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Comparative Phylogenetic Perspectives on the Evolutionary Relationships in the Brine Shrimp *Artemia* Leach, 1819 (Crustacea: Anostraca) Based on Secondary Structure of *ITS1* Gene

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Abstract

This is the first study on phylogenetic relationships in the genus *Artemia* Leach, 1819 using the pattern and sequence of secondary structures of internal transcribed spacer 1 (*ITS1*). Significant intraspecific variation in the secondary structure of *ITS1* rRNA was found in *Artemia tibetiana*. In the phylogenetic tree based on joined primary and secondary structure sequences, *Artemia urmiana* and parthenogenetic populations displayed new lineages, and two New World species (*Artemia franciscana* and *Artemia persimilis*) were located in a basal clade that was not detected in previous studies. The close evolutionary relationship between *A. franciscana* and *A. persimilis* are expressively supported by the previous empirical and experimental investigation on the ability of hybridization (in natural habitats and lab conditions) and analysis on allozyme markers.

Keywords: Phylogenetic; Primary sequence; Secondary structures; Internal transcribed spacer 1; *Artemia*

Introduction

Phylogenetics is the study of evolutionary history and relationships of biological taxa using mostly morphological, genetic and molecular characters. Sometimes the results due to different phylogenetic methods are paradoxical. The genus *Artemia* leach, 1819 (Crustacea: Anostraca) is one taxon representing this kind of paradox. *Artemia* includes three bisexual species in the New World (*Artemia franciscana* Kellogg, 1906, *Artemia persimilis* Piccinelli & Prosdocimi, 1968 and *Artemia monica* Verrill, 1869), four bisexual species in the old world (*Artemia salina* (Linnaeus, 1758), *Artemia urmiana* Günther, 1899, *Artemia sinica* Cai, 1989 and *Artemia tibetiana* Abatzopoulos *et al.*, 1998) (Asem *et al.*, 2010), and a large number of parthenogenetic populations including di-, tri-, tetra-, penta- and also heteroploids or even mixtures of different ploidies (Sun *et al.*, 1999; Abatzopoulos *et al.*, 2002a,b; Amat *et al.*, 2007; Zheng and Sun 2013). Although recent analyses based on mitochondrial DNA data confirmed that Asian bisexual species had a common ancestor (Maniatsi *et al.*, 2011; Asem *et al.*, 2016), a previous morphological study demonstrated that *A. urmiana* significantly distinguished from the other Asian species as well as the Mediterranean *A. salina* and the American *A. franciscana* (Triantaphyllidis *et al.*, 1997). Baxevanis *et al.* (2005) claimed there was no consistency between results of genetic distance and morphometric characters of bisexual *Artemia*. They proved that *A. urmiana* and *A. tibetiana* were genetically close but significantly dissimilar in the morphology, while *A. urmiana* and *A. persimilis* had obvious genetic differentiation but were close in morphometric patterns. On the other hand, different genetic methods also showed inconsistent results for evolutionary relationships of genus *Artemia*. Analysis using the sequence of the nuclear internal transcribed spacer 1 (*ITS1*) region confirmed that *A.*

persimilis formed a distinct clade and was well differentiated from the others, and *A. franciscana* was placed as a clade sister to Asian bisexuals and parthenogenetics. (Baxevanis *et al.*, 2006; Hou *et al.*, 2006; Kappas *et al.*, 2009; Vikas *et al.*, 2012; Eimanifar *et al.*, 2014). According to the results of *16S* rDNA RFLP analyses (Unrooted NJ), *A. franciscana* located in a cluster, and *A. salina* + *A. persimilis* and three Asian bisexual species in two others separately (Baxevanis *et al.*, 2005). Maniatsi *et al.* (2011) confirmed that *COI* (Cytochrome *c* Oxidase subunit I) data displayed the similar result with *ITS1* data. Moreover, the analysis on *COI* sequences of 541 individuals showed that the Mediterranean *A. salina*, rather than the South American *A. persimilis*, was placed in a separate phylogenetic clade (Eimanifar *et al.*, 2014). These consequences indicate that systematics of *Artemia* is still puzzling and therefore a comprehensive review is needed.

In the past decade, several studies have demonstrated the application of nuclear rRNA secondary structure models (mostly *SSU-rRNA*, *ITS1* and *ITS2*) could clarify the evolutionary history of taxa (Gottschling & Plotner 2004; Campbell *et al.*, 2005; Sun *et al.*, 2010; Reblova *et al*., 2013; Yosefzadeh *et al*., 2012; Coleman, 2013; Hodac *et al.*, 2014; Wang *et al*., 2015; Hosseinzadeh Colagar *et al.*, 2016). For example, Wang *et al.* (2015) proved that using the sequence of secondary structure of *SSU-rRNA* gene could give different information than its primary sequence to better understand phylogenetic relationships among members of family Pseudokeronopsidae in the ciliates.

In this study, the secondary structures of the first partial *ITS1* region of bisexual/parthenogenetic *Artemia* are predicted and compared. Phylogenetic trees are constructed based on the primary and primary+ secondary sequences. The aims of this study are to model *ITS1* secondary structures and examine the contribution of secondary sequence in understanding the evolutionary relationships in the genus *Artemia*.

Materials and Methods

Taxa and sequences

Sequences of the internal transcribed spacer 1 (*ITS1*) region were downloaded entirely from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Our dataset contained 313 *ITS1* sequences including seven bisexual species and parthenogenetic populations with different ploidy degrees (i.e. di-, tri-, tetra- and pentaploidy) (Table 1). Sequences were aligned using Muscle in MEGA ver. 6.00 with default parameters (Tamura *et al*., 2013). The total sequences (including 111 haplotypes) were collapsed by DNAsp ver. 5.00 (Librado and Rozas, 2009). *Streptocephalus proboscideus* (AY519840) was used as an outgroup (Baxevanis *et al.*, 2006; Eimanifar *et al.*, 2014).

