890
1890
1780
1878
hzsA_1829R
hzsA_1857R
hzsA_1829R Scalindua
1,900
1,920
1,940
1,960
1957
1960
1960
1850
1948


K. stuttgartiensis
Strain KSU-1
Jettenia asiatica
Brocadia fulgida
Scalindua sp.
1,980
2,000
2,020
2027
2030
2030
1920
2018
2,040
2,060
2,080
2,100
2097
2100
2100
1990
2088
2,120
2,140
2,160
2167
2170
2170
2060
2158
2,180
2,200
2,220
2,240
2237
2240
2240
2130
2228
hzsA_2210R

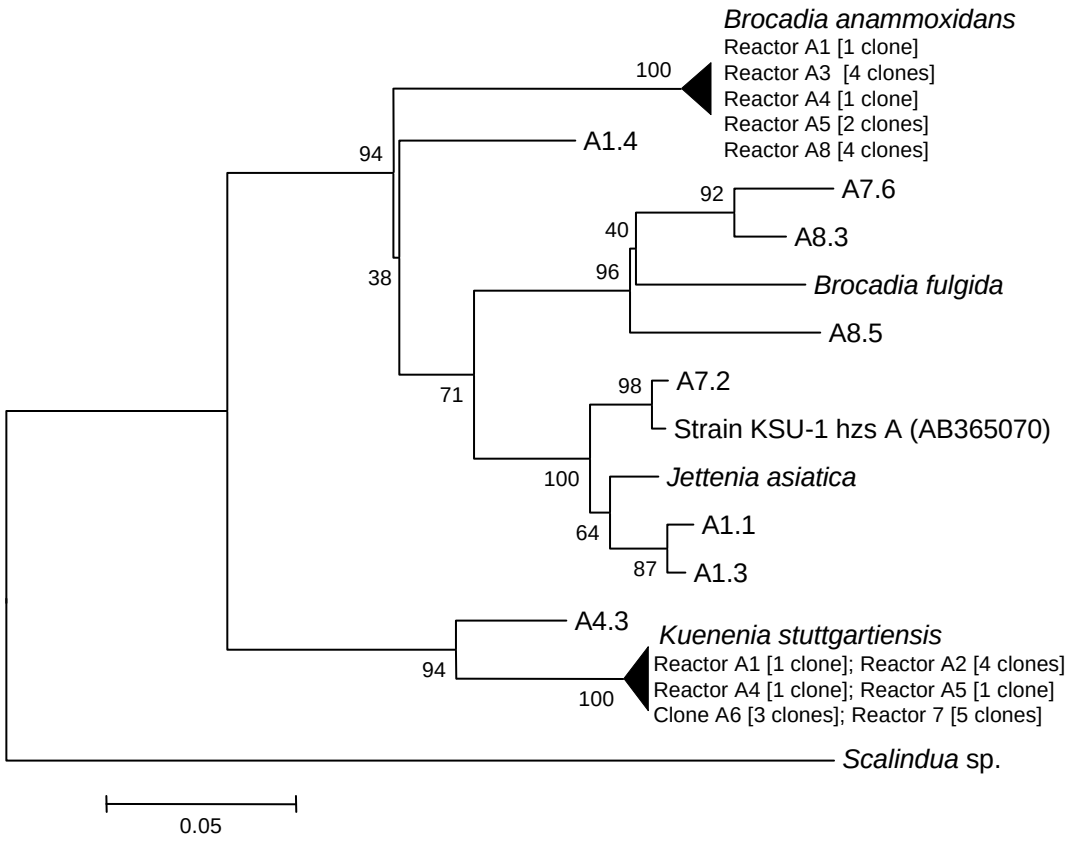


Supplementary Figure S1: ClustalW alignment of the *hzsA* genes of five different anammox bacteria. The Forward and Reverse primers designed are indicated by arrows.

