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MOLECULAR DETECTION OF *SARCOCYSTIS CRUZI* IN SLAUGHTERED CATTLE AT BAGHDAD CITY IN IRAQ

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Abstract

The study was conducted to estimate the prevalence of *Sarcocystis* spp. in slaughtered cattle, at Baghdad city by using of 200 slaughtered cattle esophagus samples collected during the period from beginning of December 2018 to the end of September 2019. All samples were examined by molecular methods and positive samples collected were molecular detection as first study in Iraq by using sequencing and phylogenic to identity the species of *Sarcocystis spp.* infection and to compared it with other *Sarcocystis spp* in Gene Bank which gives an idea about the new strain of Iraqi *Sarcocystis* spp. Results of DNA sequencing were checked by using references of *18S rRNA* gene (615bp and 574bp) of *Sarcocystis* parasite. Phylogenetic topology of 14 samples bootstraps of our samples revealed high sequence similarity to identified species, and BLAST searches revealed that the *18S rRNA* sequences shared 99.5- 100% identity to *S. cruzi.* The results of the phylogenetic tree was showed similarities between Iraqi strain and the distant world. In conclusion PCR methods followed by phylogenic tree analysis a good methods for diagnosis and identification of genetic variants studies.

Key words: Molecular detection, *Sarcocystis cruzi*, cattle.

Introduction

Sarcocystis is a genus of intracellular, coccidian parasites belonging to the protozoa (Sporozoa: Apicomplexa) with an obligate two host life cycle between predators (carnivores) as final hosts and prey animals (herbivores) as intermediate hosts, species of *Sarcocystis* are generally more specific for their prey hosts than for their predator hosts (Badree, 2009; Hornok *et al*., 2015). *Sarcocystis* spp. are highly prevalent in livestock such as cattle and are considered to be host specific (Fayer, 2004).

Cattle are mainly infected with *Sarcocystis cruzi*, *Sarcocystis hominis*, *Sarcocystis hirsuta S. heydorni*, *S. rommeli* and *S. sinensis* has been reported from cattle (Moré *et al*., 2014). *Sarcocystis cruzi* is the most common and important species affecting cattle **(**Dubey *et al*., 2015).

Sarcocystiosis in bovine intermediate hosts is characterized by encephalitis, inflammation of the brain and spinal cord, fever, anorexia, anemia, diarrhea, cachexia, weight loss, accelerated heart rate, abortion, myositis and occasionally may lead to death (Jehle *et al*., 2009; Januskevicius *et al*., 2019). Diagnosis of blood parasites depend on microscopic examination and molecular assay are a range of DNA based methods for the detection of blood parasites (Gjerde *et al*., 2016 Mohamed *et al*., 2016). Due to the few studies that related to the molecular diagnosis of Sarcocystis spp. infected cattle in Iraq. The present study was designed to detection of *Sarcocystis cruzi* in slaughtered cattle at Baghdad city, using molecular diagnosis and phylogenic analysis.

Materials and Methods

Collection of samples:

Two hundred specimens were collected from esophagus of slaughtered cattle in different area of Baghdad city, during the period from beginning of December 2018 to the end of September 2019. Their ages were ranged between less than 1 year to up 4 year. 60mg of each sample were considered for DNA extraction.

DNA Extraction

Genomic DNA of bradyzoites *Sarcocystis* isolate was extracted by using tissue DNA extraction kit Geneaid/ USA and (Bioneer)/Korea according manufacturer

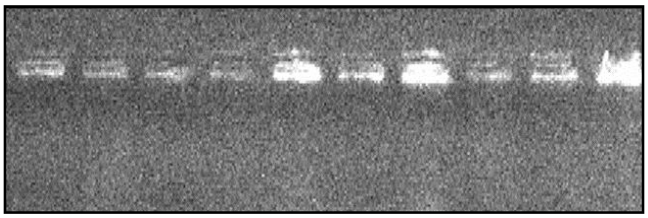

Fig. 1: Gel electrophoresis of agarose gel stained with ethidium bromide for DNA extraction of *Sarcocystis*.

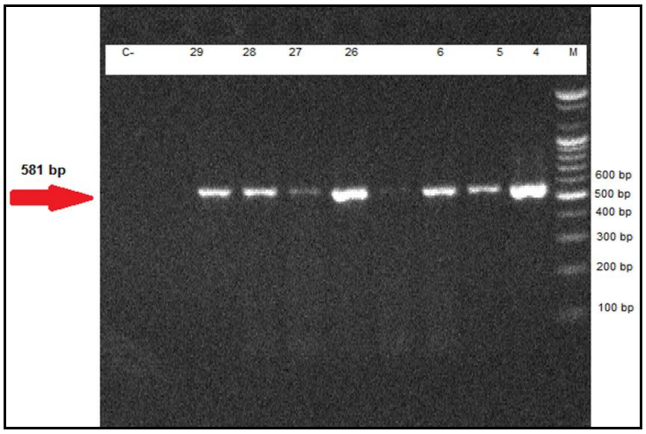

Fig. 3: Agarose gel electrophoresis M: molecular marker (100bp), C: control negative showed the PCR product analysis of 18S rRNA gene in *Sarcocystis spp.* positive samples line 4,5,6,26,27,28.29 (Sar2), 581 bp in 1.5% agrose gel, 60 volt and 1.5 hr.

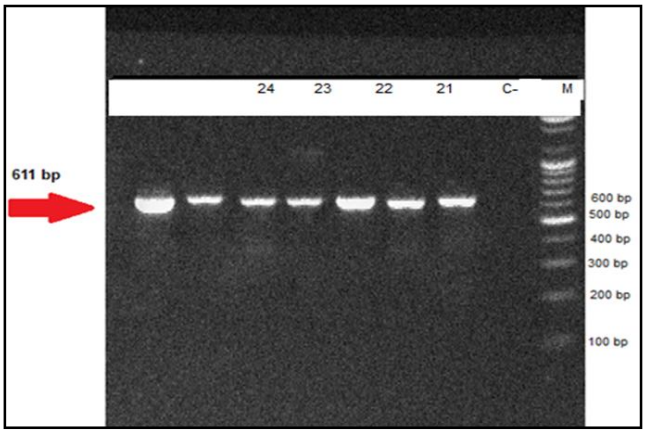

Fig. 2: Agarose gel electrophoresis M: molecular marker (100bp),C: control negative, showed the PCR product analysis of 18S rRNA gene in *Sarcocystis spp.* positive samples line 21,22,23,24, (Sar1), 611 bp in 1.5% agrose gel, 60 volt and 1.5 hr.

protocol from tissue cells extraction of digestion test.

Table 2: The NCBI-BLAST Homology Sequence identity (%) between Iraqi *Sarcocystis cruzi* of slaughtered cattle isolates and NCBI-BLAST submitted *Sarcocystis* cruzi. isolates.

No.	*Sarcocystis spp.* isolate	Genbank accession No.	Amplicon size	NCBI-BLAST Homology sequence identity (%)	
				Identical *Sarcocystis Spp.*	Genbank Accession No.
1	*Sarcocystis spp.*slaughtered cattle isolate No.4	MN096331	581 bp	*Sarcocystis cruzi*	MG787078.1(13 – 35) + (574 – 593) -
2	*Sarcocystis spp.*slaughtered cattle isolate No.5	MN096332	581 bp	*Sarcocystis cruzi*	MG787078.1(13 – 35) + (574 – 593) -
3	*Sarcocystis spp.*slaughtered cattle isolate No.6	MN096333	581 bp	*Sarcocystis cruzi*	MG787078.1(13 – 35) + (574 – 593) -
4	*Sarcocystis spp.*slaughtered cattle isolate No.21	MN197848	611 bp	*S. cruzi*	LC171829.1(699 – 716)+(1291 – 1309) -
5	*Sarcocystis spp.*slaughtered cattle isolate No.22	MN197849	611 bp	*S. cruzi*	LC171829.1(699 – 716)+(1291 – 1309) -
6	*Sarcocystis spp.*slaughtered cattle isolate No.23	MN197850	611 bp	*S. cruzi*	LC171829.1(699 – 716)+(1291 – 1309) -
7	*Sarcocystis spp.*slaughtered cattle isolate No.24	MN197851	611 bp	*S. cruzi*	LC171829.1(699 – 716)+(1291 – 1309) -
8	*Sarcocystis spp.*slaughtered cattle isolate No.26	MN197853	581 bp	*S. cruzi*	MN096333.1(1 – 23)+(562 – 581)-
9	*Sarcocystis spp.*slaughtered cattle isolate No.27	MN197854	581 bp	*S. cruzi*	MN096333.1(1 – 23)+(562 – 581)-
10	*Sarcocystis spp.*slaughtered cattle isolate No.28	MN197855	581 bp	*S. cruzi*	MN096333.1(1 – 23)+(562 – 581)-
11	*Sarcocystis spp.*slaughtered cattle isolate No.29	MN628312	581 bp	*S. cruzi*	MN096333.1(1 – 23)+(562 – 581)-
12	*Sarcocystis spp.*slaughtered cattle isolate No.30	MN628313	581 bp	*S. cruzi*	MN096333.1(1 – 23)+(562 – 581)-
13	*Sarcocystis spp.*slaughtered cattle isolate No.31	MN628314	581 bp	*S. cruzi*	MN096333.1(1 – 23)+(562 – 581)-
14	*Sarcocystis spp.*slaughtered cattle isolate No.32	MN628315	581 bp	*S. cruzi*	MN096333.1(1 – 23)+(562 – 581)-