The first partial *ITS1* region which ranged from 294 bp to 340 bp (started with a conserved sequence of GTTT and stopped with TCKC) was chosen for secondary structure analysis followed by secondary structure model for *ITS1* suggested by Gottschling and Plötner (2004), using ΔG minimization, similarity and constraint folding (Mathews *et al.*, 1999; Reuter and Mathews, 2010) using mfold onlen software (Zuker, 2003). Additionally, tree topology from the primary sequence of this part was the same as that from the whole *ITS1* sequence (for more information see results and discussion sections).

Secondary structure prediction

The secondary structures were predicted for each haplotype with respect to same shapes for conserved parts between species/populations and minimum free-energy optimization (Zuker, 1989, Hofacker *et al.*, 2002) using the mfold web server (http://unafold.rna.albany.edu/?q= mfold/RNA-Folding-Form) (Zuker, 2003). The structures were aligned and further edited by 4SALE ver. 1.7 (Seibel *et al.*, 2006). The sequence alignments were manually edited via comparison between primary and secondary positions to find the best homogeneous arrangements. 4SALE ver. 1.7 (Seibel *et al.*, 2006) was used to draw the general patterns of secondary structure based on the results of conservation rates. The lengths of helices and single strands and the number of paired nucleotides were counted for each general pattern (Table 2).

Table 1. Sampling information of *Artemia* specimens/sequences (all downloaded from GenBank) used in the present study.

Species/Population	Abbreviation	Haplotype names	Accession numbers	References
Artemia urmiana	URM	URM1	DQ201275	Baxevanis *et al.,* 2006
		URM2	DQ201276	Baxevanis *et al.,* 2006
		URM3	DQ201277	Baxevanis *et al.,* 2006
		URM4	KF736251	Maccari *et al.,* 2013
		URM5	KF736252	Maccari *et al.,* 2013
		URM6	KF703810-15	Eimanifar *et al.,* 2014
		URM6	KF703820	Eimanifar *et al.,* 2014
		URM6	KF703822-23	Eimanifar *et al.,* 2014
		URM7	KF703816	Eimanifar *et al.,* 2014
		URM8	KF703817	Eimanifar *et al.,* 2014
		URM9	KF703818	Eimanifar *et al.,* 2014
		URM10	KF703819	Eimanifar *et al.,* 2014
		URM11	KF703821	Eimanifar *et al.,* 2014
		URM12	KF703824	Eimanifar *et al.,* 2014
		URM13*	DQ069926	Hou *et al.,* 2006
		URM13*	DQ084193	Hou *et al.,* 2006
		URM13*	KF736249-50	Maccari *et al.,* 2013
Artemia tibetiana	TIB	TIB1	DQ201269-70	Baxevanis *et al.,* 2006
		TIB2	KF736290-95	Maccari *et al.,* 2013
		TIB3	KF703778	Eimanifar *et al.,* 2014
		TIB4	KF703785	Eimanifar *et al.,* 2014
		TIB5	KF703798	Eimanifar *et al.,* 2014
Artemia sinica	SIN	SIN1	DQ069929	Hou *et al.,* 2006
		SIN1	DQ069930	Hou *et al.,* 2006
		SIN2	DQ069931	Hou *et al.,* 2006
		SIN3	DQ084196	Hou *et al.,* 2006
		SIN4	DQ084197	Hou *et al.,* 2006
		SIN5	DQ084198	Hou *et al.,* 2006
		SIN6	DQ201285	Baxevanis *et al.,* 2006
		SIN7	DQ201286	Baxevanis *et al.,* 2006
		SIN8	DQ201287	Baxevanis *et al.,* 2006
		SIN9	FJ004945	Kappas *et al.,* 2009
		SIN10	KF736296-97	Maccari *et al.,* 2013
		SIN11	KF703766	Eimanifar *et al.,* 2014
		SIN11	KF703790	Eimanifar *et al.,* 2014
		SIN12	KF703796	Eimanifar *et al.,* 2014
Artemia sp.	SP	SP1	DQ084194	Hou *et al.,* 2006
Artemia salina	SAL	SAL1	DQ201302	Baxevanis *et al.,* 2006
		SAL2	DQ201303	Baxevanis *et al.,* 2006
		SAL3	DQ201304	Baxevanis *et al.,* 2006
		SAL4	DQ201305	Baxevanis *et al.,* 2006
		SAL5	DQ201306	Baxevanis *et al.,* 2006
		SAL6	DQ201307	Baxevanis *et al.,* 2006
		SAL7	DQ201308	Baxevanis *et al.,* 2006
		SAL8	DQ201309	Baxevanis *et al.,* 2006
		SAL9	FJ004946	Kappas *et al.,* 2009
		SAL10	KF703762	Eimanifar *et al.,* 2014
Artemia persimilis	PER	PER1	DQ069925	Hou *et al.,* 2006
		PER2	DQ084192	Hou *et al.,* 2006
		PER3	DQ201263	Baxevanis *et al.,* 2006
		PER4	DQ201264	Baxevanis *et al.,* 2006
		PER5	DQ201265	Baxevanis *et al.,* 2006
		PER6	DQ201266	Baxevanis *et al.,* 2006
		PER7	DQ201267	Baxevanis *et al.,* 2006
		PER8	DQ201268	Baxevanis *et al.,* 2006
		PER9	FJ004922-23	Kappas *et al.,* 2009
		PER10	FJ004924	Kappas *et al.,* 2009
Artemia franciscana	FRA	FRA1	DQ069923	Hou *et al.,* 2006
		FRA1	DQ084190	Hou *et al.,* 2006
		FRA1	DQ201297	Baxevanis *et al.,* 2006
		FRA2	DQ069924	Hou *et al.,* 2006
		FRA2	FJ004935-36	Kappas *et al.,* 2009
		FRA2	FJ004938-39	Kappas *et al.,* 2009
		FRA2	FJ004941-42	Kappas *et al.,* 2009
		FRA2	GU252106	Maniatsi *et al.,* 2009
		FRA2	GU323291	Vikas *et al.,* 2012
		FRA2	GU323293-94	Vikas *et al.,* 2012
		FRA2	GU323296-97	Vikas *et al.,* 2012
		FRA2	GU323309-12	Vikas *et al.,* 2012
		FRA2	GU323314	Vikas *et al.,* 2012
		FRA2	GU323316	Vikas *et al.,* 2012
		FRA3	DQ084191	Hou *et al.,* 2006

