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An RFLP database for authentication of commercial cyst samples of the brine shrimp *Artemia* spp. (International Study on *Artemia* LXX)

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Abstract

Artemia cysts, originating from a variety of locations, are currently commercially exploited. The overall quality of the cysts depends on a lot of features including their intrinsic nutritional quality, harvesting and processing conditions, diapause characteristics and size, all influencing their commercial value. In order to mediate authentication of commercial *Artemia* cyst samples, a database containing eight RFLP patterns of a 1500 bp mitochondrial rDNA fragment was constructed. The database contains 53 samples, covering most of the geographical distribution of *Artemia.* On the basis of a band sharing index these 53 samples could be clustered into five groups. These groups coincide to a great extent with the currently accepted species or species complexes. Within each cluster diversity between samples is still considerable, reflecting the genetic diversity within each species. The developed method allows to assign samples to these clusters, facilitating their authentication at the species level.

Keywords: Artemia; Cyst; Authentication; Mitochondrial rDNA; RFLP

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1. Introduction

Artemia are branchiopod crustaceans that inhabit hypersaline habitats. They have been recorded in over 600 coastal and inland sites worldwide (Triantaphyllidis and Sorgeloos, 1998; Van Stappen, 2002). *Artemia* have the ability to produce storable dormant embryos, or cysts, that can hatch swiftly into live nauplii. For this reason *Artemia* is the most widely used live feed item in the larviculture of fish and shellfish.

Until recently the Great Salt Lake (Utah, USA), inhabited by *A. franciscana*, has been the largest supplier of the cyst market. Poor yields at this site from 1996 to1999 (Lavens and Sorgeloos, 2000) have stimulated harvesting companies to explore alternative resources. Several populations, especially in continental Asia, are now regularly exploited.

The recovery of *Artemia* cyst harvests from the Great Salt Lake around the turn of the millennium (9000 tons of raw cyst product harvested in season 2000–2001; Dhont and Sorgeloos, 2002) may have temporarily alleviated the need for alternative *Artemia* harvesting sites. Nevertheless, since the mid-1990's various non-*franciscana Artemia* species and strains have been introduced to the world cyst market. These new species are dominated by a variety of parthenogenetic populations, originating from sites such as the Karabogaz-Gol in Turkmenistan, a vast hypersaline bay of the Caspian Sea, but also from a plethora of small or medium-sized lakes occurring within an area, stretching from the Kurgan area in Southwest Siberia, over the Altai area (South Siberia) and Kazakhstan, eastwards into continental and coastal China. The range of applications for these strains is sometimes quite analogous to the Great Salt Lake (GSL) strain, which is unofficially considered as a 'standard' in aquaculture practices. Other strains, however, display several characteristics that deviate markedly from GSL cysts, which affects supplier and customer. These characteristics include diapause features, chorion colour, cyst and naupliar biometrics, nutritional (HUFA) profile, buoyancy, decapsulation requirement and behavior, hatching percentage and hatching rate, separation of instar I nauplii in hatching vessel, enrichment requirements, etc. This applies also to several *Artemia* sources with only local of regional commercial importance, such as *A. sinica* from several lakes in continental China and adjacent territories, and *A. urmiana* from Lake Urmia, Iran. Finally, in addition to the 'archetypes' of *Artemia* history, viz. *A. franciscana* from San Francisco Bay (SFB; California, USA) and from GSL, exploitation of hypersaline biotopes in areas like Central America and southern-hemisphere South America or inoculation and harvesting of brine shrimp (generally SFB) in coastal saltworks with or without autochthonous brine shrimp population (e.g. coastal China and monsoon Southeast Asia, resp.) has resulted in the commercial availability of 'new' *A. franciscana* strains. Although these have a limited market share in terms of tonnage, they may fetch a high price per weight unit, thanks to, e.g. low cyst diameter and/or interesting HUFA pattern, making them particularly attractive on the cyst market.

With the increased variety of *Artemia* strains reaching the market, it has become necessary to develop suitable and practical procedures to assess the overall quality of a cyst sample (Lavens and Sorgeloos, 1996). So far, no technique is available that unequivocally establishes the species or strain (an important quality feature) present in a commercial *Artemia* sample. A variety of DNA fingerprinting techniques have been used for describing the diversity within the genus *Artemia*, namely RAPD (Random Amplified

Polymorphic DNA) (Badaracco et al., 1995; Sun et al., 1999a; Camargo and Sorgeloos, 2002), AFLP (Amplified Fragment Length Polymorphism) (Triantaphyllidis et al., 1997a,b,c; Sun et al., 1999b), cyt.C I and cyt.B gene sequences comparison (Perez et al., 1994), digestion of genomic DNA with EcoRI and AluI (Badaracco et al., 1991) and analysis of nuclear 5S rRNA (Cruces et al., 1989). None of these techniques have been performed on a large set of cyst samples in order to validate their usefulness in authenticating samples.

