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Artemia spp. (Crustacea, Anostraca) in Crimea: New Molecular Genetic Results and New Questions without Answers

Anastasia Lantushenko [1], Yakov Meger [1], Alexandr Gadzhi [1], Elena Anufriieva [1,2,*] and Nickolai Shadrin [1,2]

1 Sevastopol State University, 33 Universitetskaya Street, 299053 Sevastopol, Russia
2 A.O. Kovalevsky Institute of Biology of the Southern Seas of RAS, 2 Nakhimov Ave., 299011 Sevastopol, Russia
* Correspondence: lena.anufriieva@gmail.com; Tel.: +7-8692-54-5550

Abstract: Many works have been devoted to the study of the molecular genetic diversity of *Artemia* in different regions; however, there are regions such as Crimea, the largest peninsula in the Black Sea, which has seen few studies. *Artemia* specimens from several Crimean hypersaline lakes were analyzed using the mitochondrial marker cytochrome oxidase C (COI). The analyzed individuals from bisexual populations formed clades with the species *A. salina*, *A. urmiana*, *A. sinica*, and *A. monica* (=*A. franciscana*). *A. sinica* and *A. monica* had not been recorded in Crimea previously. In Lake Adzhigol, the three species *A. urmiana*, *A. sinica*, and *A. monica* were found at the same time, which has not been noted anywhere before. In the Crimean lakes, a total of 10 haplotypes were found, six of them for the first time: Once for *A. monica*, once for *A. sinica*, and four for *A. salina*. Those haplotypes may be regarded as endemic to Crimea. In the 1990s, experiments were carried out in Lake Yanyshskoe using mainly purchased cysts of *Artemia*, so *A. monica* and *A. sinica* were introduced into Crimea and could then have easily been spread by birds to other Crimean lakes.

Keywords: *Artemia*; cytochrome oxidase C (COI); haplotypes; hypersaline lakes; invasive species

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1. Introduction

Other than in Antarctica, *Artemia* spp. are the most common and abundant animals in hypersaline waters worldwide [1,2]. They belong to Anostraca, the most primitive and ancient group among living crustaceans and have one of the most advanced osmoregulation systems among all animals, which allows them to exist in an extremely wide range of salinity [3,4]. Due to this, they play a key multidimensional role in most ecosystems of hypersaline waters of the planet [1,5–8]. The existence of several water bird species depends on the development of *Artemia* populations [9–11]. *Artemia* biomass and its cysts are of great commercial value [1,12]. These crustaceans are also considered convenient test objects in ecotoxicology [13,14], as well as model species to study various issues in different branches of biology [3,4,15,16].

It is therefore not difficult to understand the existing theoretical and practical interest in the study of *Artemia*, including the study of its diversity and the factors that determine it [5,6,8,17]. Recent studies show that, along with parthenogenetic populations, there are five species of bisexual *Artemia* in the world: *A. salina* (Linnaeus, 1758), *A. urmiana* Günther, 1890, *A. monica* Verrill, 1869 (=*A. franciscana* Kellogg, 1906), *A. sinica* Cai, 1989, and *A. persimilis* Piccinelli and Prosdocimi, 1968. Regarding the species *A. monica* and *A. franciscana*, there is currently no consensus, as some researchers believe that both species are valid while others believe that this is one species.

Many works have been devoted to the study of the molecular genetic diversity of *Artemia* in different regions [5,18,19]; however, there are still some practically unexplored regions. One of these is Crimea, the largest peninsula in the Black Sea (27,000 km^2). The existence of *Artemia* in Crimea, thanks to P. Pallas, was already determined in the 18th century [20]. In the 19th century, there were four different species described, including the species *A. salina*, *A. arietina* Fischer, 1851, *A. milhausenii* Fischer de Waldheim, 1834, and *A. koeppeniana* Fischer, 1851 [21,22]. *A. arietina* is now recognized as a variety of *A. salina*, and *A. milhausenii* and *A. koeppeniana* are recognized as synonyms of *A. urmiana* [8].