PCR Amplification

PCR primers for detection *Sarcocystis* spp. based on 18S rRNA, two primers were used in this study. The lyophilized primers were purchased from Bioneer (Bioneer, Daejeon, South Korea), which almost amplify at

Table1: show the primer used in the study according there reference:

Primers	Primer sequence (5- to 3-)		Product size (bp)	Annaling	References
Sar1	F1	GCACTTGATGAATTCTGG	615	52	(Ufuk *et al*., 2018
	R1	CACCACCCATAGAATCAAG			
Sar2	F2	AACCGTGGTAATTCTATGGCTAA	574	56	NCBI primer Blast
	R2	TGCCAGAATTCATCAAGTGC			

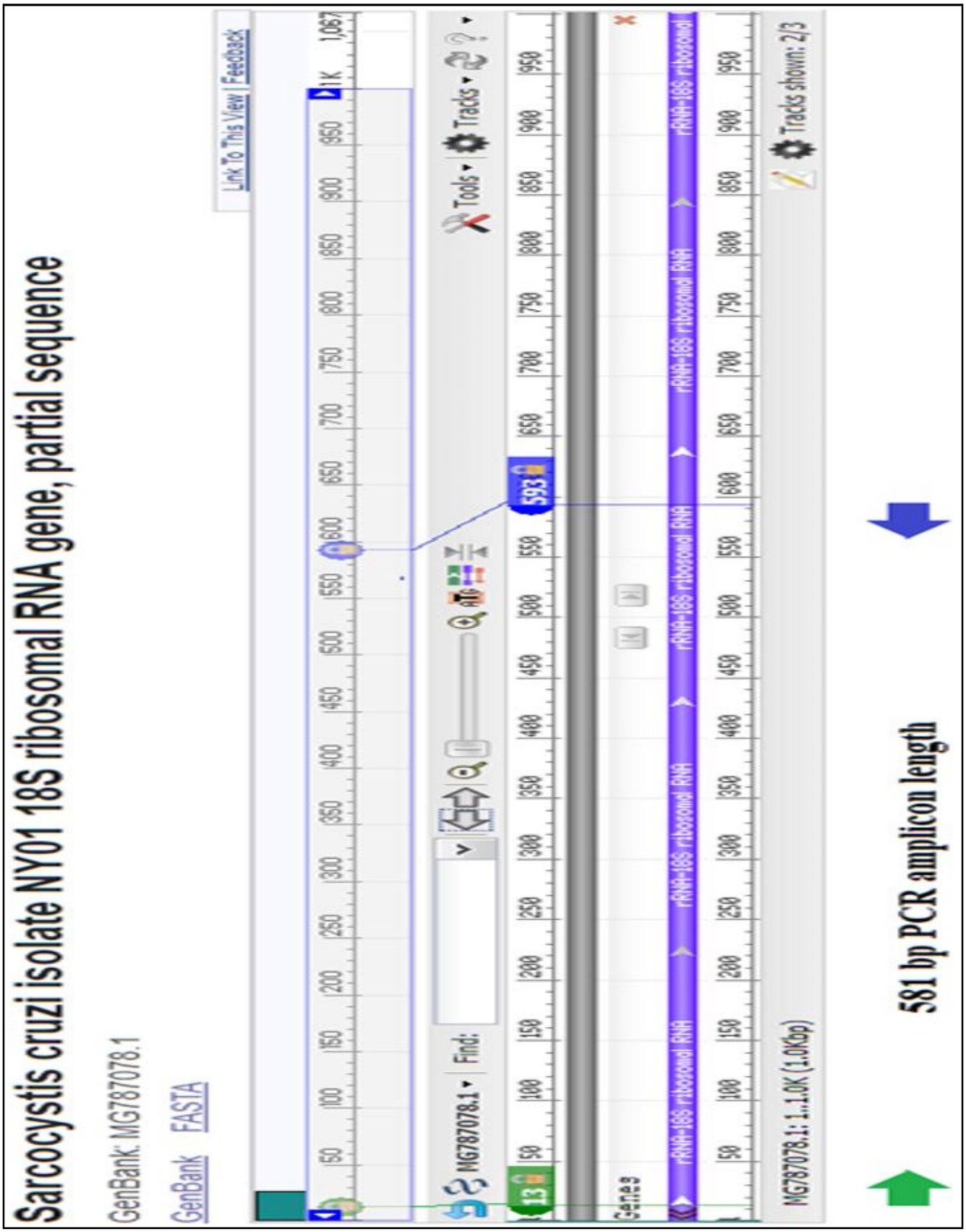

Fig. 4: The exact position of the retrieved 581 bp amplicon that entirely covered a portion of the *18S rRNA* locus within the *Sarcocystis cruzi* DNA genomic sequences (acc no. MG787078.1). The green arrow refers to the starting point of this amplicon while the cyan arrow refers to its end point.