Table 1. Continued

Species/Population	Abbreviation	Haplotype names	Accession numbers	References
Artemia franciscana	FRA	FRA3	DQ201298	Baxevanis *et al.,* 2006
		FRA4	DQ201289	Baxevanis *et al.,* 2006
		FRA4	DQ201291	Baxevanis *et al.,* 2006
		FRA4	DQ201295	Baxevanis *et al.,* 2006
		FRA4	FJ004933-34	Kappas *et al.,* 2009
		FRA4	GU252102-04	Maniatsi *et al.,* 2009
		FRA4	GU323298	Vikas *et al.,* 2012
		FRA4	GU323301-02	Vikas *et al.,* 2012
		FRA4	GU323304-06	Vikas *et al.,* 2012
		FRA4	GU323308	Vikas *et al.,* 2012
		FRA4	GU323315	Vikas *et al.,* 2012
		FRA4	GU323317	Vikas *et al.,* 2012
		FRA5	DQ201290	Baxevanis *et al.,* 2006
		FRA6	DQ201292	Baxevanis *et al.,* 2006
		FRA7	DQ201293	Baxevanis *et al.,* 2006
		FRA8	DQ201294	Baxevanis *et al.,* 2006
		FRA9	DQ201296	Baxevanis *et al.,* 2006
		FRA9	FJ004925-31	Kappas *et al.,* 2009
		FRA9	GU323299	Vikas *et al.,* 2012
		FRA10	DQ201299	Baxevanis *et al.,* 2006
		FRA11	DQ201300	Baxevanis *et al.,* 2006
		FRA12	DQ201301	Baxevanis *et al.,* 2006
		FRA13	FJ004932	Kappas *et al.,* 2009
		FRA14	FJ004937	Kappas *et al.,* 2009
		FRA15	FJ004940	Kappas *et al.,* 2009
		FRA16	GU252105	Maniatsi *et al.,* 2009
		FRA17	GU252107	Maniatsi *et al.,* 2009
		FRA18	GU323289	Vikas *et al.,* 2012
		FRA19	GU323290	Vikas *et al.,* 2012
		FRA19	GU323292	Vikas *et al.,* 2012
		FRA20	GU323295	Vikas *et al.,* 2012
		FRA21	GU323300	Vikas *et al.,* 2012
		FRA22	GU323303	Vikas *et al.,* 2012
		FRA23	GU323307	Vikas *et al.,* 2012
		FRA24	GU323313	Vikas *et al.,* 2012
		FRA25	KF703763	Eimanifar *et al.,* 2014
		FRA26	KF703765	Eimanifar *et al.,* 2014
		FRA26	KF703770	Eimanifar *et al.,* 2014
		FRA26	KF703781	Eimanifar *et al.,* 2014
		FRA26	KF703787-88	Eimanifar *et al.,* 2014
		FRA26	KF703808	Eimanifar *et al.,* 2014
		FRA27	KF703767	Eimanifar *et al.,* 2014
		FRA27	KF703795	Eimanifar *et al.,* 2014
		FRA28	KF703771	Eimanifar *et al.,* 2014
		FRA28	KF703773	Eimanifar *et al.,* 2014
		FRA28	KF703777	Eimanifar *et al.,* 2014
		FRA28	KF703801	Eimanifar *et al.,* 2014
		FRA28	KF703826	Eimanifar *et al.,* 2014
		FRA28	KF703836	Eimanifar *et al.,* 2014
		FRA29	KF703776	Eimanifar *et al.,* 2014
		FRA30	KF703779	Eimanifar *et al.,* 2014
		FRA31	KF703784	Eimanifar *et al.,* 2014
		FRA32	KF703786	Eimanifar *et al.,* 2014
		FRA33	KF703791	Eimanifar *et al.,* 2014
		FRA34	KF703797	Eimanifar *et al.,* 2014
		FRA35	KF703799	Eimanifar *et al.,* 2014
		FRA35	KF703827	Eimanifar *et al.,* 2014
		FRA35	KF703834	Eimanifar *et al.,* 2014
		FRA36	KF703800	Eimanifar *et al.,* 2014
		FRA37	KF703806	Eimanifar *et al.,* 2014
		FRA36	KF703800	Eimanifar *et al.,* 2014
		FRA38	KF703848	Eimanifar *et al.,* 2014
		FRA38	KF703854	Eimanifar *et al.,* 2014
Parthenogenetic populations	PART	PART1	DQ201271-72	Baxevanis *et al.,* 2006
		PART1	DQ201274	Baxevanis *et al.,* 2006
		PART2	DQ201273	Baxevanis *et al.,* 2006
		PART3	DQ201278	Baxevanis *et al.,* 2006
		PART4	DQ201279	Baxevanis *et al.,* 2006
		PART5	DQ201280	Baxevanis *et al.,* 2006
		PART6	DQ201281-83	Baxevanis *et al.,* 2006
		PART6	KF703804	Eimanifar *et al.,* 2014
		PART6	KU183830-36	Asem *et al.,* 2016
		PART7*	FJ004943-44	Kappas *et al.,* 2009