In the second half of the 19th century, it was experimentally shown that salinity causes a high level of *Artemia* morphological variability [23–25], and, proceeding from this, all *Artemia* species in Crimea were reduced to one species, *A. salina* [26]. A revision of the diversity of *Artemia* in Crimea using electron microscopy showed that bisexual brine shrimp on the peninsula mainly belong to the species *A. salina*, but several males in Lake Sasyk-Sivash belonged to another species [27]. Later, another species was found in Lake Koyashskoe, identified by morphological characteristics as *A. urmiana* [28], which was confirmed using molecular genetic methods [29]. Previously, based on morphological similarity, it was suggested that *A. mulhausinii* corresponds to *A. urmiana* described from Lake Urmia [30]. Crimea was regarded as unique due to having a relatively small territory, and its hypersaline lakes host at least two bisexual *Artemia* species and their parthenogenetic populations [30]. So, the conclusion was made that Crimea may be considered a remnant of the center of the *Artemia* biodiversity origin near the ancient Tethys Ocean [30,31]. To date, it is known that *Artemia* populations exist in Crimea in more than fifty water bodies, including Bay Sivash, the world's largest *Artemia* habitat (2560 km^2), which are represented by two bisexual native species and parthenogenetic populations of different ploidy [7,32–34]. Nevertheless, the existence of only one bisexual species, *A. urmiana*, was confirmed using the molecular genetic approach [29].

The main objectives of this study are (1) to analyze *Artemia* specimens from different lakes of Crimea using the mitochondrial marker cytochrome oxidase C (COI), and (2) to test the hypotheses about the existence of at least two bisexual *Artemia* species on the peninsula and the possibility of coexistence of two bisexual *Artemia* species in one water body.

2. Materials and Methods

On the Crimean Peninsula, there are many hypersaline water bodies (Figure 1), which differ in size, ranges of salinity fluctuations, and biological diversity [35,36].

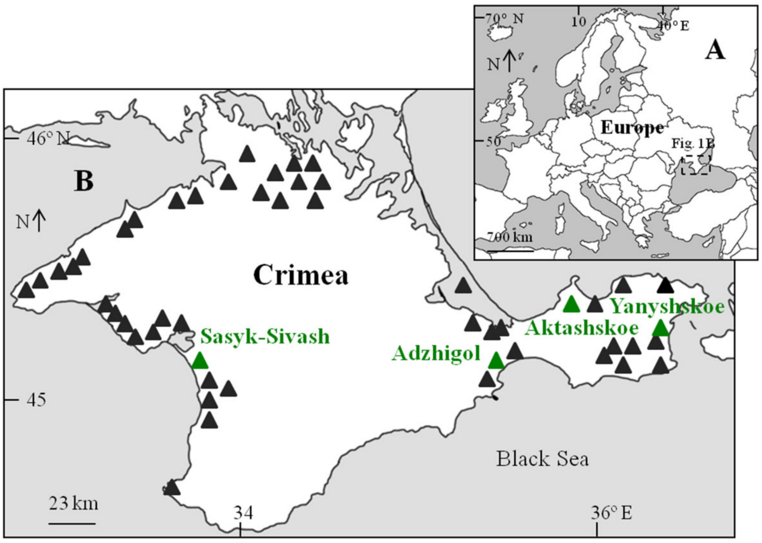

Figure 1. Distribution of the hypersaline lakes in Crimea, including the location of the studied lake. ((**A**) — the European scale, (**B**) — the Crimean scale).

All lakes are shallow (up to 1.5 m deep), polymyxic, and characterized by high seasonal and long-term variability. In this study, 14 specimens of bisexual *Artemia* from three lakes were analyzed, the general characteristics of which are given in Table 1. Sampling was carried out by standard methods, by filtering water through a plankton net [34,35]. Live crustaceans were delivered to the laboratory. Simultaneously with sampling in lakes, water salinity and temperature were measured using a WZ212 portable refractometer (Kelilong Electron Co. Ltd., Fuan, China) and a PHH-830 electronic pH meter (OMEGA Engineering Inc., Norwalk, CT, USA), respectively.

Table 1. General characteristics of the studied Crimean hypersaline lakes where *Artemia* was taken.