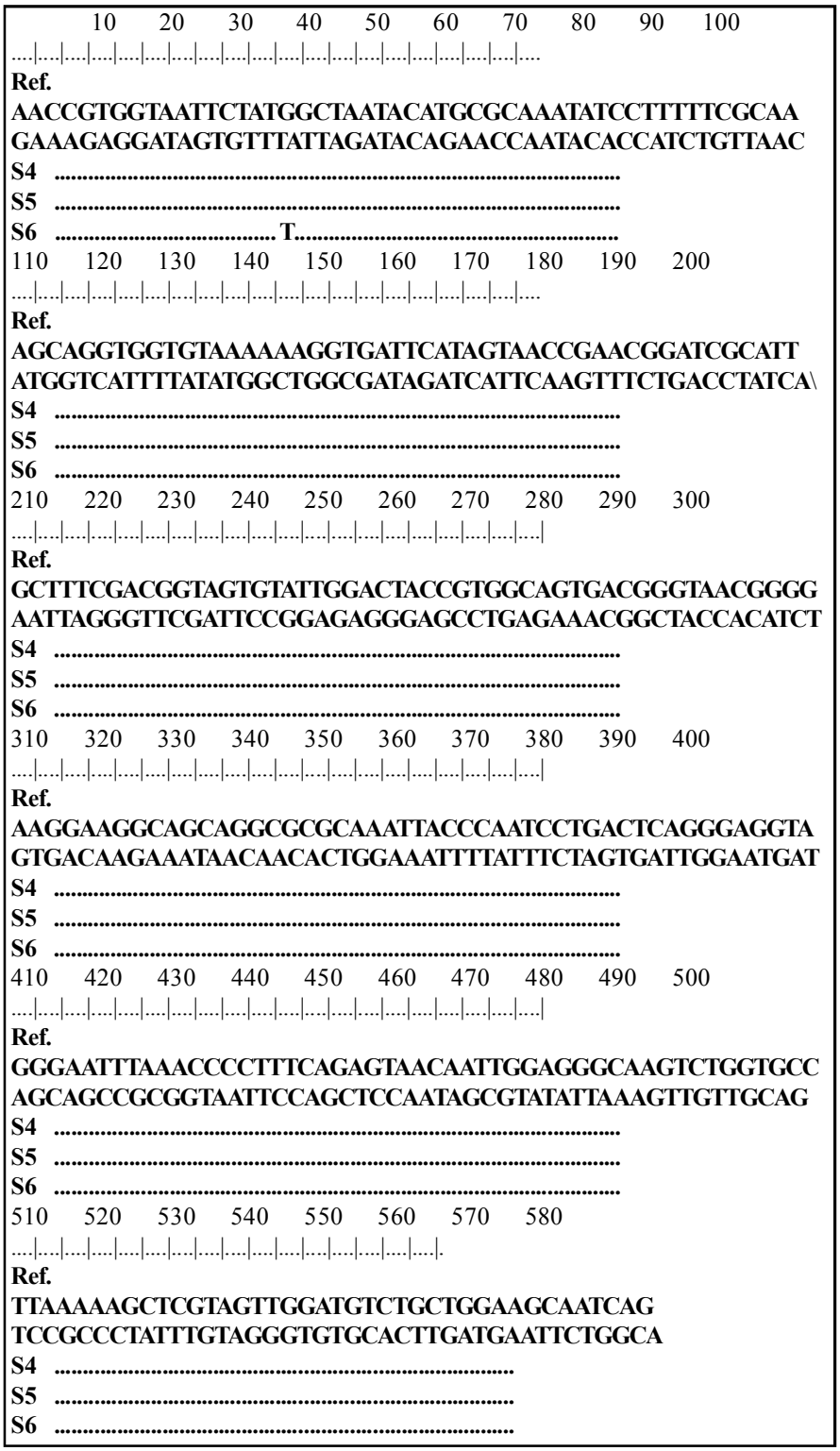

Fig. 5: DNA sequences alignment of 3 local protozan samples with their corresponding reference sequences of the 581 bp amplicons of the *18S rRNA* locus within the genomic DNA sequences of *Sarcocystis cruzi*. The symbol "ref" refers to the NCBI reference sequences, while S4, S5 and S6 refer to the sample No. 4, 5 and 6 respectively.

615 bp segment (Sar1) (Ufuk *et al*., 2018). Another primer (Sar2) amplify at 574 bp using the primer BLAST tool on the NCBI of the Genbank.These primers were prepared according to the information of the company Table 1.

Components of 20 PCR Master Mix Reaction for 18s rRNA gene were 10 ìl of Master Mix, 2 µl of Primer, 3 µl of dionized water, 5 µl of DNA template. Initial denaturation of Sar1 at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec., annealing at 52°C for 30 sec and extension at 72°C for 30 sec and then final extension at 72°C for 5 min. The Sar2 was the same program of Sar1 with exception of the Annealing temperature was 56°C. The amplification reactions were carried out in PCR Thermocycler (Bioneer / Korea), electrophoresed on 1.5% agarose gel,stained with ethidium bromide, examined using a UV transilluminator and photographed.

Sequence and genotyping of Sarcocystis Isolate from Iraq

The resolved PCR amplicons were commercially sequenced from termini, forward and reverse, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from Applied Biosystem extension (ABI) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local samples with the retrieved DNA sequences of *Sarcocystis spp.*, the virtual positions and other details of the retrieved PCR fragments were identified. The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using Bio Edit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA).

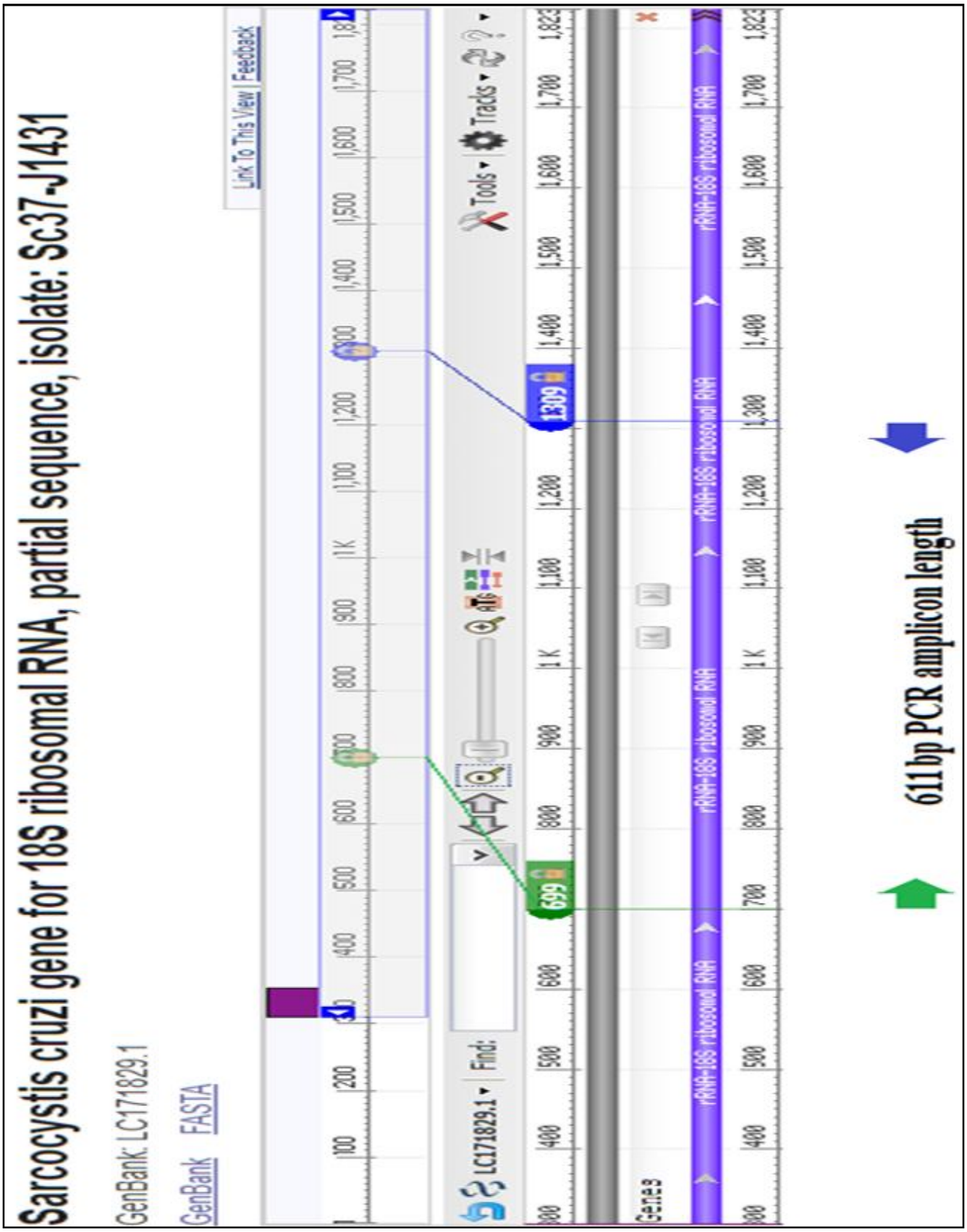

Fig. 6: The exact position of the retrieved 611 bp amplicon that entirely covered a portion of the *18S rRNA* locus within the *Sarcocystis cruzi* DNA genomic sequences (access no. KR186121.1). The green arrow refers to the starting point of this amplicon while the cyan arrow refers to its endpoint.