Table 1. Continued

Species/Population	Abbreviation	Haplotype names	Accession numbers	References
Parthenogenetic populations	PART	PART7*	KF736253-73	Maccari *et al.*, 2013
		PART7*	KF736276-89	Maccari *et al.*, 2013
		PART7*	KF703764	Eimanifar *et al.*, 2014
		PART7*	KF703803	Eimanifar *et al.*, 2014
		PART7*	KF703807	Eimanifar *et al.*, 2014
		PART7*	KF703809	Eimanifar *et al.*, 2014
		PART7*	KF703825	Eimanifar *et al.*, 2014
		PART7*	KF703830	Eimanifar *et al.*, 2014
		PART7*	KF703832-33	Eimanifar *et al.*, 2014
		PART7*	KF703835	Eimanifar *et al.*, 2014
		PART7*	KF703837-39	Eimanifar *et al.*, 2014
		PART7*	KF703844	Eimanifar *et al.*, 2014
		PART7*	KF703851	Eimanifar *et al.*, 2014
		PART7*	KF703853	Eimanifar *et al.*, 2014
		PART7*	KU183800-04	Asem *et al.*, 2016
		PART7*	KU183815-19	Asem *et al.*, 2016
		PART7*	KU183820-24	Asem *et al.*, 2016
		PART7*	KU183825-29	Asem *et al.*, 2016
		PART7*	KU183805-09	Asem *et al.*, 2016
		PART7*	KU183810-14	Asem *et al.*, 2016
		PART7*	KU183843-47	Asem *et al.*, 2016
		PART8	KF736274-75	Maccari *et al.*, 2013
		PART9	KF703768	Eimanifar *et al.*, 2014
		PART10	KF703769	Eimanifar *et al.*, 2014
		PART10	KF703774-75	Eimanifar *et al.*, 2014
		PART10	KF703780	Eimanifar *et al.*, 2014
		PART10	KF703782	Eimanifar *et al.*, 2014
		PART10	KF703828	Eimanifar *et al.*, 2014
		PART10	KF703840	Eimanifar *et al.*, 2014
		PART10	KF703843	Eimanifar *et al.*, 2014
		PART10	KF703845-46	Eimanifar *et al.*, 2014
		PART11	KF703772	Eimanifar *et al.*, 2014
		PART12	KF703783	Eimanifar *et al.*, 2014
		PART13	KF703792	Eimanifar *et al.*, 2014
		PART14	KF703805	Eimanifar *et al.*, 2014
		PART15	KF703831	Eimanifar *et al.*, 2014
		PART16	KF703841	Eimanifar *et al.*, 2014
		PART17	KF703802	Eimanifar *et al.*, 2014
		PART17	KF703852	Eimanifar *et al.*, 2014
		PART18	DQ201284	Baxevanis *et al.*, 2006
		PART19	DQ201288	Baxevanis *et al.*, 2006
		PART20	KF703789	Eimanifar *et al.*, 2014
		PART21	KU183838-42	Asem *et al.*, 2016
		PART22	KU183837	Asem *et al.*, 2016

* URM13 and PART7 share the same haplotype.

Table 2. A statistic for the composition of the secondary structures proposed in this study. Data shown as number of nucleotides (L and S) or number of Nucleotide pairs (P). H_x: The xth helix; L: Length; P: Paired nucleotides; S_{x-y}: Single strand between the xth and yth helix. Abbreviations of species/populations are defined in Table S1.

Sp./P.	H_I		S_{I-II}	H_{II}		S_{II-III}	H_{III}		S_{III-IV}	H_{IV}		S_{IV-V}	H_V		S_{V-VI}	H_{VI}		S_{VI-VII}	H_{VII}		$S_{VII-VIII}$	H_{VIII}	
	L	P		L	P		L	P		L	P		L	P		L	P		L	P		L	P
FRA	18	7	3	46	17	16	58	22	7	28	10	2	27	8	4	20	8	1	62	22	1	24	7
PER	22	7	1	22	8	14	65	23	3	28	7	3	30	9	2	20	5	3	81	27	3	13	4
SAL	51	19	2	27	7	4	57	18	6	29	10	2	29	7	3	19	6	0	58	22	2	25	7
TIB[1*]	51	17	2	27	11	1	56	19	6	29	10	2	27	11	3	21	7	0	55	20	4	24	8
TIB[2*]	49	17	1	10	3	3	56	19	6	29	10	2	27	11	3	21	7	0	55	20	4	24	8
SIN	50	18	2	27	9	1	56	19	6	29	10	2	26	9	3	21	8	0	55	22	4	24	8
URM	48	17	2	27	11	1	51	20	6	29	10	2	27	9	2	20	7	0	51	17	4	24	8
SP	51	17	2	27	11	1	56	21	6	29	10	2	27	9	3	21	7	0	55	20	4	24	8
PART	51	17	2	27	11	1	56	19	6	29	10	2	27	9	3	21	7	0	55	20	4	24	8

Sp.: Species, P.: Populations

* TIB[1]: Haplotypes TIB2-5; TIB[2]: Haplotype TIB1

Phylogenetic analyses

Phylogenetic analyses were performed based on the alignment of primary sequences for both the first partial and whole *ITS1* region, as well as the alignment consisting of sequence information of joined primary and secondary structure. Phylogenetic trees of haplotypes were designed by Maximum Likelihood (ML) in RAxML-HPC BlackBox 8.2.3 on XSEDE (Miller *et al.*, 2010), Bayesian Inference (BI) as implemented in MrBayes 3.2.2 on XSEDE (Miller *et al.*, 2010), and Neighbor-Joining (NJ) in MEGA ver. 6.00 (Tamura *et al.*, 2013). For ML and NJ, the robustness of branches was assessed by default setting and 1000 bootstrap replicates, respectively. For BI the best nucleotide substitution model of DNA was selected based on MrModeltest 2.2 (Nylander, 2004). Phylogenetic trees based on the primary sequences and sequences of secondary structures (hereinafter referred to as 'sequence-structure') of the partial *ITS1* region were constructed via ProfDistS 0.9.9 (Wolf *et al.*, 2008) with 1000 bootstrap replicates. All trees were visualized using FigTree v 1.4.0 (Rambaut, 2012). For the Maximum Likelihood and Neighbor-Joining bootstraps, the values <70 were regarded as low, 70-94 as moderate, and ≥95 as high (Hillis & Bull, 1993). For the Bayesian posterior probabilities, the values <0.94 were considered as low, and ≥0.95 as high following (Alfaro *et al.*, 2003).