Lake	Coordinates	Area, km^2	Date of Sampling	Salinity (during Sampling), g L^{-1}	Temperature, °C	Number of Analyzed Individuals (Female/Male)	Total *Artemia* Abundance, Ind. m^{-3}
Aktashskoe	45°22′31″ N 35°49′45″ E	26.8	1 July 2021	173	28.5	*A. urmiana*—2 f *A. monica*—1 m	34,820
Adzhjigol	45°06′32″ N 35°27′58″ E	0.6	1 July 2021	50	34.5	*A. monica*—2 m *A. sinica*—2 f *A. urmiana*—1 f	902,960
Sasyk-Sivash	45°09′21″ N 33°31′09″ E	75.3	3 July 2021	245	36.0	*A. salina*—4 m/2 f	220

Before DNA isolation, crustaceans were placed overnight in distilled water, then the intestines were removed from each individual, and DNA was isolated from each sample. DNA isolation was performed using a DNA-Extran 2 reagent kit (Sintol, Russia) according to the manufacturer's instructions. Quantitative determination of the obtained genomic DNA and assessment of its purity were carried out on an Inplen nanophotometer (Inplen, Munich, Germany) using gel electrophoresis in 1% agarose gel. The PCR reaction was carried out using primer pairs jgLCOI490 and jgHCO2198 for the COI gene [37]. The PCR reaction was carried out in a volume of 25 µL using ScreenMix reagents (Evrogen, Moscow, Russia) and consisted of the following steps: 94 °C—2 min, 30 cycles (94 °C—1 min, 48 °C—1 min, 72 °C—1 min), and a final elongation of 5 min at 72 °C. The sequencing of the obtained fragments was carried out on the NANOFOR-05 sequencer (Sintol, Moscow, Russia) at the Center for Collective Use "Molecular Structure of Matter" of the Sevastopol State University. The generated DNA sequences were stored at GenBank (accession numbers ON872198-ON872211) and compared with those available in the National Center for Biotechnology Information (NCBI) database. The analysis used a large dataset containing bisexual and parthenogenetic *Artemia* sequences from all geographic locations. GenBank codes for sequences previously obtained by other researchers [6,38–44] and used in the work are presented in Table A1. Phylogenetic reconstruction was performed using a Bayesian Inference approach implemented in MrBayes version 3.2.6 [45]. When constructing a phylogenetic tree, the HQ972028 *Daphnia tenebrosa* sequence for the CO1 gene was used as an outgroup.

3. Results

The analyzed individuals from bisexual populations formed clades with the species *A. salina*, *A. urmiana*, *A. sinica*, and *A. monica* (Figure 2). In Lake Sasyk-Sivash, among the analyzed individuals, only *A. salina* was found, while in Lake Aktashskoe, representatives of two species were found, *A. urmiana* and *A. monica*, and in Lake Adzhigol, three species of *A. urmiana*, *A. sinica*, and *A. monica*. Contemporaneously, bisexual individuals of *A. urmiana* from lakes Adzhigol and Aktashskoe formed a common clade with parthenogenetic populations. In the Crimean lakes, a total of 10 haplotypes were found during this study (Table 2). Two haplotypes (H2 and H3) were shared between parthenogenetic and bisexual individuals of *A. urmiana*, (3W2, 3W3, and 4W2). The rather high nucleotide variability of the COI genes of samples from Crimea was noted: *A. salina* specimens (H7-10) from Sasyk-Sivash Lake formed separate haplotypes, and *A. sinica* samples from Adzhigol Lake were also not included in the haplotypes of previously studied samples and formed a separate (H6) group. Two sequences of *A. monica* from Lake Adzhigol form joint haplotypes with other *A. monica* (H4 and H5), and one is allocated to a separate H1 group.