The aim of this experimental work was to construct an *Artemia* database of identifying RFLP patterns on a mitochondrial rDNA fragment and to evaluate the relevance of the developed method as a quick and reliable molecular tool for the authentication of commercial samples.

2. Material and methods

2.1. Samples

Sample material was available as dried cysts at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University (Belgium) or at INVE Technologies NV (Baasrode, Belgium). Fifty-three samples have been used in the analysis (Table 1), covering almost the entire natural distribution area of *Artemia* species. Strict criteria were applied to assign a species name to a sample, i.e. only if cross breeding tests, morphological or molecular data described in literature, had proven or strongly evidenced the species status of that specific sample. In all other cases, the cyst sample was designated as 'unidentified species'. Yet circumstantial evidence of the species status of these 'unidentified' samples is often available (Table 1). Firstly, field observations may be an indication of the species status (e.g. in the case of parthenogenetic populations). Secondly, some 'unidentified' samples originated from the same site as 'identified' cysts, suggesting that they belong to the same species, in case there are no indications for co-occurrence of species in this habitat. Finally, literature data are available on the species status of some populations mentioned in Table 1, however without exact justification of this species designation. Thus, if less strict criteria are applied, the vast majority of samples can be assigned a species name with high probability. The species status of samples from the Lake Urmia area was uncertain, with the exception of sample ARC1230 (*A. urmiana*), as literature data evidence the existence of mixed populations in this area. Finally, the cyst samples originating from Vinh Chau (Vietnam) (Hoa, 2002) and Macau (Brazil) (Camara, 2001) were assigned the species name *A. franciscana* because the latter species was used to inoculate these sites.

2.2. DNA extraction

Dried cysts (10 mg) were weighed and suspended in 50 μl of distilled water for 2 h. The hydrated cysts were then crushed on ice with a sterile pestle directly in a 1.5 ml eppendorf tube and the DNA was extracted using either the Wizard® Genomic DNA Purification kit (Promega™, mouse tail protocol) or a CTAB method. For the latter, 600 μl of 2 × CTAB

Table 1
Sources of *Artemia* used for RFLP analysis and literature references on species status (*: unknown lake; ° samples not included in Fig. 1)

Species status	Site	ARC number	Literature references with species status evidenced	Circumstantial evidence on species status
A. franciscana	San Francisco Bay, CA, USA	1258	Gajardo et al., 2001	Kellogg, 1906
A. franciscana	Great Salt Lake, USA	1287	Triantaphyllidis et al., 1997c	Bowen et al., 1978, 1980; Belk and Bowen, 1990
A. franciscana	Macau, Brazil	1300	Camara, 2001	
A. franciscana	Vinh Chau, Vietnam	1301	Hoa, 2002	
A. franciscana	Vinh Chau, Vietnam	1454	Hoa, 2002	
A. franciscana	Vinh Chau, Vietnam	1455	Hoa, 2002	
A. franciscana	Vinh Chau, Vietnam	1456	Hoa, 2002	
A. franciscana	Vinh Chau, Vietnam	1457	Hoa, 2002	
A. persimilis	Argentina*	1321	Gajardo et al., 2001	
A. salina	Larnaka, Cyprus	1011	Triantaphyllidis et al., 1997a	
A. salina	Larnaka, Cyprus	1148	Triantaphyllidis et al., 1997a	
A. salina	Mégrine, Tunisia	1268	Triantaphyllidis et al., 1997a,c	
A. salina	Sfax, Tunisia	1269	Triantaphyllidis et al., 1997a,c	
A. salina	Wadi Natrun, Egypt	1290	Triantaphyllidis et al., 1997a	
A. sinica	Yimeng area, Inner Mongolia, China*	1188	Triantaphyllidis et al., 1997a,c	
A. sinica	Xiechi Lake, Yuncheng, Shanxi, China	1218	Triantaphyllidis et al., 1997b	
Parthenogenetic Artemia	Vineta Swakopmund, Namibia	1186	Triantaphyllidis et al., 1996b, 1997a,b	
Parthenogenetic Artemia	Aibi Lake, Xinjiang, China	1236	Triantaphyllidis et al., 1997a	
Parthenogenetic Artemia	Citros, Pieria, Greece	1280	Triantaphyllidis et al., 1997b	
Parthenogenetic Artemia	Ankiembe saltworks, Madagascar	1314	Triantaphyllidis et al., 1996a	
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1322	Prouva, 1997	
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1407	Nyonye, 2000	
Unidentified Artemia	Kazakhstan*	1016		
Unidentified Artemia	Uzbekistan*	1162		
Unidentified Artemia	Xiechi Lake, Yuncheng, Shanxi, China	1206		Originating from same site as 1218 (*A. sinica*)