Results

Secondary structure

All the analyzed *Artemia* shared a similar fingers-pattern of secondary structure with eight helices (Fig. 1), contrast *Streptocephalus proboscideus* (out group) in the same alignment length have a significant difference in secondry structure with six helices (Fig. 2). According to the rate of conservation, a significant intraspecific difference was only observed in the second helix of *A. tibetiana* (Fig. 3).
Statistics of the numeric characters of the general secondary structure are shown in Table 2. Helices IV and VI were conservative in length (28 to 29 bp and 19 to 21 bp, respectively), while helices II and I showed high variability (10 to 46 bp and 18 to 51 bp, respectively). The substitution rate of paired nucleotides had almost same pattern with the variation of helical length; the lowest substituted numbers of paired nucleotides were observed in Helices IV and VI (7 to 10 bp and 5 to 8 bp, respectively), but the highest rate belonged to helices II and I (3 to 17 bp and 7 to 19 bp, respectively). The highest length variations of the single strand between helix were present in S_{II-III} (1 to16 bp; with the longest ones appearing in FRA (16 bp) and PER (14 bp), respectively). TIB displayed highly intraspecific variation in the length of helix II (27 bp *vs* 10 bp) and paired nucleotides (11 bp *vs* 3 bp) (Figs. 1 and 3).

Comparison of phylogenies based on primary and secondary structure sequences

All methods of ML, NJ, and BI demonstrated uniform tree topology for primary sequences of the first partial *ITS1* region (Fig. 4a). The genus *Artemia* was divided into two distinct and well-supported clusters. Cluster I was further divided into four clades, with either of the Mediterranean *A. salina* and American *A. franciscana* constituting a separate clade, and the Asian bisexual species and parthenogenetic populations constituting the other two clades. The South American *A. persimilis* is placed in a basal position with long branch (Fig. 4a). In addition, the ML (Baxevanis *et al.*, 2006; Eimanifar *et al.*, 2014), BI (Baxevanis *et al.*, 2006; Eimanifar *et al.*, 2014) and NJ (this study, result not shown) analyses based on the complete primary sequence of *ITS1* also generated correspondent tree topologies. Therefore, the first partial region, which ranged from 294 bp to 340 bp, was likely to have the same evolutionary pattern as the total sequence of *ITS1*.
The sequence-structure tree, by the profile neighbor-joining (PNJ) method, displayed same general pattern for parthenogens, Asian and Mediterranean bisexual *Artemia*; whereas *A. franciscana* and *A. persimilis* were clustered into a basal clade in the tree (Fig.4b). While no significant intra-specific variation was determined with primary sequences for the bisexual species (Fig. 4a), the results of sequence-structure showed markedly intra-specific variation within *A. urmiana*, which was divided into two different sub-clades (support values = 95) (Fig. 4b). For the parthenogenetic *Artemia*, $PART_{19,20}$ were collected with SINs; the others were collected

with Asian bisexual species in trees based on primary sequence (Fig. 4a). In contrast, they were divided into four major groups in the sequence-structure tree, with the PART$_{6,15,16,18,22}$ placed with *A. tibetiana*, PART$_{1-5,7-14,17}$ placed with *A. urmiana*, PART$_{19,20}$ placed with *A. sinica*, and PART$_{21}$ located separately (Fig. 4b).

Discussion

This study provides the first evidence of phylogeny of the Anostraca *Artemia,* using the sequence of RNA secondary structure.

Even if the general secondary structure of *ITS1* shows a fingers-pattern in all the studied species, interspecific variation is considerable in the length of helices, the paired structure and the length of single strands (Fig. 1). Though phylogenetic trees of total primary sequence of *ITS1* (Baxevanis *et al*., 2006, Hou *et al.*, 2006; Kappas *et al.*, 2009; Vikas *et al.*, 2012, Eimanifar *et al.*, 2014), partial primary sequence (Fig. 4a) and sequence-structure (Fig. 4b) showed a single collection for *A. tibetiana,* a remarkable intraspecific variation was detected in the second helix of *A. tibetiana* (Figs. 1 and 3).

Asem *et al.* (2016) proved that the nuclear marker *ITS1* could not clearly sort *A. urmiana*, *A. tibetiana* and parthenogenetic populations in phylogenetic trees (see also Maccari *et al*., 2013; Eimanifar *et al*., 2014; this study Fig. 4a); but the phylogenetic tree based on sequence-secondary of *ITS1* displayed an appreciable differentiation for these groups in this study (Fig. 4b). *Artemia tibetiana* clearly located in a separated clade.