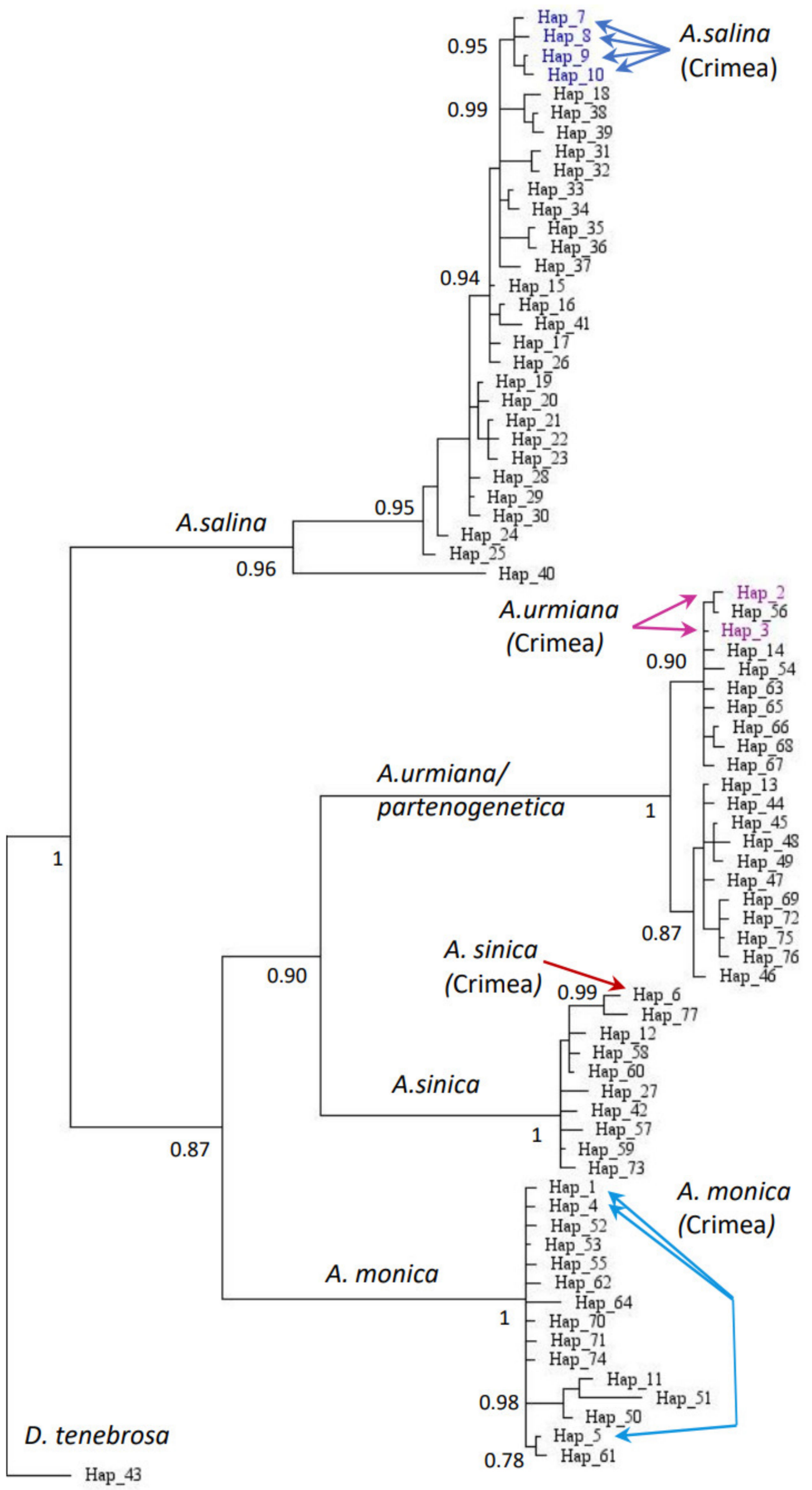

Figure 2. Phylogenetic relationship of analyzed *Artemia* revealed by Bayesian analysis based on cytochrome c oxidase subunit I (COI). The bootstrap values at the nodes higher than 75% are shown next to the branches.

Table 2. *Artemia* haplotypes found in the Crimean hypersaline lakes.

Species	Abbreviation/Haplotypes	Lake	GenBank Number
A. monica (=*A. franciscana*)	3M2/H1	Aktashskoe	ON872200
A. urmiana	3F2/H2	Aktashskoe	ON872198
A. urmiana	3F3/H3; 4F2/H3	Aktashskoe, Adzhigol	ON872199 ON872202
A. monica (=*A. franciscana*)	4M1/H4	Adzhigol	ON872204
A. monica (=*A. franciscana*)	4M2/H5	Adzhigol	ON872205
A. sinica	4F1/H6; 4F3/H6	Adzhigol	ON872201 ON872203
A. salina	5M1/H7	Sasyk-Sivash	ON872208
A. salina	5M2/H8,5M3/H8,5M5/H8	Sasyk-Sivash	ON872209 ON872210 ON872211
A. salina	5F1/H9	Sasyk-Sivash	ON872206
A. salina	5F2/H9	Sasyk-Sivash	ON872207

4. Discussion

The obtained data show the presence of four bisexual *Artemia* species in Crimea, and such value is very high for so small an area as Crimea. In general, in the hypersaline water bodies of the Western Mediterranean region, all these species have also been previously noted but in a much larger area [8]. This fact supports the suggestion that Crimea may be regarded as one of *Artemia* biodiversity hotspots [30]. Individuals of three bisexual species were simultaneously found in Lake Adzhigol, something that has never been noted anywhere before.