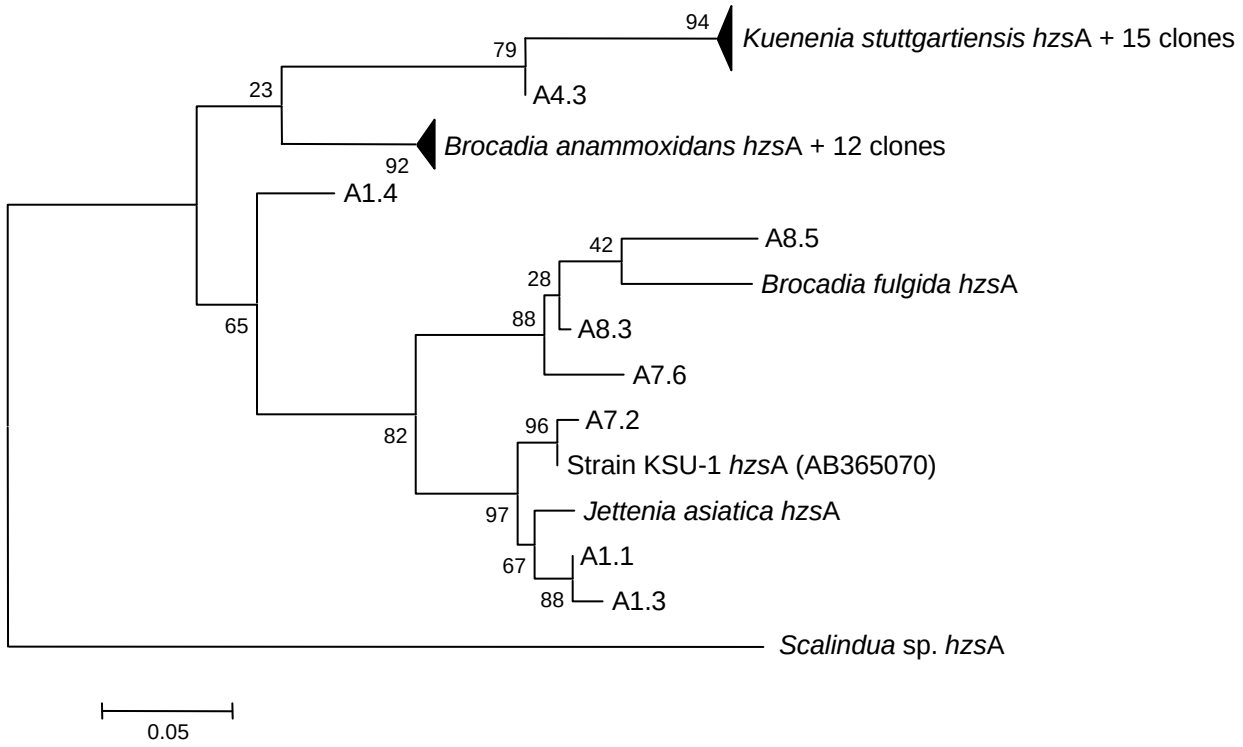


Supplementary Figure S2:
Agarose gel electrophoresis of the PCR products amplified using gDNA extracted from highly enriched reactor biomass with the hzsA_526F / hzsA_1857R primer pair.

M = Marker lane
1 = *Scalindua*-dominated reactor (1000 x diluted)
2 = *Brocadia*-dominated reactor (1000 x diluted)
3 = *Kuenenia*-dominated reactor (10 x diluted)
4 = *Jettenia*-dominated reactor (10 x diluted)