Unidentified Artemia	Urmia Lake area, Iran	1226	Van Stappen, 2002 (occurrence of both *A. urmiana* and parthenogenetic Artemia in Urmia Lake area)
Unidentified Artemia	Urmia Lake area, Iran	1229	Van Stappen, 2002 (occurrence of both *A. urmiana* and parthenogenetic Artemia in Urmia Lake area); *A. urmiana* according to Abatzopoulos et al., 2002 and Triantaphyllidis et al., 1997a
Unidentified Artemia	Gahai Lake, Qinghai, China	1265	Xin et al., 1994; Thomas, 1995; Beardmore et al., 1996 (parthenogenetic)
Unidentified Artemia	Mono Lake, CA, USA	1277	Bowen et al., 1985; Browne and Bowen, 1991; Lenz and Browne, 1991. (*A. monica*, closely resembling *A. franciscana*)
Unidentified Artemia	Great Salt Lake, UT, USA, harvest 1996	1320	Bowen et al., 1978, 1980; Belk and Bowen, 1990; originating from same site as 1287 (*A. franciscana*)
Unidentified Artemia	Sayten Lake, Kazakhstan	1367	Baitchorov and Nagorskaya, 1999 (parthenogenetic)
Unidentified Artemia	Karabogaz-Gol, Turkmenistan	1374	Originating from same site as 1322 and 1407 (parthenogenetic)
Unidentified Artemia	Karabogaz-Gol, Turkmenistan	1380	Originating from same site as 1322 and 1407 (parthenogenetic)
Unidentified Artemia	Haolebaoji, Inner Mongolia, China	1415	Xin et al., 1994, 1999; Thomas, 1995, Triantaphyllidis et al., 1997c (*A. sinica*)
Unidentified Artemia	Megalon Embolon, Greece	1420	Abatzopoulos et al., 1986, 1989, 1993 (parthenogenetic)
Unidentified Artemia	Blenheim saltworks, New Zealand	1422	
Unidentified Artemia	Xiechi Lake, Yuncheng, Shanxi, China	1434	Originating from same site as 1218 (*A. sinica*)
Unidentified Artemia	Urmia Lake area, Iran	1444	Van Stappen, 2002 (occurrence of both *A. urmiana* and parthenogenetic Artemia in Urmia Lake area)
Unidentified Artemia	Tuhum Lake, Mongolia	1463	
Unidentified Artemia	San Francisco Bay, CA, USA	1470	Kellogg, 1906; originating from same site as 1258

(continued on next page)

Table 1 (*continued*)

Species status	Site	ARC number	Literature references with species status evidenced	Circumstantial evidence on species status
(*A. franciscana*)				
Unidentified Artemia	San Francisco Bay, CA, USA	1472		Kellogg, 1906; originating from same site as 1258
(*A. franciscana*)				
Unidentified Artemia	Karabogaz-Gol, Turkmenistan	1504		Originating from same site as 1322 and 1407 (parthenogenetic)
Unidentified Artemia	Urmia Lake area, Iran	1505		Van Stappen, 2002 (occurrence of both *A. urmiana* and parthenogenetic Artemia in Urmia Lake area)
Unidentified Artemia	Medvezhe (Bear) Lake, Kurgan area, Russia	1507		Parthenogenetic according to own field observations
Unidentified Artemia	Great Salt Lake, UT, USA, harvest 1999	1508		Bowen et al., 1978, 1980; Belk and Bowen, 1990; originating from same site as 1287 (*A. franciscana*)
Unidentified Artemia	Great Salt Lake, UT, USA, harvest 2000	1509		Bowen et al., 1978, 1980; Belk and Bowen, 1990; originating from same site as 1287 (*A. franciscana*)
Unidentified Artemia	Great Salt Lake, UT, USA, harvest 2001	1520		Bowen et al., 1978, 1980; Belk and Bowen, 1990; originating from same site as 1287 (*A. franciscana*)
Unidentified Artemia	Bolshoe Yarovoe, Altai area, Russia	1552		Baitchorov and Nagorskaya, 1999 (parthenogenetic)
Unidentified Artemia	Bolshoe Yarovoe, Altai area, Russia	1553		Baitchorov and Nagorskaya, 1999 (parthenogenetic)
Unidentified Artemia	Bonaire Duinmeer, Netherlands Antilles	28		Vanhaecke et al., 1987 (*A. franciscana*)
Unidentified Artemia	Estuario de Virrila, Peru	479		Vanhaecke et al., 1987 (*A. franciscana*)
Unidentified Artemia	Curaçao Fuik, Netherlands Antilles	502		Vanhaecke et al., 1987 (*A. franciscana*)
Unidentified Artemia	Port Araya, Venezuela	554		Vanhaecke et al., 1987 (*A. franciscana*)
A. urmiana°	Urmia Lake, Iran	1230	Triantaphyllidis et al., 1997c	
A. tibetiana°	Lagkor Co, Tibet, China	1347	Abatzopoulos et al., 2002	