How and when did *A. monica* and *A. sinica* appear in Crimea? This is a question that can hardly be answered unambiguously. *A. monica* (=*A. franciscana*) is known as a highly invasive species, having its native range in the Americas and currently found in Australia, Asia, Europe, and Africa [6,19,46–48]. Displacing native bisexual and parthenogenetic populations of *Artemia*, this species is rapidly expanding its presence on all continents except Antarctica. The main vector of distribution of *A. monica* is the widespread use of cysts, which were initially harvested mainly in American water bodies, in aquaculture of fish and shrimp [12,19]. Pond cultivation of *A. monica* has begun in several regions [12,19], which significantly accelerates their expansion into new territories. After a species enters a new region, its cysts within it are rapidly spread by birds over thousands of kilometers [9,49,50]. In the 1990s, experiments were carried out in Lake Yanyshskoe using mainly purchased cysts of *A. monica*, so the species could have entered Crimea, and later could have been easily spread by birds to other Crimean lakes.

However, one of the finds makes it doubtful that the species could have been brought to Crimea only by humans, and other scenarios for its entry into Crimea are unbelievable. Near Lake Adzhigol, where the species was found, at a distance of 2–3 km, there is another lake, Kuchuk-Adzhigol (salinity 5–7 g L^{-1}), where three species of cyclops from Southeast Asia were previously found, transported here by birds [35]. Both of these lakes are intensively used by some aquatic bird species making various migrations, and this fact does not preclude the idea that *Artemia* cysts were also transported here from outside Crimea by birds. Nevertheless, looking at Figure 2 the authors also can assume an earlier migration of the species into Crimea (tens to hundreds of thousands of years ago). In this case, it is impossible to imagine any other way for the species to enter Crimea, except by an accidental introduction by birds.

Once in a new region, *A. monica* begins to change rapidly, adapting to the conditions of the new region [18,19,51,52]. The rapid variability and adaptability of *A. monica* under new conditions are facilitated by the fact that the species has different alternative gene expression patterns [16,53]. So, the existence of the alternative patterns provides the possibility to shift from one homeostatic strategy to another in a novel environment, and this may enhance the invasiveness and fitness of the species in the new habitat. Based on this, it can be assumed that, most likely, the species was brought to Crimea rather recently by humans or birds.

It is highly likely that one of those two scenarios can be assumed for *A. sinica*. Its cysts could be among those purchased and used in Lake Yanyshskoe by fish farmers. However, the possibility of transport by birds cannot be ruled out. At the same time, of course, it is difficult to imagine that the same birds, within the framework of one migration, brought cysts directly from China to Crimea. One can easily imagine that the transport was carried out in the form of a kind of relay race by different birds, for example, through Transbaikalia (between China and Crimea), where *A. sinica* was also found [54]. Relatively recent finds of *A. sinica*, thanks to molecular genetic studies, in the West Mediterranean [8], allow the authors to suggest other possible ways for the species to enter Crimea.

Ten *Artemia* haplotypes were found in Crimea and 77 haplotypes globally [6,38–40]. Among the 10 haplotypes found in Crimea, 6 were found for the first time: One for *A. monica*, one for *A. sinica*, and four for *A. salina*. Those haplotypes may be regarded as endemic to Crimea. This fact may also be explained in two ways: First, the introduction of *A. monica* and *A. sinica* occurred before the 1990s, or second, those species evolved quickly in Crimea as was shown for other regions [19].

Where did *A. monica* and *A. sinica* appear earlier in the Mediterranean or Crimea? How did each species first enter Europe? At present, there are no answers to these questions; new, deeper studies of both the genetic structure of local populations and bird migrations are needed. Another question, which is likely difficult to answer without answering the previous ones is how long have all four bisexual species coexisted, and will they continue to coexist for a long time in Crimea? The large number and variety of hypersaline water bodies, as well as their high seasonal and interannual variability, only suggest a possibility of long-term coexistence. New comprehensive studies on the Crimean *Artemia* populations are needed to find answers to these questions.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. The list of COI haplotypes for all *Artemia* populations analyzed in the present study (Figure 2). The Crimea populations studied in this work are highlighted in bold.