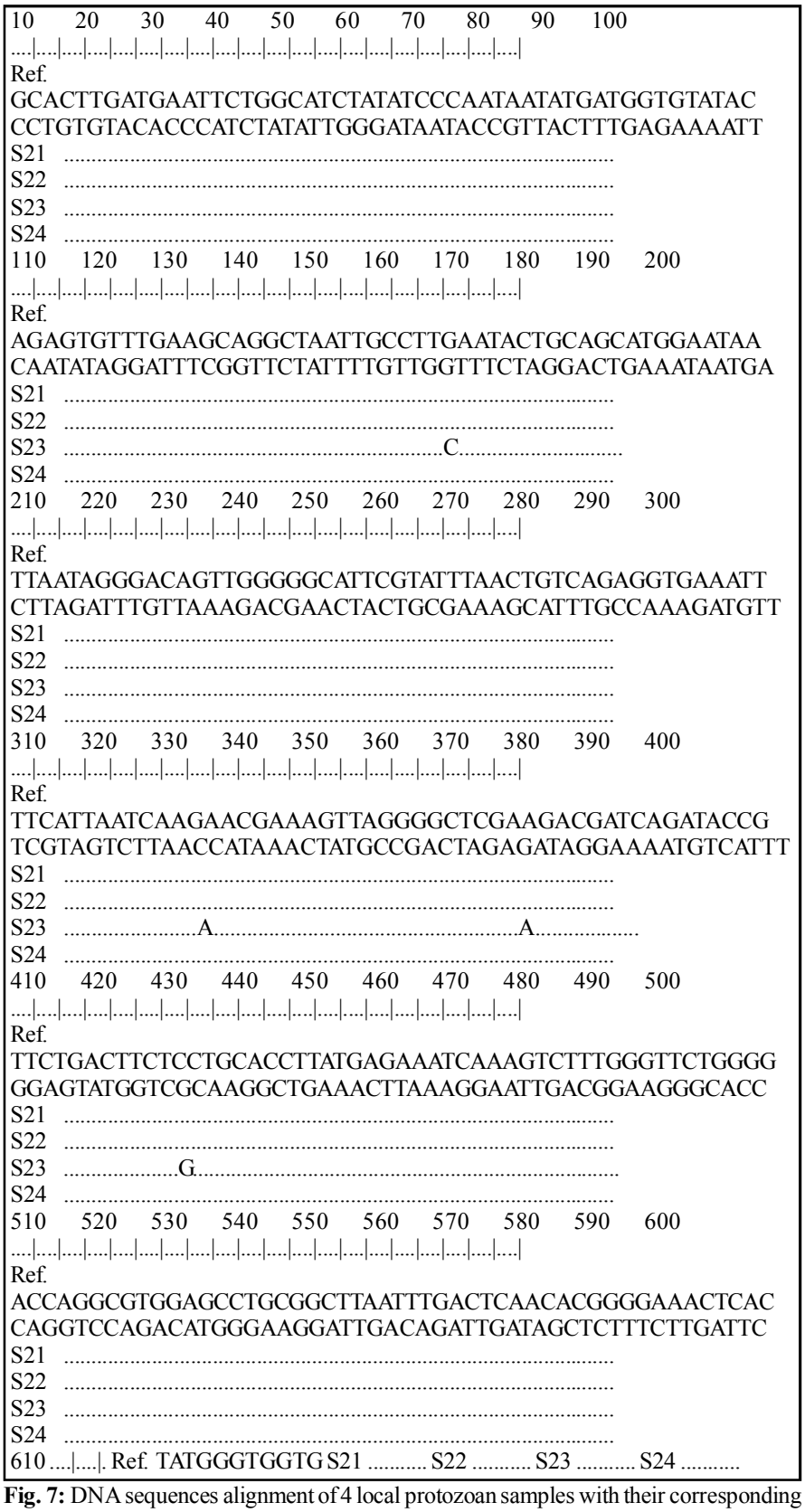

Fig. 7: DNA sequences alignment of 4 local protozoan samples with their corresponding reference sequences of the 611 bp amplicons of the *18S rRNA* locus within the genomic DNA sequences of *Sarcocystis cruzi*. The symbol "ref" refers to the NCBI reference sequences, while S21 – S24, refer to the sample No. 21 - 24 respectively.

Result and Discussion

After DNA extracted from 200 organ sample (esophagus), which were checked by using Nanodrop spectrophotometer (ActGene USA). The concentration of DNA was measured between (98-100) ng/µl and the purity of DNA through reading the absorbance at wavelength (260 /280 nm) was 1.6 - 1.8 DNA extraction of *Sarcocystis* which appeared as compact bands (Fig. 1).

The PCR results revealed that 4 samples found positive for *S.cruzi* with (611 bp) using first primer and 10 samples found positive for *S.cruzi* with (571 bp) using second primer as demonstrated in (Figure 2,3). Results indicated that molecular method could detected DNA of *Sarcocystis* spp. subspecies present and it an accurate technique. Another previous molecular biological assay studies improved many genetic parasites detection (Hamidinejat *et al*., 2015; Akhlaghi *et al*., 2016; El-kady *et al*., 2018, Januskevicius *et al*., 2019).

Sequencing Analysis

Two PCR fragment was selected for amplification, which supposed to partially cover 574 bp and 611 fragment that indented to partially amplify *18S rRNA* locus within *Sarcocystis spp.* genomic DNA sequences. The sequence analysis revealed that the partial sequences of the 18S rRNA gene of Sarcocystis cysts isolated from slaughtered cattle were identical with the published data of MG787078.1, MN096333.1 and LC171829.1 to 98 -100%.

The resolved PCR amplicons were commercially sequenced from termini, forward and reverse, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South

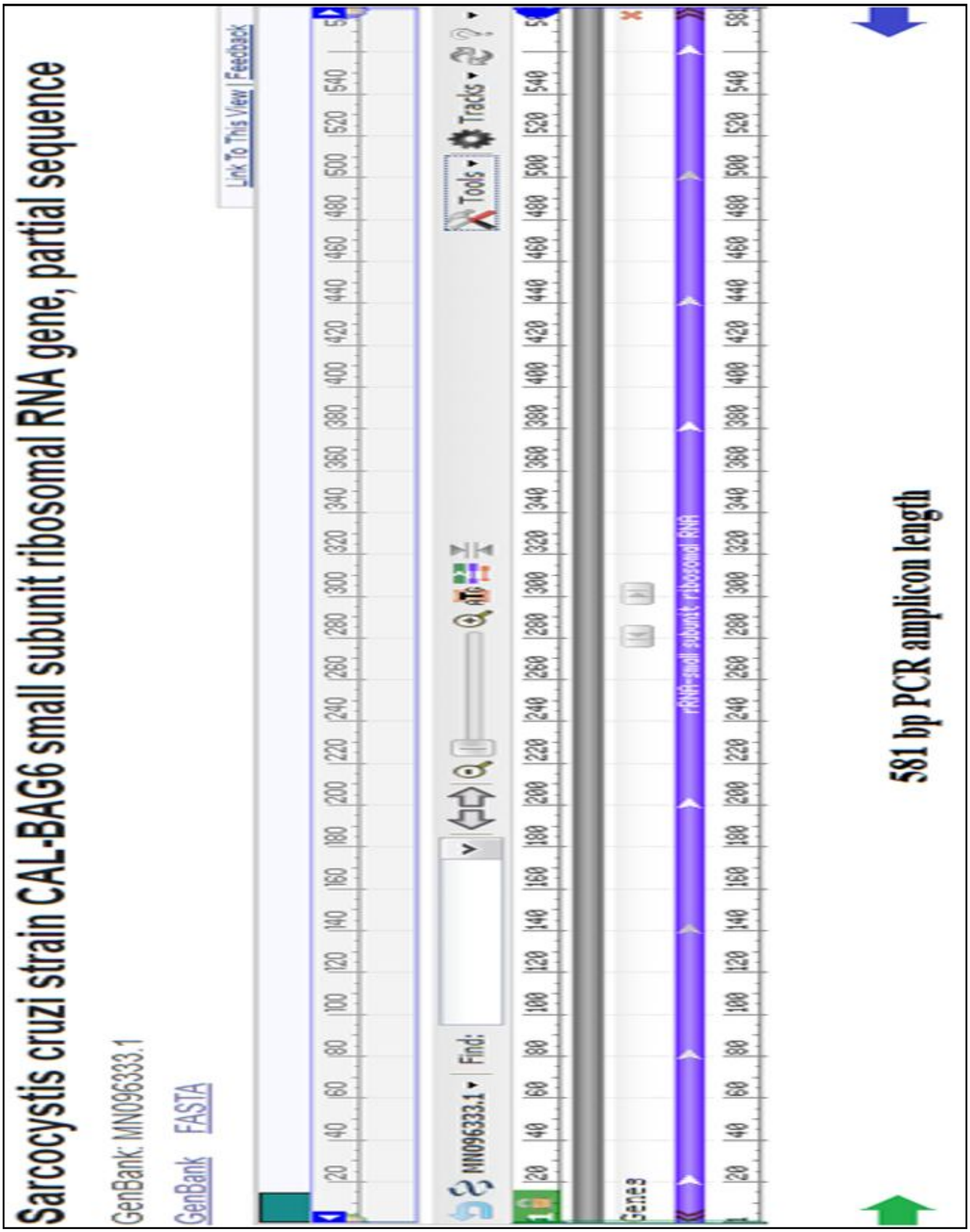

Fig. 8: The exact position of the retrieved 581 bp amplicon that entirely covered a portion of the *18S rRNA* locus within the *Sarcocystis cruzi* DNA genomic sequences (access no. MN096332.1). The green arrow refers to the starting point of this amplicon while the cyan arrow refers to its endpoint.