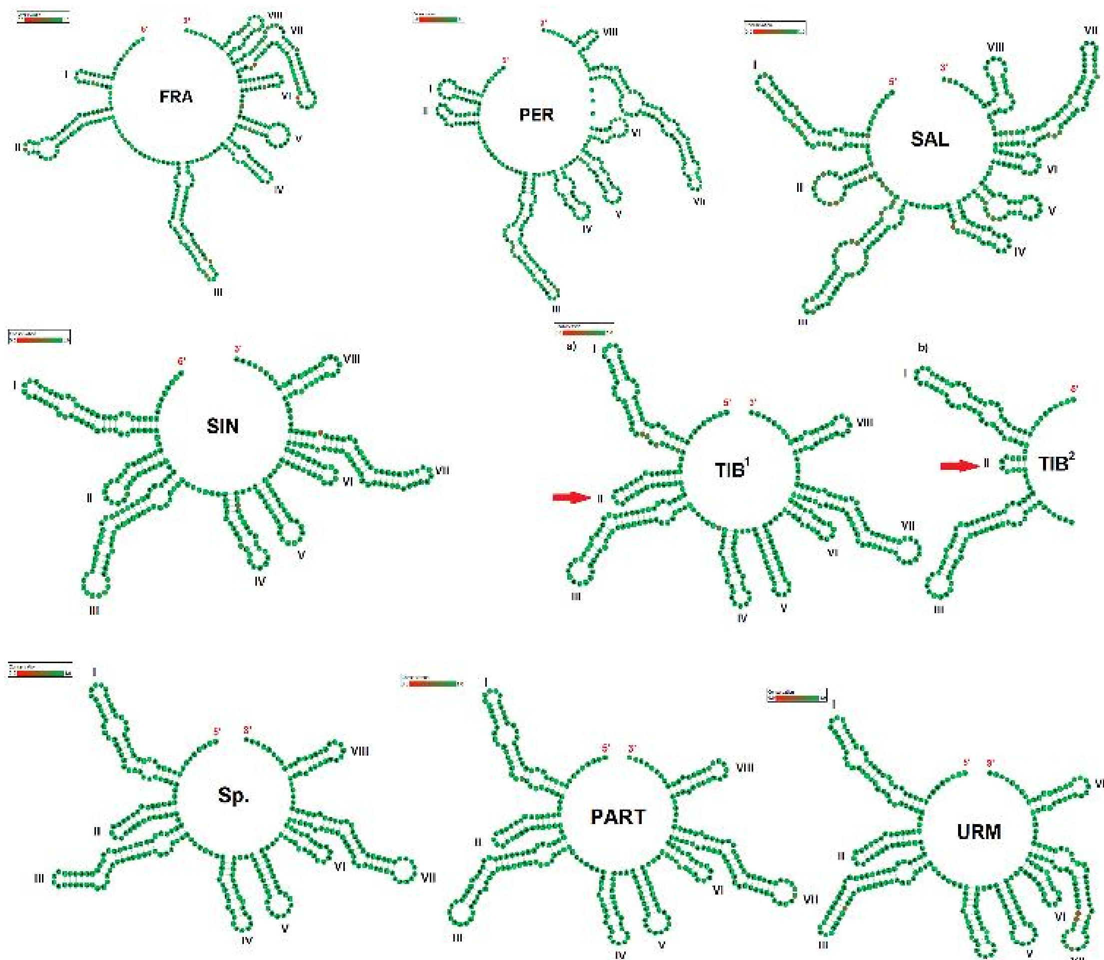

Fig. 1. Predicted general secondary structure for the *ITS1* partial regions of genus *Artemia*: TIB^1; Haplotypes TIB2-5; TIB^2; Haplotype TIB1; Arrows point to the region with different patterns between two secondary structures of TIB. (Abbreviations of species/population are defined in Table 1).

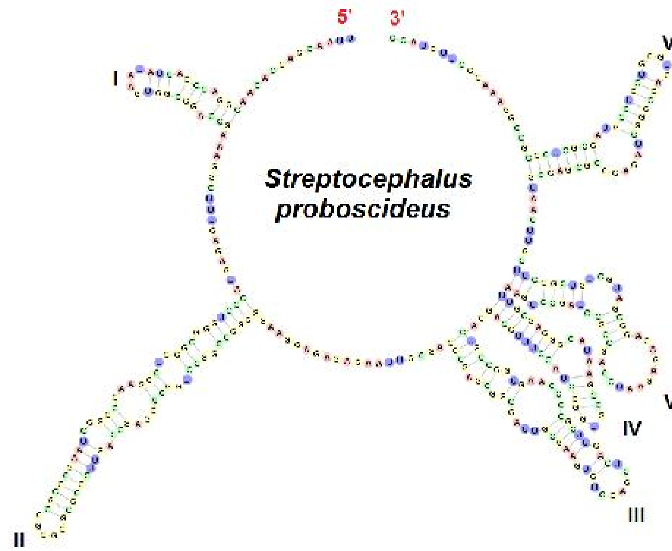

Fig. 2. Predicted general secondary structure for the *ITS1* partial regions of *Streptocephalus proboscideus* (outgroup).

Fig. 3. The primary (upper) and secondary (lower) sequences in Helix II of *ITS1* of *Artemia tibetiana* (boxes show the position of Helix II). TIB[1]: Haplotypes TIB2-5; TIB[2]: Haplotype TIB1. (Abbreviations of species/population are defined in Table 1).

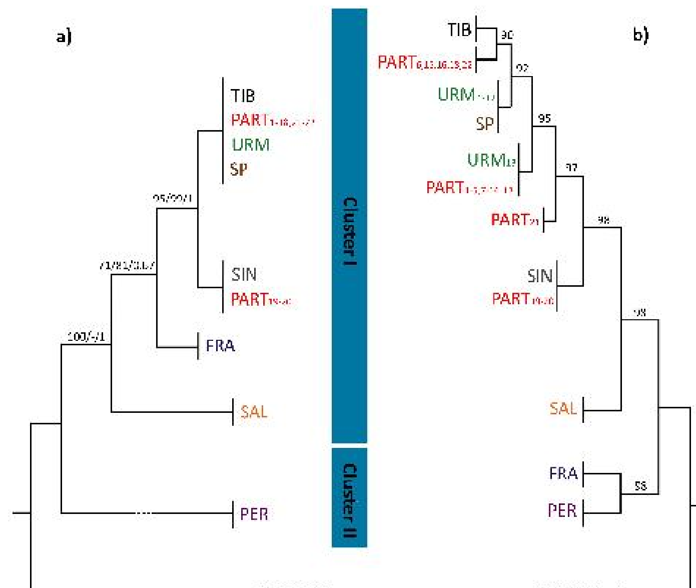

Fig. 4. Simplified phylogenetic trees of the genus *Artemia* based on 313 *ITS1* sequences. *Streptocephalus proboscideus* was used as an out-group. **a**) ML/BI/NJ trees inferred from primary sequence. Numbers on the nodes are: the bootstrap value from maximum-likelihood / that of neighbor-joining / the Bayesian posterior probability values. **b**) PNJ tree based on sequence-structure. Numbers at the nodes represent the bootstrap values from profile neighbor-joining. (Abbreviations of species/population are defined in Table 1).