Haplotypes	Species	GenBank Number	Geographical Locality	Reference
H1	***A. franciscana***	**ON872200**	**Crimea (Aktashskoe)**	**this work**
H2	***A. urmiana***	**ON872198**	**Crimea (Aktashskoe)**	**this work**

Table A1. *Cont.*

Haplotypes	Species	GenBank Number	Geographical Locality	Reference
	A. parthenogenetica	KF691333-337,343,345,357,358,359,361,	Iran	[6]
	A. parthenogenetica	KF691530-532	Turkmenistan	[6]
	A. urmiana	**ON872199, ON872202**	**Crimea (Aktashskoe, Adzhjigol)**	**this work**
	A. parthenogenetica	KF691148-153,166-172,187-189,208-212,224-226,233-236,238,287-290,	China	[6]
H3	*A. parthenogenetica*	KF691338-342,344,346,348	Iran	[6]
	A. parthenogenetica	KF691373-375	Iraq	[6]
	A. parthenogenetica	KF691391-434	Kazakhstan	[6]
	A. parthenogenetica	KF691442-448	Pakistan	[6]
	A. parthenogenetica	KF691455,456,458-461,465,467-475,477,478,480,485-491,493,495-497	Russia	[6]
	A. parthenogenetica	KF691534	Turkmenistan	[6]
	A. parthenogenetica	KF691548-553,555	Uzbekistan	[6]
	A. franciscana	**ON872204**	**Crimea (Adzhjigol)**	**this work**
H4	*A. franciscana*	KF691154-156,174-175,176,179,181,184,185,186,190,192,206,222,223,231,232,252,255,258,261,264,266,267,281,292,303,315,	China	[6]
	A. franciscana	KF691351,353,355,	Iran	[6]
	A. franciscana	KF691439-441	Pakistan	[6]
	A. franciscana	KF691508	Sri Lanka	[6]
	A. franciscana	KF691568	Vietnam	[6]
	A. franciscana	**ON872205**	**Crimea (Adzhjigol)**	**this work**
H5	*A. franciscana*	KF691191,205,239,240,242–244,250,251,253,259,260,262,263,278,280,294,295,296,297,304,305,307,308,309–314,	China	[6]
	A. franciscana	KF691378-382	Iraq	[6]
	A. franciscana	KF691449-454	Portugal	[6]
	A. franciscana	KF691503-507	Sri Lanka	[6]
	A. franciscana	KF691556-567	Vietnam	[6]
H6	***A. sinica***	**ON872201, ON872203**	**Crimea (Adzhjigol)**	**this work**
H7	***A. salina***	**ON872208**	**Crimea (Sasyk-Sivash)**	**this work**
H8	***A. salina***	**ON872209, ON872210, ON872211**	**Crimea (Sasyk-Sivash)**	**this work**
H9	***A. salina***	**ON872206**	**Crimea (Sasyk-Sivash)**	**this work**
H10	***A. salina***	**ON872207**	**Crimea (Sasyk-Sivash)**	**this work**
H11	*A. franciscana*	DQ119645	USA	[40]
H12	*A. sinica*	DQ119650	Mongolia	[40]
H13	*A. urmiana*	JX512748,755,758,762,766,769,771,774,776,778,780,783,788,790,795,796,803,804,808	Iran (Urmia)	[38]
H14	*A. parthenogenetica*	DQ426826	Spain	[40]
H15	*A. salina*	DQ426831,853,857	Spain	[40]
H16	*A. salina*	DQ426832, KF691502	Spain	[40]
H17	*A. salina*	DQ426833	Spain	[40]
H18	*A. salina*	DQ426834,856	Spain	[40]
H19	*A. salina*	DQ426836	Spain	[40]
H20	*A. salina*	DQ426837	Spain	[40]
H21	*A. salina*	DQ426841	Spain	[40]
H22	*A. salina*	DQ426845	Spain	[40]
H23	*A. salina*	DQ426846	Spain	[40]
H24	*A. salina*	DQ426847	Spain	[40]
H25	*A. salina*	DQ426848	Spain	[40]
H26	*A. salina*	DQ426858	Spain	[40]
H27	*A. sinica*	EF615592	China	[40]
H28	*A. salina*	EU543444	Spain	[40]