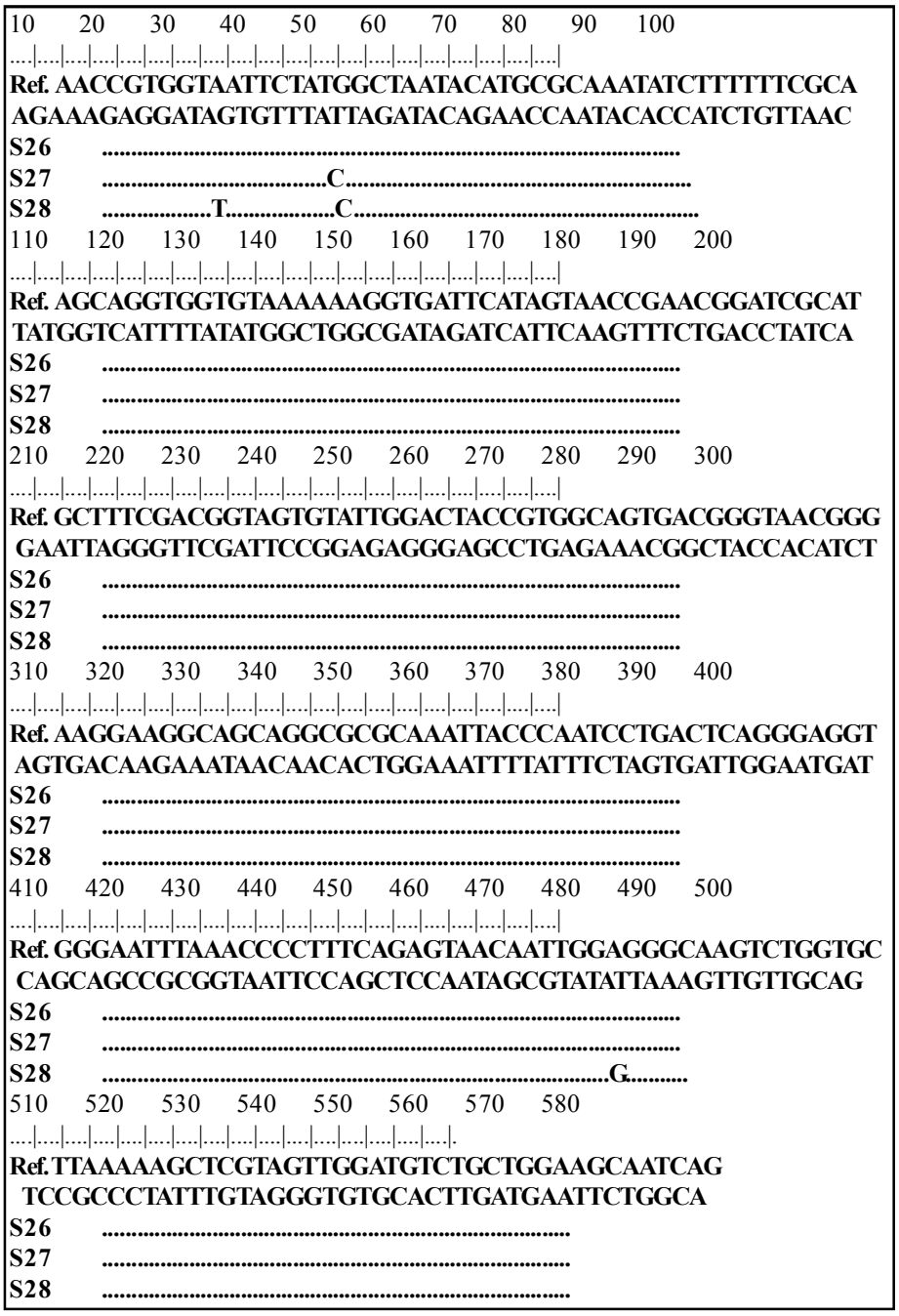

Fig. 9: DNA sequences alignment of three local protozoan samples with their corresponding reference sequences of the 581 bp amplicons of the *18S rRNA* locus within the genomic DNA sequences of *Sarcocystis cruzi*. The symbol "ref" refers to the NCBI reference sequences, while S26 - S28 refers to the sample No. 26 – 28 respectively.

Korea). Only clear chromatographs obtained from ABI. This extension is concerned with the DNA peaks, green, black, blue, and red for T, G, C and T respectively) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local samples with the retrieved DNA sequences of *Sarcocystis spp.*, the virtual positions and other details of the retrieved PCR fragments were identified in (Table 2) for slaughtered cattle.

Sequencing of the *18S rRNA* gene for samples S4, S5, and S6

With regard to the investigated samples S4, S5, and S6, the sequencing reactions indicated the exact positions after performing NCBI blast n for these PCR amplicons (Zhang *et al*., 2000). NCBI BLAST n engine has shown about 98% to 100% sequences of similarities between the sequenced samples and this target. NCBI BLASTn engine has indicated the presence of remarkable of homology with the expected target that covered a portion of the *18S rRNA* within *Sarcocystis cruzi* genomic DNA sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank access. MG957194.1), the exact positions and other details of the retrieved PCR fragment were identified in (Fig. 4).

The alignment results of the 581 bp samples revealed the presence of one mutation in only S6 out of the other analyzed samples in comparison with the referring *18S rRNA* genetic sequences, namely C40T (Fig. 5).

Sequencing of the *18S rRNA* gene for samples S21 – S24

With regard to the investigated samples S21 – S24 samples, the sequencing reactions indicated the exact positions after performing NCBI blastn for these PCR amplicons (Zhang *et al*., 2000). NCBI BLASTn engine has shown about 98% to 100% sequences of similarities between the sequenced samples and this target. NCBI BLASTn engine has indicated the presence of remarkable of homology with the expected target that covered a portion of the *18S rRNA* within *Sarcocystis cruzi* genomic DNA sequences. By comparing the observed DNA sequences of these

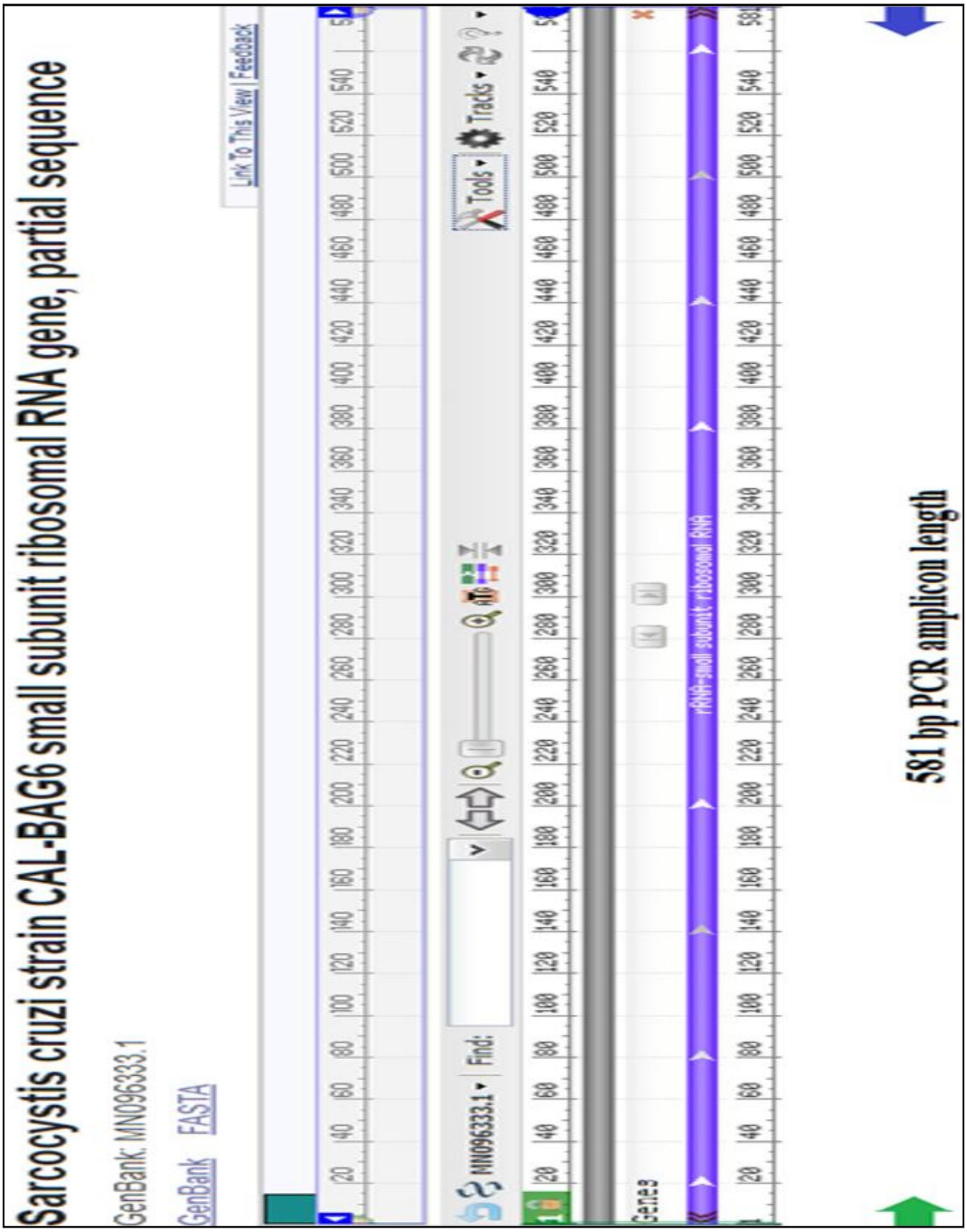

Fig. 10: The exact position of the retrieved 581 bp amplicon that entirely covered a portion of the *18S rRNA* locus within the *Sarcocystis cruzi* DNA genomic sequences (MN096332.1). The green arrow refers to the starting point of this amplicon while the cyan arrow refers to its endpoint.