buffer (Tris–HCl 100 mM, pH 8.0, NaCl 1.4 M, EDTA 20 mM, CTAB 2%) was added to the hydrated cysts and the mixture was incubated for 30–60 min at 60 °C. After centrifugation (14 000 × *g* for 15 min), the supernatant was extracted with phenol/chloroform (1:1), centrifuged (14 000 × *g* for 5 min) and extracted again with chloroform-isoamyl alcohol (24:1). After centrifugation (14 000 × *g* for 5 min) 2.5 volumes of 100% ethanol was added to the supernatant and the DNA was allowed to precipitate at − 20 °C for 2 h. This was centrifuged at 14 000 × *g* for 15 min and then the DNA pellet was washed with 70% ethanol, left to dry at room temperature and redissolved in 50 μl of HPLC water. The CTAB extraction method produced higher quality DNA as judged by the more consistent success in PCR-amplifying the desired fragment. Hence this method was applied on all samples. A Chelex method was used to extract DNA from single cysts. Therefore one cyst suspended in 20 μl of 5% Chelex-100 (Biorad, Belgium) for 2 h was crushed with a sterile pestle directly in the test tube and incubated at 60 °C for 30 min (with intermittent vortexing every 10 min), followed by incubation at 100 °C for 8 min. After centrifugation (14 000 × *g* for 5 min), the supernatant was collected and used directly for PCR.

2.3. PCR

PCR was performed in a Hybaid PCR Express (Labsystems™, Belgium). The amplification reactions were carried out in a final volume of 50 μl containing a mixture of 50–100 ng of DNA, Tris–HCl 5 mM (pH 8.3), $MgCl_2$ 5 or 2.5 mM, dNTP's 0.2 mM, 1.75U. DNA polymerase mixture (Expand™ High Fidelity PCR system, Roche™ Molecular Biochemicals), and 25 μM of each primer. The thermal cycler PCR conditions were as follows: 1 cycle of 94 °C for 2 min, 34 cycles of 1 min 15 s at 94 °C, 45 s at 52 °C, 2 min at 72 °C and a final extension cycle of 72 °C for 4 min.

The primer combinations 12SA/16Sbr, 12S-R/16S-F and 12S-SP/16S-SP have been used in order to amplify the fragment of DNA to be studied, namely a 1500 bp fragment of mitochondrial rDNA. The primer sequences were as follows: 12SA: ctaggattagataccctattag, 16Sbr: ccggtctgaactcagatcacg, 12S-R: ctaggattagataccctattagTT, 16S-F: ccggtctgaactcagatcacgTAG, 12S-SP: CTAGGATTAGATACCCTA, 16S-SP: CCGGTCTGAACTCAGATC. All these sequences were taken from the published mitochondrial rDNA sequence of *A. franciscana* (Valverde et al., 1994) based on their very high homology to the universal primers (16Sbr-3′ and 12SA-5′) for mitochondrial rDNA (Palumbi, 1996). The 16S-SP primer was identical to the universal primer, while the 12S-SP primer differed in two positions from the universal primer. All primers amplified the same 1500 bp fragment as they only differ from each other at the 3′ end. It was determined via experimental procedure that DNA extracted using the CTAB protocol, followed by PCR amplification in the presence of 2.5 mM $MgCl_2$ and the primer combination 12S-SP/16S-SP yielded good amplification from all samples.

2.4. Restriction digestion and data analysis

The PCR products, verified for the appropriate size by agarose gel electrophoresis, were purified using the WIZARD® DNA Purification System (Promega™). The restriction

enzymes (used according the manufacturer's instructions at 5 units per reaction) HaeIII, NdeII, HpaII, HinfI and DdeI (Roche), MseI, Tsp 509I (Biolabs), TaqI (Promega) were used to detect polymorphism in the samples. Digested products were electrophoretically separated on agarose gel (2.5%) in a 1 × TAE buffer. An UV transilluminator was used to visualise the fragments stained with ethidium bromide. Agarose gels were photographed with a Polaroid film. The images, scanned by a flatbed scanner (HP™ Scanjet II cx), were processed with the Gelcompar II®software (Applied Maths, Sint-Martens-Latem, Belgium). The automatic band searching facility of the software was used. Any band that represented more than 1% of the total surface under the densitometric curve was scored. The scoring of bands was then edited manually. Bands smaller than 100 bp were eliminated, as they could not be scored consistently. Bands were then automatically assigned to band classes. For this purpose, the position tolerance (the maximal shift, in percentage of the pattern length, between two bands) and the optimisation (the shift allowed between two patterns) for each restriction enzyme was calculated by the software. It was possible that the procedure of automated band class assignment, without further manual editing, introduced some erroneous allocation of bands to certain band classes. This procedure (automated band class assignment) was however sustained because of the speed it allowed in processing RFLP results and building matrices of similarities, a characteristic that is desirable for quick authentication of commercial samples. For each restriction enzyme, a matrix of band class sharing coefficients between the generated patterns was calculated based on the Dice index [$2n_{AB}/(n_A + n_B)$: in which n_{AB} is the number of band classes with the same electrophoretic mobility shared by sample A and B and n_A and n_B are respectively the number of band classes present in sample A and B]. The eight matrices were averaged, serving as input for the construction of an unweighted pair-group method arithmetic average (UPGMA) dendrogram. By calculating the final UPGMA dendrogram in this way, the contribution of each restriction enzyme was equal, irrespective of the amount of bands that had been generated by each one.