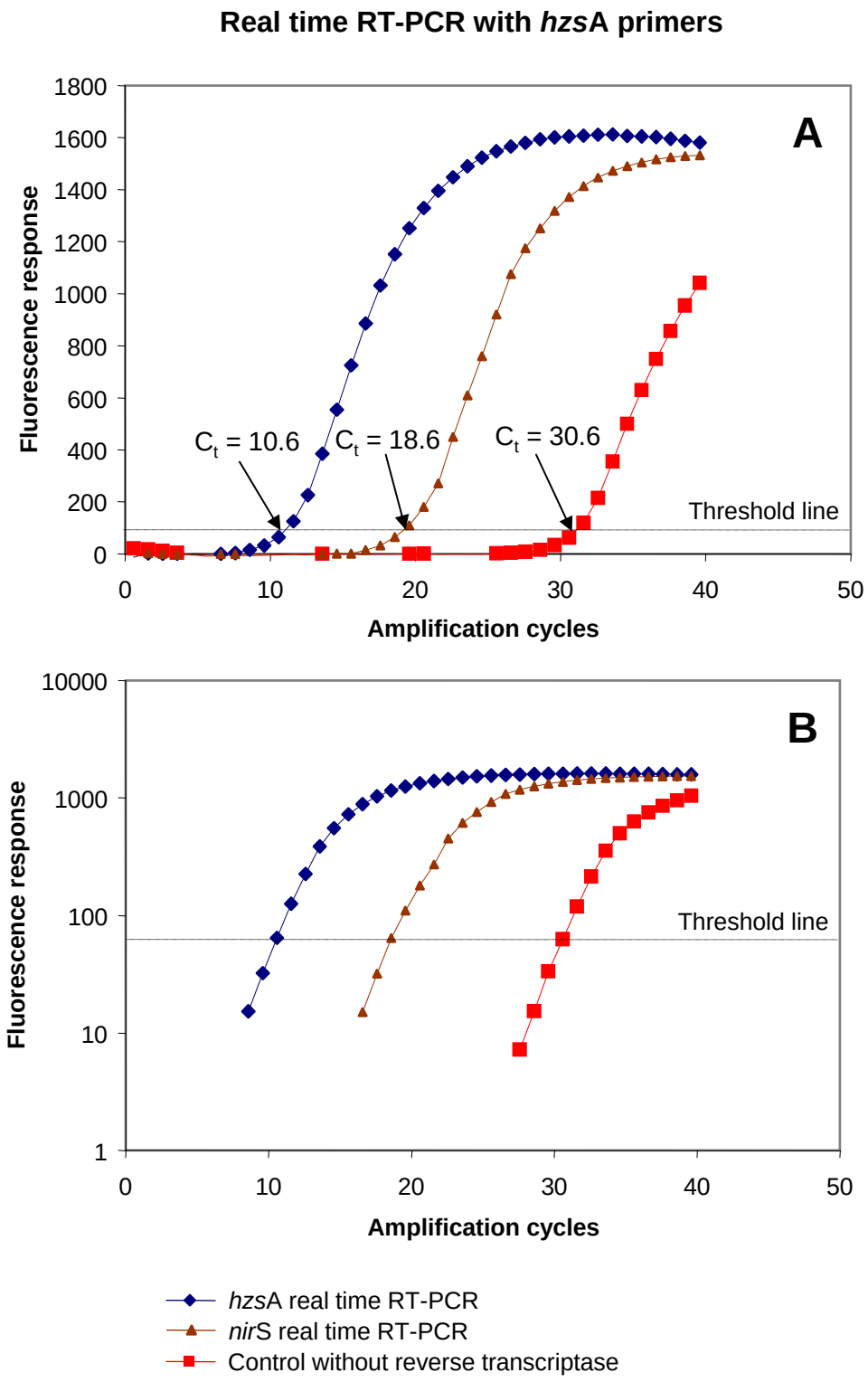


Supplementary Figure 3A. Phylogenetic tree of the *hzs*A short qPCR sequences (260 nt) and known anammox bacteria. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.02205903 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 42 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair.



Supplementary Figure 3B. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-1651.9420) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 42 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.



Supplementary Figure 4. Real time RT-PCR to analyze the mRNA expression level of the *hzs*A and *nir*S gene in *Kuenenia stuttgartiensis* using the primers pair hzsA_1597F/hzsA_1857R and nirSF/nirSR. Amplification cycles are plotted against the fluorescence respons; **A.** linear scale, **B.** logarithmic scale. To check for contaminating DNA, control without reverse transcriptase were performed.