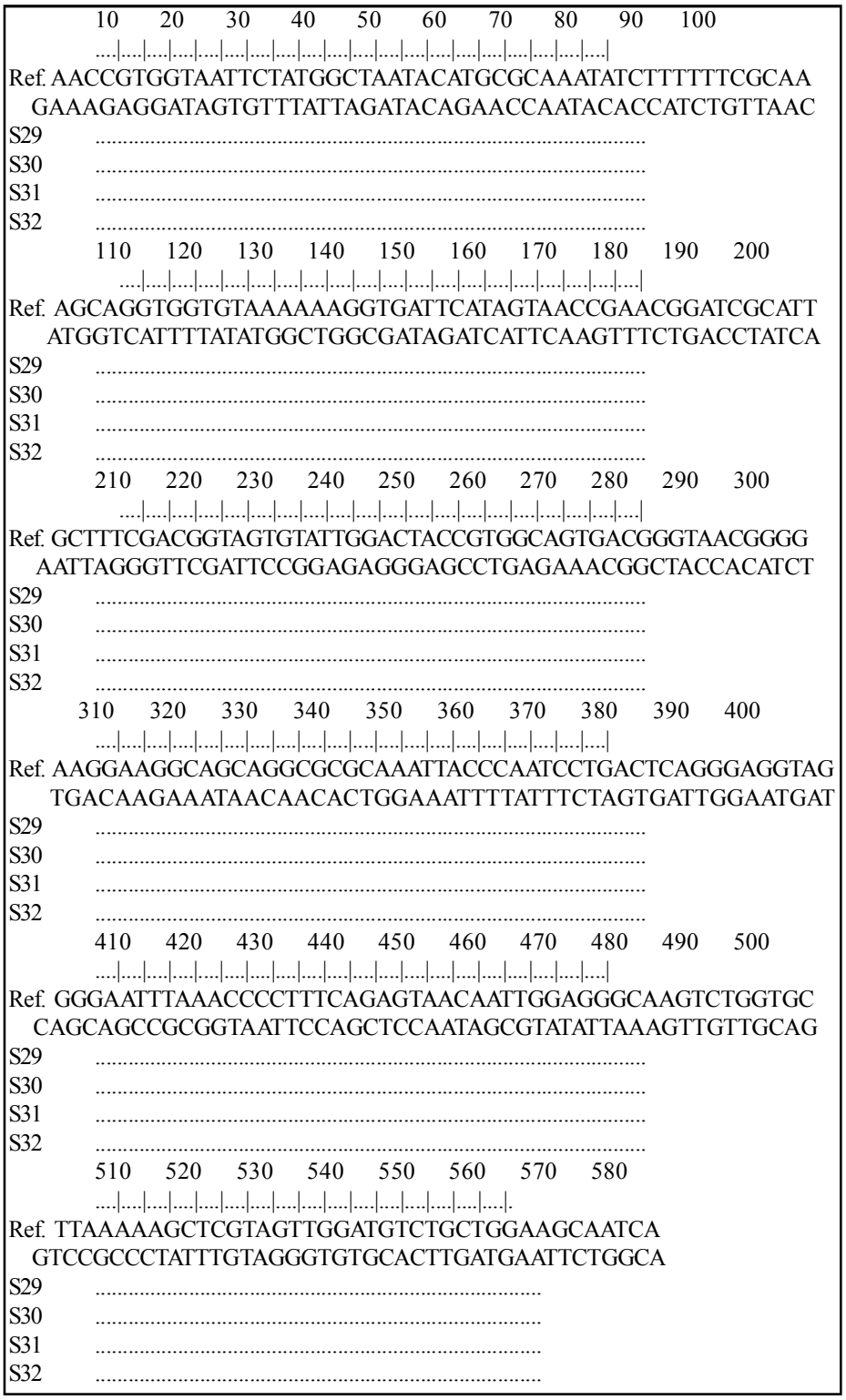

Fig. 11: DNA sequences alignment of four local protozoan samples with their corresponding reference sequences of the 581 bp amplicons of the *18S rRNA* locus within the genomic DNA sequences of *Sarcocystis cruzi*. The symbol "ref" refers to the NCBI reference sequences, while S29 - S32 refers to the sample No. 29 – 32 respectively.

local samples with the retrieved DNA sequences (GenBank acc. LC171829.1), the exact positions and other details of the retrieved PCR fragment were identified (Fig. 6).

The alignment results of the 611 bp samples revealed the presence of three mutations in some of the analyzed samples in comparison with the referring *18S rRNA* genetic sequences. These 3 mutations were taken a different distribution in the analyzed samples. S23 was shown all of these substitution mutations, while other samples, S21, S22 and S24, had not exhibited any mutation in comparison with their corresponding reference sequences of *S. cruzi* (Fig. 7).

Sequencing the *18S rRNA* gene for samples S26-S28

With regard to the investigated samples S26 – S28, the sequencing reactions indicated the exact positions after performing NCBI blast for these PCR amplicons. NCBI BLAST n engine has shown about 98 – 100 % sequences of similarities between the sequenced samples and this target. NCBI BLAST n engine has indicated the presence of remarkable of homology with the expected target that covered a portion of the *18S rRNA* within *S. cruzi* genomic DNA sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (Gen Bank acc. MN096332.1), the exact positions and other details of the retrieved PCR fragment were identified (Fig. 8).

The alignment results of the 581 bp samples revealed the presence of three mutations in S26-S28 sample in comparison with the referring *18S rRNA* genetic sequences. These 3 mutations were distributed in two samples, including S27 and S28, while S26 had not shown any detectable mutation (Fig. 9).

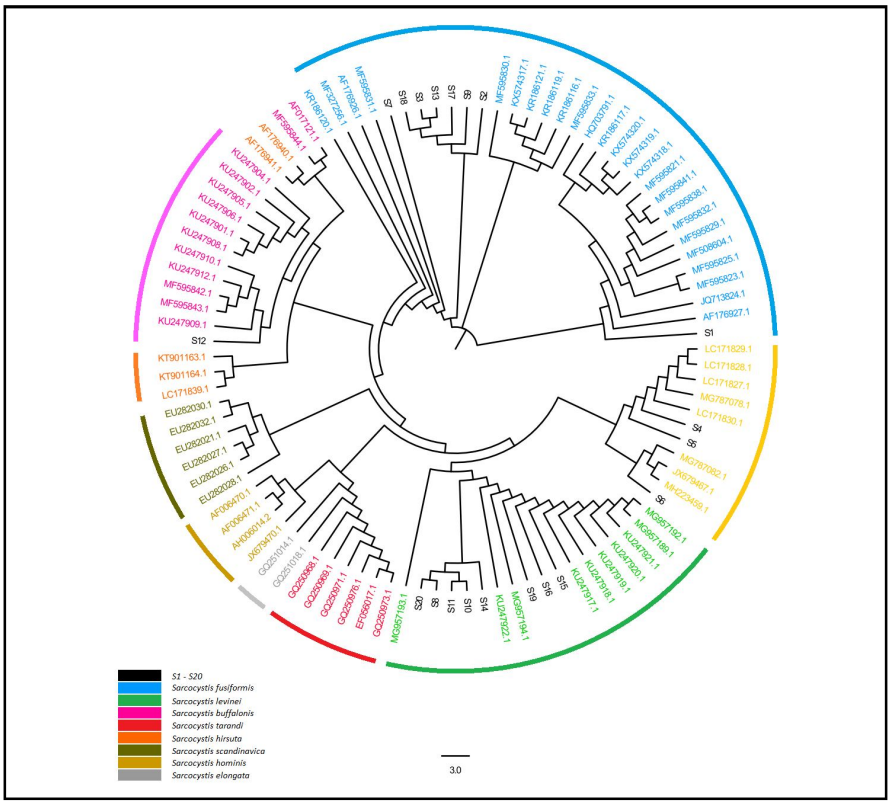

Fig. 12: The comprehensive phylogenetic tree of genetic variants of *18S rRNA* gene fragment of *Sarcocystis* local isolate.