3. Results

3.1. The 1500 bp mitochondrial rDNA fragment

Little is currently known about the genetic variability of the selected 1500 bp mitochondrial rDNA fragment and its usefulness for authenticating commercial *Artemia* samples. Neither the intra-nor interpopulation genetic variability at a given time is known for all tested samples, nor its temporal stability. Given the fact that mitochondrial rDNA polymorphism is normally found at the genus or species level (Palumbi, 1996), and due to allopatric distribution of *Artemia*, it was anticipated that this marker would be able to differentiate samples at the species or sub-species level. Fig. 1 shows the *Nde* II pattern of the 1500 bp fragment generated using gDNA extracted from a batch of cysts (sample ARC 1154). The sum of the size of the bands of this pattern is higher than 1500 bp, suggesting either a technical problem (e.g. partial digestion) or the presence of PCR products with different DNA sequences (hereafter called "double pattern"). In order to investigate the possibility of a technical problem, the experiment was repeated several times with different

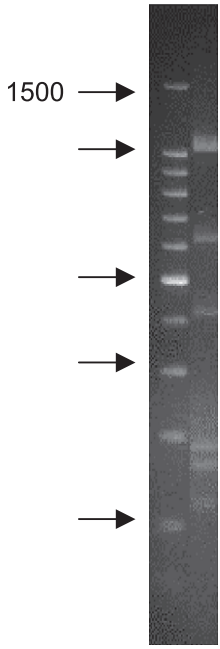

Fig. 1. Restriction digest of the 1500 bp mt rDNA fragment with the enzyme Nde II. The PCR fragment was generated using DNA isolated from a batch of cysts as target DNA (strain ARC 1154). Lane 1: 100 bp ladder (top to bottom: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp), lane 2: Nde II pattern.

DNA/restriction enzyme ratio's and incubation times, always yielding the result shown in Fig. 1. This strongly suggested that this pattern was not the result of partial digestion. To further analyse the phenomenon, individual cysts were examined (Fig. 2), in order to obtain a preliminary assessment of the intra-sample variability of the chosen mt rDNA marker. The results demonstrated that in some samples no intra-sample variability could be detected with this marker (results not shown), but in other samples, cysts produced different RFLP patterns (Figs. 1 and 2). In addition it showed that even one cyst could display a restriction pattern in which the sum of the bands is more than 1500 bp (which is most likely due to the fact that the $\pm$ 1080 bp fragment in some DNA strands is further cut into $\pm$ 640 and $\pm$ 440 bp fragments) (Fig. 2, lane 3, 5 and 7). Identical results were obtained when the restriction digest conditions were changed. It is also unlikely that the presence of the 1080 bp fragment is the consequence of a partial digest, as all other putative partially digested fragments were absent. This suggested that within a cyst more than one type of fragment was amplified in the PCR reaction. This phenomenon can be explained by heteroplasmy, or by the presence of mtDNA pseudo-genes in the nuclear genome (Parfait et al., 1998; Bensasson et al., 2000; Williams and Knowlton, 2001). It was outside the scope of this study to exactly establish the nature of the observed phenomenon. However, the results did indicate that the occurrence of a double restriction pattern is not necessarily an indication of intra-population genetic variation (although it can not be

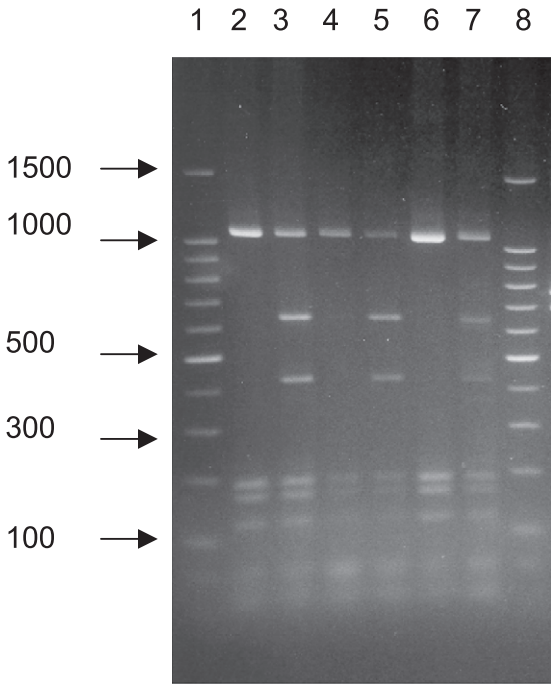

Fig. 2. Restriction digest of the 1500 bp mt rDNA fragment with the enzyme Nde II. The PCR fragment was generated using DNA isolated from a single cyst as target DNA (strain 1154). Lane 1 and 8: 100 bp ladder, lane 2 to 7: Nde II RFLP pattern from individual cysts.

excluded) of the mt rDNA marker, but that it could originate from a specific genetic background in certain cysts. Given this possible genetic background, it was decided to screen RFLP patterns carefully for double patterns, in order to avoid the incorporation of such samples in the reference database.