The highest intra-population variation was shown in the parthenogenetic *Artemia* with four lineages which two ones ($PART_{1-5,7-14,17}$ and $PART_{19-20}$) shared same subclades with *A. urmiana* and *A. sinica* respectively, and two others ($PART_{6,15,16,18,22}$ and $PART_{21}$) located in unique separated platforms. In addition, *A. urmiana* presented a level of intraspecific variation in two subclades (Fig. 4b). Our findings showed the secondary structure shapes could not support the observed intra-specific/population differentiation by sequence-secondary tree among variants of PART and URM (Figs. 4b, 5, and 6), so that different lineages of parthenogenetic populations and *A. urmiana* in the phylogenetic tree (Fig. 4b) have same secondary structures with their general predicted patterns (Figs. 1, 5, and 6). This finding confirmed that phylogenetic analysis by combined primary and secondary sequences can display remarkable diversification in comparison of using only secondary structure.

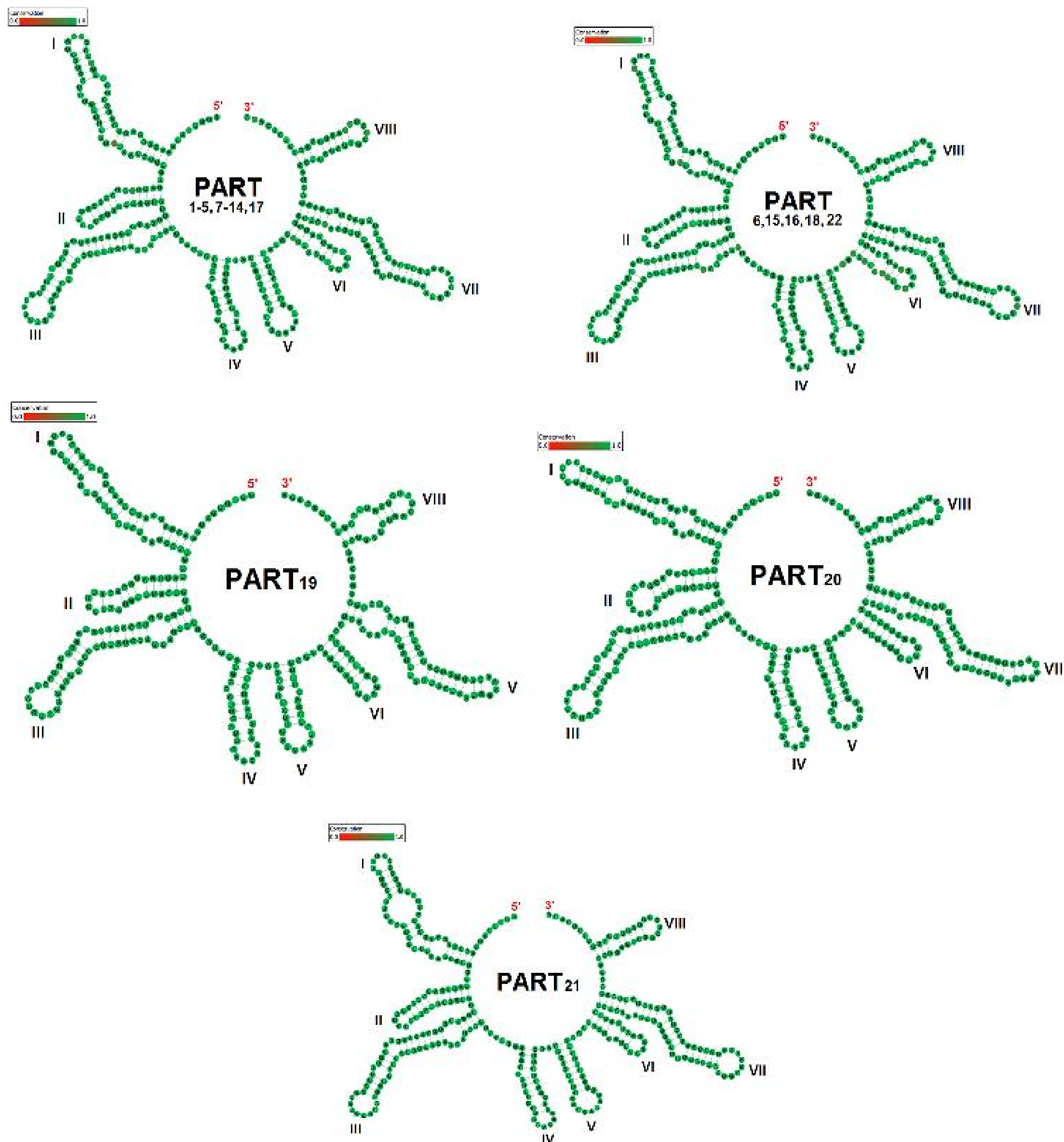

Fig. 5. Predicted secondary structure for partial *ITS1* regions of parthenogenetic *Artemia*, based on four separated lineages by sequence-structure from profile neighbor-joining (See Fig. 4b). (Abbreviations of population are defined in Table 1).