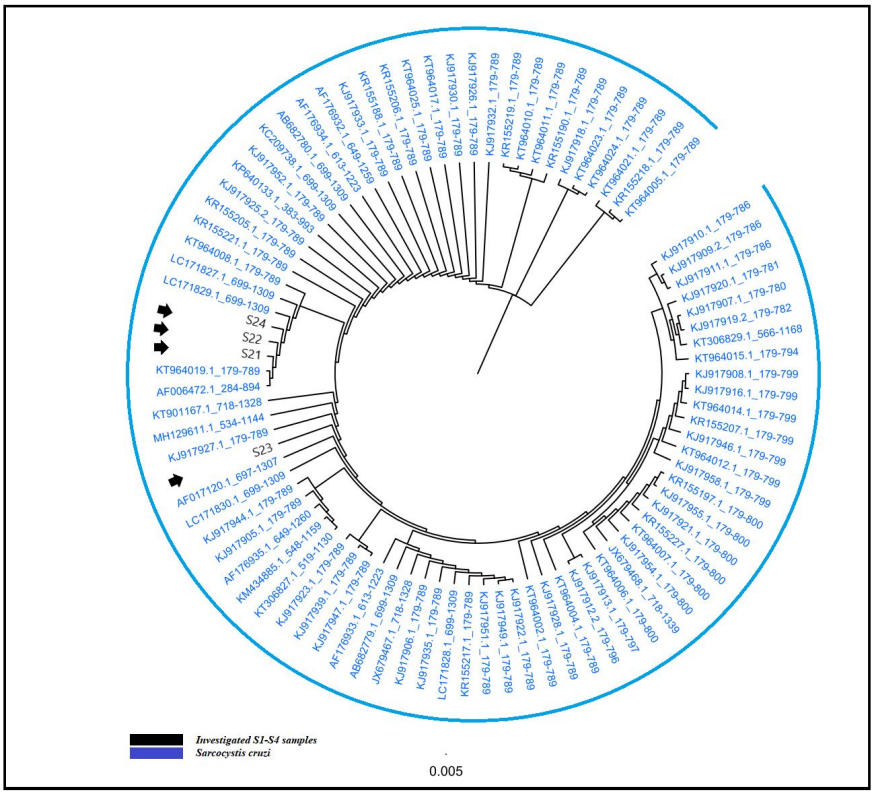

Fig. 13: The comprehensive phylogenetic tree of genetic variants of *18S rRNA* gene fragment of four *Sarcocystis cruzi* local isolate. The black color refers to the sequenced *S21 – S24* variants, while the cyan color refers to the related referring NCBI *Sarcocystis cruzi* deposited species. All the mentioned numbers referred to Genbank acc. no. of each referring species. The number "0.005" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

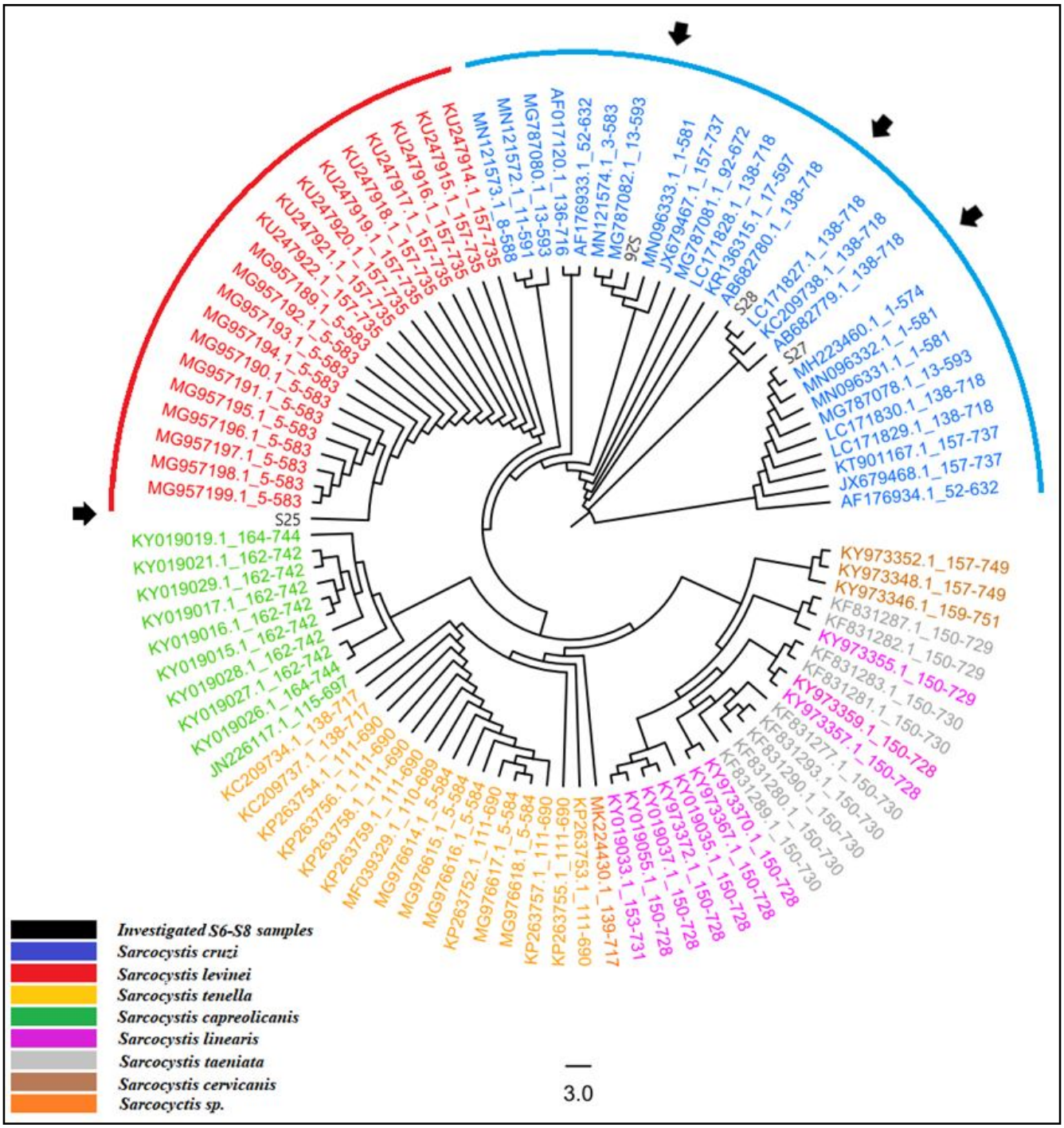

Fig. 14: The comprehensive phylogenetic tree of genetic variants of *18S rRNA* gene fragment of four *Sarcocystis* local isolate. The black color refers to the sequenced *S26 – S28* variants, while the other colors refer to the related referring NCBI *Sarcocystis* deposited species. All the mentioned numbers referred to Genbank acc. no. of each referring species. The number "3.0" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

Sequencing of the *18S rRNA* gene for samples S29-S32

With regard to the investigated samples S29 – S32, the sequencing reactions indicated the exact positions after performing NCBI blastn for these PCR amplicons. NCBI BLASTn engine has shown about 100% sequences of similarities between the sequenced samples and this target. NCBI BLAST n engine has indicated the presence of remarkable homology with the expected target that covered a portion of the *18S rRNA* within *S. cruzi*