3.2. The reference database

Based on the criteria described in the section Material and Methods, fifty-three samples were withheld (Fig. 3). Twenty-two samples had a species status sufficiently evidenced in literature, but indirect evidence allowed to identify 23 more samples (Table 1). Two samples referred to in literature as respectively *A. urmiana* (ARC1320) and *A. tibetiana* (ARC1347) (Table 1) were not retained in the reference database, as they generated double restriction patterns with at least four different enzymes (result not shown). In total 182 RFLP band classes were scored. They were all found to be polymorphic within these 53 samples. On the basis of Dice similarities between them a UPGMA dendrogram was generated, visualising the reference database (Fig. 3).

The dendrogram showed five major groups (with similarities among them lower than 32%). From top to bottom in the dendrogram these groups were: a group called ‘parthenogenetic’, containing nearly all investigated parthenogenetic strains, samples from

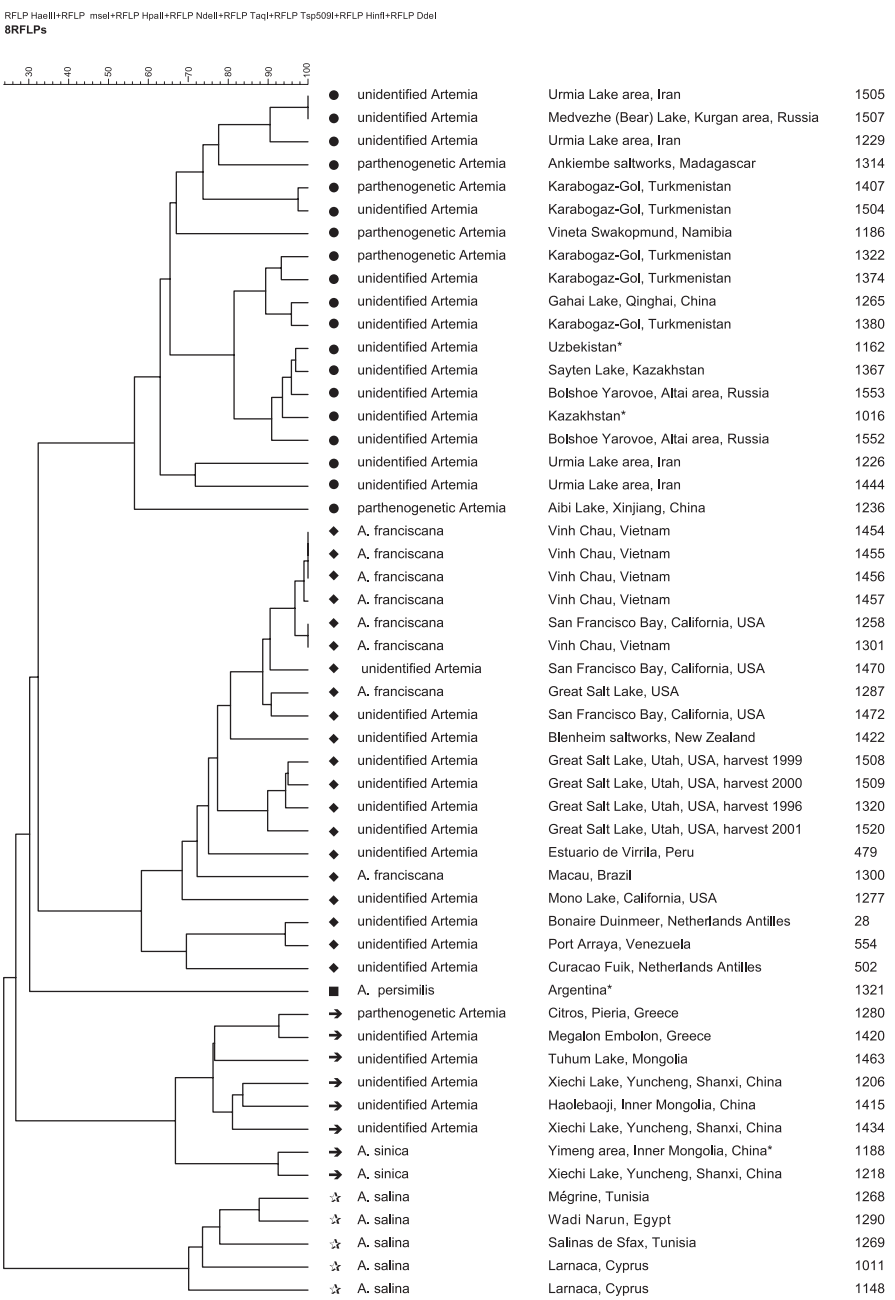

Fig. 3. UPGMA dendrogram of Dice band sharing similarities, based on 8 restriction digests of a 1500 bp mt rDNA fragment of 53 *Artemia* samples (*: unknown lake). ● parthenogenetic group, ◆ franciscana group, ■ persimilis group, → sinica group, ☆ salina group.

the Lake Urmia area, and a few unidentified strains. This group did not contain two parthenogenetic strains from Greece. Those are clustering within the 'sinica' group. A second group ('franciscana' group) joined all *Artemia* samples from North as well as South America (except ARC1321, see further), including some type strains of *A. franciscana* with verified species status, and the populations from Vietnam, produced from *A. franciscana* inoculations. A third group ('persimilis'; ARC1321) contained one *A. persimilis* type strain. The fourth group ('sinica' group) contained two *A. sinica* type strains, a series of Chinese samples with indirect evidence for being *A. sinica*, and the two parthenogenetic Greek samples. The last group ('salina') contained only samples that are known to be *A. salina*.