Table A1. *Cont.*

Haplotypes	Species	GenBank Number	Geographical Locality	Reference
H29	*A. salina*	EU543445	Spain	[40]
H30	*A. salina*	EU543448	Spain	[40]
H31	*A. salina*	EU543452	Morocco	[40]
H32	*A. salina*	EU543453	Morocco	[40]
H33	*A. salina*	EU543456	Tunisia	[40]
H34	*A. salina*	EU543457	Tunisia	[40]
H35	*A. salina*	EU543467	Algeria	[40]
H36	*A. salina*	EU543468	Algeria	[40]
H37	*A. salina*	EU543470	Egypt	[40]
H38	*A. salina*	EU543480	Italy	[40]
H39	*A. salina*	EU543481	Italy	[40]
H40	*A. salina*	EU543485	South Africa	[40]
H41	*A. salina*	GU248381	Italy	[41]
H42	*A. sinica*	HM998990	China	[42]
H43	*D. tenebrosa*	HQ972028	-	-
H44	*A. urmiana*	JX512751	Iran (Urmia)	[38]
H45	*A. urmiana*	JX512756,764,	Iran (Urmia)	[38]
H46	*A. urmiana*	JX512775,791,805	Iran (Urmia)	[38]
H47	*A. urmiana*	JX512777,801	Iran (Urmia)	[38]
H48	*A. urmiana*	JX512782	Iran (Urmia)	[38]
H49	*A. urmiana*	JX512792	Iran (Urmia)	[38]
H50	*A. franciscana*	KF691137-141	Canada	[6]
H51	*A. franciscana*	KF691143-147	Cape Verde	[6]
H52	*A. parthenogenetica*	KF691159,257	China	[6]
H53	*A. franciscana*	KF691160-165,173,177,178,180,182,207,227-230,237,241,256,279,282-286,291,293	China	[6]
	A. franciscana	KF691328-332	India	[6]
	A. franciscana	KF691347,349,354,356,	Iran	[6]
	A. franciscana	KF691376,377,381,383,	Iraq	[6]
	A. franciscana	KF691384-390	Jamaica	[6]
	A. franciscana	KF691535,537,538,543,544,546	USA	[6]
H54	*A. parthenogenetica*	KF691183	China	[6]
	A. parthenogenetica	KF691462	Russia	[6]
H55	*A. franciscana*	KF691196,219	China	[6]
H56	*A. parthenogenetica*	KF691199-204,265,268,	China	[6]
H57	*A. sinica*	KF691270,271	China	[6]
H58	*A. sinica*	KF691272	China	[6]
H59	*A. sinica*	KF691274,276,277,300,302	China	[6]
H60	*A. sinica*	KF691275,299	China	[6]
H61	*A. franciscana*	KF691306	China	[6]
H62	*A. franciscana*	KF691320,322	Columbia	[6]
H63	*A. parthenogenetica*	KF691360,367,369-372	Iran	[6]
H64	*A. franciscana*	KF691435,437,438	Mexico	[6]
H65	*A. parthenogenetica*	KF691457	Russia	[6]
H66	*A. parthenogenetica*	KF691464,466,476	Russia	[6]
H67	*A. parthenogenetica*	KF691479,482,484,492,494	Russia	[6]
H68	*A. parthenogenetica*	KF691481,483	Russia	[6]
H69	*A. parthenogenetica*	KF691520,522-525,528,529	Turkey	[6]
H70	*A. franciscana*	KF691536,540,542,545	USA	[6]
	A. franciscana	KJ863431,438,439,443,455,460,466,467,471,474,479,481,482,487,489		[38]
H71	*A. franciscana*	KF691539	Canada	[6]
	A. franciscana	KJ863454	USA	[38]
H72	*A. urmiana*	KF707695	Iran	[43]
H73	*A. sinica*	KF707886-889	China	[43]
H74	*A. franciscana*	KJ863484	USA	[38]

Table A1. *Cont.*

Haplotypes	Species	GenBank Number	Geographical Locality	Reference
	A. parthenogenetica	KU053797-802	Djarylpach	[39]
H75	*A. parthenogenetica*	KU053803-807	Sakskoye	[39]
	A. parthenogenetica	KU053808-818	Dzharylhach	[39]
H76	*A. parthenogenetica*	KU053811,814	Ukraine	[39]
H77	*A. sinica*	LC195586	Mongolia	[44]

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