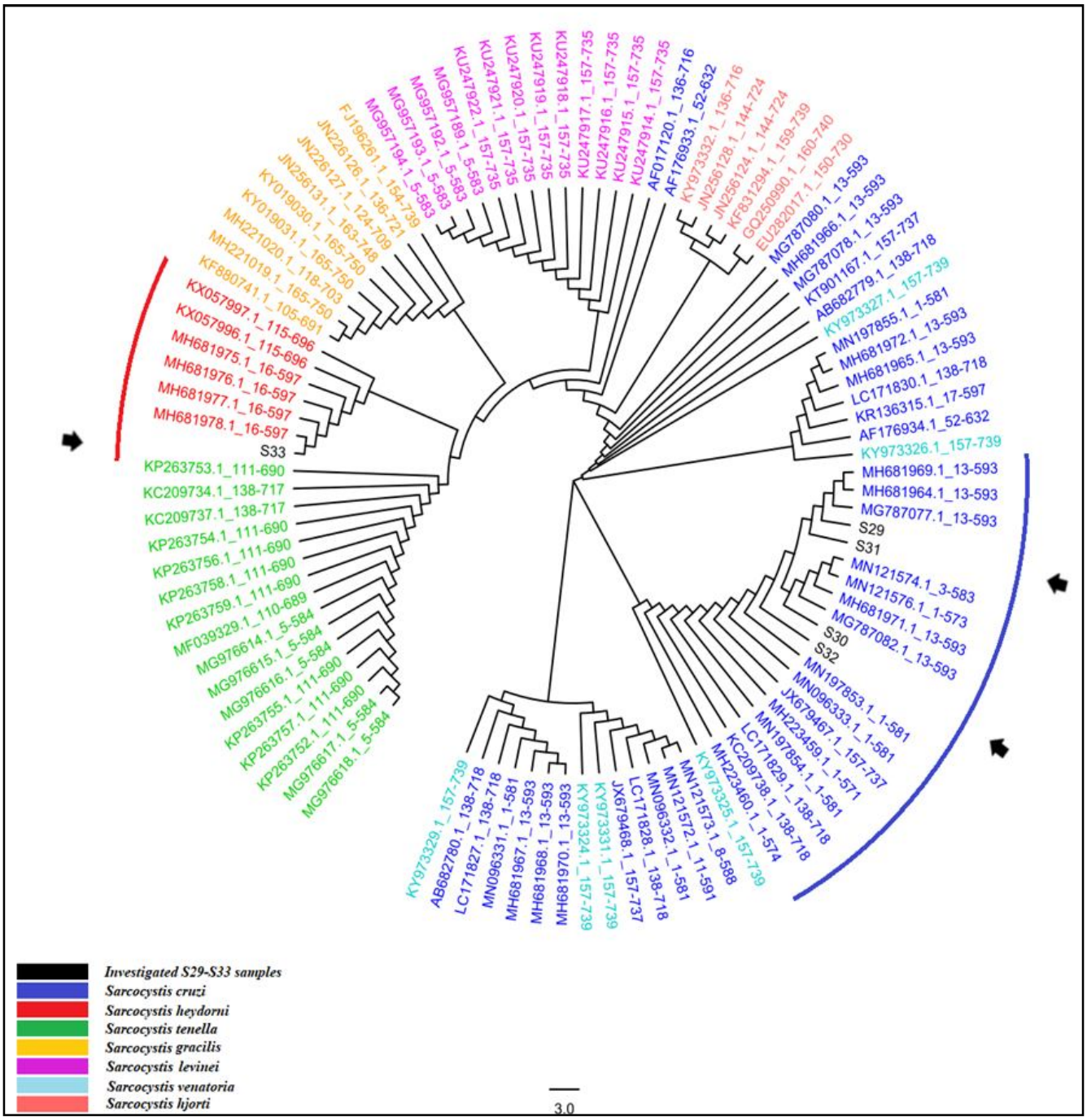

Fig. 15: The comprehensive phylogenetic tree of genetic variants of the *18S rRNA* gene fragment of five *Sarcocystis* local isolate. The black color refers to the sequenced *S29 – S33* variants, while other colors refer to the related referring NCBI *Sarcocystis* deposited species. All the mentioned numbers referred to Genbank acc. no. of each referring species. The number "3.0" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

genomic DNA sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank access MN096332.1), the exact positions and other details of the retrieved PCR fragment were identified (Fig. 10).

The alignment results of the 581 bp samples revealed the absence of any mutation in S29-S32 samples in comparison with the referring *18S rRNA* genetic sequences (Fig. 11).

DNA sequence and phylogenetic analysis are considered the main important approach to identity the species of *Sarcocystis spp*. infection and to compared it

with other *Sarcocystis spp* in Gene Bank which gives an idea about the new strain of Iraqi *Sarcocystis* spp. in Baghdad city in the slaughtered cattle. The phylogenic tree were clustered with the *S. cruzi*. With regard to either close positioning was detected beside two Japanese strains, Romanian ROBT11 strain, Malaysian *S. cruzi* strain, Japanese *S. cruzi* strain, Chinese *S. cruzi* strains, Indian strain and Australian *S. cruzi* (Fig. 12, 13, 14, 15).

References

Akhlaghi, M., M. Razavi and A. Hosseini (2016). Molecular differentiation of bovine sarcocysts. *Parasitol Res.,* **115:** 2721-8.

Badree, M. (2009). Seroprevalence of antibodies against Sarcocystis gigantea in different hosts in Ninevah governorate. *J. Iraqi veterinary science,* **23:** 107-112.

Dubey, J.P., E. Wilpe, R. Calero-Bernal and S.K. Verma (2015). *Sarcocystis heydorni*, (Apicomplexa: Sarcocystidae) with cattle (Bos taurus) and human (Homo sapiens) cycle. *Parasitology Research,* **114:** 4143–4147.

El-kady, A., N. Hussein and A. Hassan (2018). First molecular characterization of Sarcocystis spp. in cattle in Qena Governorate, Upper Egypt. *J. Parasit Dis.,* **42:** 114–121.

Fayer R. (2004). Sarcocystis spp. in human infections. *Clin. Microbiol. Rev.,* **17:** 894-902.

Gjerde, B. (2016). Molecular characterisation of Sarcocystis bovifelis, Sarcocystis bovini n. sp., *Sarcocystis hirsuta* and *Sarcocystis cruzi* from cattle (Bos taurus) and *Sarcocystis sinensis* from water buffaloes (Bubalus bubalis). *Parasitol Res.,* **115:** 1473-92.

Hamidinejat, H., M.H.R. Jalali, D. Gharibi and P.H. Molayan (2015). Detection of *Sarcocystis spp*. in cattle (Bos taurus) and water buffaloes (Bubalus bubalis) in Iran by PCR–RFLP. *J. Parasit. Dis.,* **39:** 658–662.

Hornok, S., A. Mester, N. Takacs, F. Baska, G. Majoros, E. Fok, I. Biksi, Z. Nemet, A. Hornyak, S. Janosi and R. Farkas (2015). *Sarcocystis* infection of cattle in Hungary. *Parasites Vectors,* **8:** 8-69.

Januskevicius, V., G. Januskeviciene, P. Prakas, D. Butkauskas and I.S. Petkevicius (2019). Prevalence and intensity of Sarcocystis spp. infection in animals slaughtered for food in Lithuania. *Veterinarni Medicina.,* **64 :** 149–157.

Jehle, C., A. Dinkel, A. Sander, M. Morent, T. Romig, P.C. Luc, T.V. De, V.V. Thai and U. Mackenstedt (2009). Diagnosis of Sarcocystis spp. in cattle (Bos taurus) and water buffalo (Bubalus bubalis) in Northern Vietnam. *Vet Parasitol.,* **23:** 314-320.

Mohamed, M., M. Fatma, A. Hiekal, M. Samia, M. Marwa, M. Radwan and S.H.A. (2016). Characterization of *Sarcocystis* Species Based on Traditional and Molecular Methods in imported Frozen Buffalo Meat in Egypt. *Alexandria Journal of Veterinary Sciences,* October **51(1):** 155-161.

More, G., P. Abrahamovich, S. Jurado, D. Bacigalupe, J.C. Marin, M. Rambeaud, L. Venturini and M.C. Venturini (2011). Prevalence of Sarcocystis spp. in Argentinean cattle. *Vet. Parasito.,* **177:** 162-165.

Ufuk, K., Z.M. Arslan, G. Glbaz, G. Taþkýn and A. Aka (2018). Identification of Sarcocystis spp. by polymerase chain reaction and microscopic examination in various beef products (minced meat, meatballs, fermented sausage). *Turk. J. Vet. Anim. Sci.,* **42:** 1-6.

Zhang, Z., S. Schwartz, L. Wagner and W.A. Miller (2000). Greedy algorithm for aligning DNA sequences. *J. Comput. Biol.,* **7:** 203-14.