3.3. Validation

The reference database contained the *A. franciscana* sample ARC1258 originating from San Francisco Bay (USA). This sample has been used to inoculate salt ponds in Vietnam. Five samples (ARC 1454,1455,1456,1457 and ARC1301) from different year classes (see Table 1 and Baert et al., 1997) were investigated. Among them the similarity was higher than 96% (Fig. 3). As the rDNA fragment is a conservative marker, this high similarity was expected and underscores the robustness of the data generating procedure. It also underlines that the described RFLP-based technique is able to group very related samples together, which allows to confirm the authenticity of the Vietnam samples as *A. franciscana*.

The robustness of the technique was further verified by performing a sensitivity analysis with respect to the number of restriction enzymes necessary to form the described groups. It was found that four restriction enzymes (namely HaeIII, MseI, HpaII and NdeII) were sufficient to group the 53 samples as shown in Fig. 3. Even two restriction enzymes (namely the combinations MseI/HpaII and HpaII/HaeIII) were sufficient to maintain the same grouping. Finally using only one restriction enzyme (HpaII) the integrity of the 'franciscana', 'sinica' and 'parthenogenetic' clusters was maintained (results not shown). These results indicated that the RFLP database with eight restriction enzymes contained information redundant for grouping the reference samples as shown in Fig. 3. It also suggested that only a limited number of restriction enzymes may be sufficient for authentication, depending on the desired resolution of the analysis. For instance if it were only necessary to confirm the presence of *A. franciscana* in a sample, an RFLP analysis with an indicative restriction enzyme (like HpaII) should be sufficient. The HpaII restriction patterns displayed two fragments, namely 1200 and 240 bp (Fig. 4), that were present in all strains from the ''franciscana'' group and absent in all other samples, making them putative markers for that group. In addition all *Artemia* samples originating from North America displayed an unique 541 bp HinfI fragment. Other groups also displayed unique restriction fragments: samples from the ''sinica'' group originating from China showed unique 1486 bp TaqI and 464 bp Hinf I fragments. The 317 bp HinfI, the 273 DdeI and the 205 bp HaeIII fragments were characteristic for the ''salina'' group. Finally the ''parthenogenetic'' group was characterised by the presence of a typical 297 bp HpaII fragment. *A. persimilis* was represented in the dendrogram by one sample. Yet, two HpaII (656 and 611 bp) and two DdeI fragments (453 and 421 bp) were unique for this sample. It

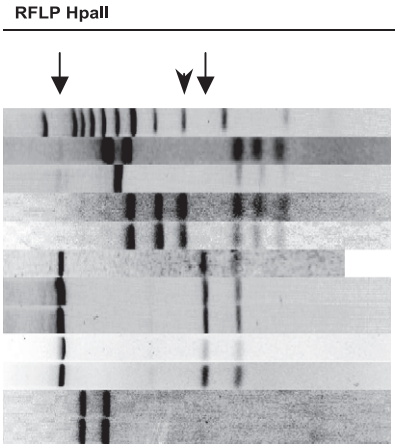

Fig. 4. HpaII digest of the 1500 bp mitochondrial rDNA fragment of samples from the reference database, showing the typical patterns of the various groups. Arrows indicate the unique 1200 and 240 bp restriction marker fragments of the "franciscana" group. The arrowhead indicates the 297 bp marker fragment unique to the parthenogenetic group. DNA ladder (first lane) as in Figs. 1 and 2.

remains to be established if these fragments are real markers for this group, through the analysis of other *A. persimilis* populations.

The applicability of the technique for authenticating samples was verified in those cases where particular samples show "double" restriction patterns. A small number of samples (Table 2), with known species status, in which "double" RFLP patterns had been detected with only one or two restriction enzymes, was treated as unknown and authenticated. For

Table 2
Samples containing parthenogenetic *Artemia* according to literature evidence, displaying double RFLP patterns, used to validate the database