With regards to the results of mitochondrial markers, parthenogenetic *Artemia* is a polyphyletic group, a fact which, di- and tetraploid parthenogenetic *Artemia* originated from *A. urmiana* and *A. sinica*, and tri- and pentaploids divided from diploid and tetraploid *Artemia*, respectively (Maniatsi *et al.*, 2011; Asem *et al.*, 2016). Contrary to the primary sequence of *ITS1*, sequence-structure was also able to differentiate parthenogenetic populations into different lineages, but not able to distinguish ploidy levels. The members of two major groups (i.e. $PART_{1-5,7-14,17}$ and $PART_{6,15,16,18,22}$) include all ploidy degrees, besides that two other lineages ($PART_{19,20}$ and $PART_{21}$) keep only tetraploids (Table 1, for more information about ploidy levels, see Baxevanis *et al.*, 2006; Kappas *et al.*, 2009; Maniatsi *et al.*, 2011; Asem *et al.*, 2016).

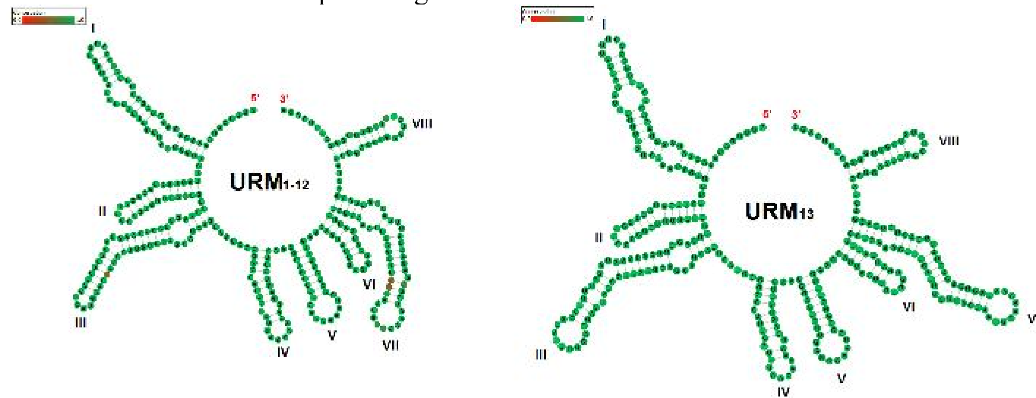

Fig. 6. Predicted secondary structure of *ITS1* partial regions for *Artemia urmiana* based on two separated lineages by sequence-structure from profile neighbor-joining (See Fig. 4b).

Different genetic methods showed almost opposed evolutionary history for taxa of genus *Artemia*, especially regarding the position of *A. salina* and *A. persimilis* (see Introduction section). While all genetic studies indicated that there was no close phylogenetic relationship between *A. franciscana* and *A. persimilis* (Baxevanis *et al.*, 2005; 2006; Hou *et al.*, 2006; Kappas *et al.*, 2009; Maniatsi *et al.*, 2011; Vikas *et al.*, 2012; Eimanifar *et al.*, 2014), phylogenetic analysis based on allozyme markers showed that *A. franciscana* and *A. persimilis* were located in the basal clade together (Beardmore and Abreu-Grobois, 1983). This finding agrees with the occurrence of natural hybridization between *A. persimilis* and *A. franciscana*. Several morphologic, genetic and cytogenetic studies have documented the existence of occasional degrees of natural hybridization and/or introgression between *A. franciscana* and *A. persimilis* in the Las Tunas Lagoon population (Córdoba Province, Argentina) (Papeschi *et al.*, 2000; Amat *et al.*, 2004; Cohen, 2012). Morphometric analyses of adults (Papeschi *et al.*, 2000; Amat *et al.*, 2004) and phylogenetic analyses using genetic markers including *16S*, *COI*, *ITS1* and *p26* (Ruiz *et al.*, 2008; Maniatsi *et al.*, 2009) grouped Las Tunas with *A. franciscana*. Meanwhile, the cytogenetic study proved that most meiotic cells of adult males had 21 haploid chromosomes; others had 22 or 23 chromosomes with irregular meiosis. This abnormality was attributed to a hybridization/introgression between *A. franciscana* (n = 21) and *A. persimilis* (n = 22) (Papeschi *et al.*, 2000). Another controversial case was documented for the population of Pichilemu saltworks (Cardenal Caro Province, Chile). Based on allozymes (Gajardo *et al.*, 1995), morphometric data of adults (Zuñiga *et al.*, 1999) and 42 diploid chromosomes (Parraguez *et al.*, 2009), this population has been imputed to *A. franciscana*, whereas analyses referring to chromocenter numbers (Gajardo *et al.*, 2001a), *16S* rRNA RFLP patterns (Gajardo *et al.*, 2004), and *ITS1* sequence (Baxevanis *et al.*, 2006) referred this population to *A. persimilis*. The possibility of hybridization between *A. franciscana* and *A. persimilis* has previously been observed in cross-fertility laboratory experiments (Gajardo *et al.*, 2001b). Since natural hybridization usually take place between very closely related species or sister taxa (Coyne and Orr, 1997; Agatsuma *et al.*, 2000; Price and Bouvier 2002; Seehausen, 2004; Mallet, 2005; Mallet *et al.*, 2007; Kovalev *et al.*, 2016), the existence of

natural hybridization between *A. franciscana* and *A. persimilis* further emphasizes that these species might have close evolutionary relationship. Moreover, in the *ITS1* primary sequence trees, *A. franciscana* was sorted as a sister clade of the Asian bisexual species, but laboratory cross-breeding tests have documented complete infertility between *A. franciscana* and Asian bisexual species (Pilla & Beardmore, 1994; Abatzopoulos *et al.*, 2002a). Therefore, phylogenetic analysis using both primary and secondary sequences may better reveal the relationships of these taxa than using only primary sequences.

In conclusion, the secondary structure and sequence-structure of *ITS1* DNA in the genus *Artemia* could be a powerful tool for understanding phylogenetic relationships among taxa. The secondary structure shows a considerable intraspecific variation in *Artemia tibetiana,* and sequence-structure reveals new lineages for parthenogenetic populations and *A. urmiana*. The New World species in the same cluster by sequence-structure analysis agrees with the ability of natural hybridization and the result from allozyme markers.

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