Strain	Site	ARC number	Double restriction pattern with	Reference
Parthenogenetic Artemia	Yimeng area, Inner Mongolia, China	1315	HinfI	Magsodi, 1999
Parthenogenetic Artemia	Yimeng area, Inner Mongolia, China	1264	NdeII	Magsodi, 1999
Parthenogenetic Artemia	Altai area, Kazakhstan	1388	HinfI	Prouva, 1997
Parthenogenetic Artemia	Kuchukskoye Lake, Russia	1389	HinfI	Prouva, 1997
Parthenogenetic Artemia	Karasjuk Lake, Kazakhstan	1387	HpaII	Prouva, 1997
Parthenogenetic Artemia	Tanggu, Tianjin, China	1187	TaqI	Prouva, 1997
Parthenogenetic Artemia	Wu Shen Qi County, Inner Mongolia, China*	1242	TaqI	Magsodi, 1999
Parthenogenetic Artemia	Bameng area, Inner Mongolia, China*	1317	NdeII	Magsodi, 1999

Table 3
Identification of the samples listed in Table 2

Species	Site	ARC number	% identity
Parthenogenetic Artemia	*Bameng area, Inner Mongolia, China**	*1317*	*100.0*
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1322	81.9
Parthenogenetic Artemia	*Wu Shen Qi County, Inner Mongolia, China**	*1242*	*100.0*
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1322	78.8
Parthenogenetic Artemia	Vineta Swakopmund, Namibia	1186	73.1
Parthenogenetic Artemia	Ankiembe saltworks, Madagascar	1314	72.9
Parthenogenetic Artemia	*Tanggu, Tianjin, China*	*1187*	*100.0*
Parthenogenetic Artemia	Ankiembe saltworks, Madagascar	1314	77.5
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1322	72.3
Parthenogenetic Artemia	*Karasjuk Lake, Kazakhstan*	*1387*	*100.0*
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1322	71.2
Parthenogenetic Artemia	Vineta Swakopmund, Namibia	1186	70.8
Parthenogenetic Artemia	*Kuchukskoye lake, Russia*	*1389*	*100.0*
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1322	80.9
Parthenogenetic Artemia	Vineta Swakopmund, Namibia	1186	76.8
Parthenogenetic Artemia	Ankiembe saltworks, Madagascar	1314	70.1
Parthenogenetic Artemia	*Altai, Kazakhstan*	*1388*	*100.0*
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1322	84.4
Parthenogenetic Artemia	Vineta Swakopmund, Namibia	1186	72.3
Parthenogenetic Artemia	*Yimeng area, Inner Mongolia, China*	*1264*	*100.0*
Parthenogenetic Artemia	Karabogaz-Gol, Turkmenistan	1322	82.3
Parthenogenetic Artemia	Vineta Swakopmund, Namibia	1186	80.3

Tested samples are in italic; samples with the highest similarity are listed below.

this purpose, the ‘‘identification’’ feature of GelCompar software was used, using the 53 entries shown in Fig. 3 as the reference database. All samples, except one, were correctly assigned to their respective alleged species-specific group, as illustrated by their similarity to database entries (Table 3). Only one sample ARC1315, previously described as containing parthenogenetic *Artemia* (on the basis of observations made on adults) displayed an RFLP pattern very similar to those of the ‘sinica’ group.

4. Discussion

The aim of this study was to construct a reference database of RFLP patterns that would allow for the authentication of commercial *Artemia* samples. The world market is dominated by samples coming from North America (*A. franciscana*) and to less extent from Asia (either parthenogenetic *Artemia* or *A. sinica*). The ‘‘franciscana’’ group contains all samples from North and South America (except for ARC1321 which is *A. persimilis*), which corroborates the knowledge that all populations from this continent (except for the southern part of South America) belong to the superspecies *A. franciscana.* Also the sample from Mono Lake, sometimes considered as a separate species *A. monica* closely related to *A. franciscana* (Bowen et al., 1985; Browne and Bowen, 1991; Lenz and Browne, 1991) is contained within the ‘‘franciscana’’ group. The samples from Vietnam, where ARC1258 *A. franciscana* has been inoculated, are very closely related to this

original inoculation material (similarity higher then 95%). The sample from Macau (Brazil), where San Francisco Bay *A. franciscana* was used to inoculate the saltworks, is clearly situated within the "franciscana" group. Finally, a sample from Blenheim saltworks (New Zealand), where *A. franciscana* was introduced (Trotman, pers. comm.), also links up with the "franciscana" group. All these results and the fact that not a single putative *A. franciscana* sample shows up in the other groups, indicate that the RFLP procedure on the rDNA fragment can generate enough information to allocate putative *franciscana* samples to the "franciscana" group. Furthermore, samples from this group are characterised by the presence of two unique HpaII restriction fragments and, as for Great Salt Lake or San Francisco Bay samples, by a unique HinfI fragment. These fragments can hence be used as markers for the allocation of commercial samples to the "franciscana" group.

Especially since the decline of the cyst harvest at Great Salt Lake in the period 1996–1999, parthenogenetic *Artemia* strains have been introduced on the world market, mainly originating from South Siberia, Central Asian republics and Bohai Bay area in China. In terms of aquaculture application, these strains display characteristics sometimes considerably diverging from the Great Salt Lake strain: often relatively large cysts and nauplii, different diapause behaviour, decapsulation characteristics and buoyancy, cyst colour, etc. Also the diversity among parthenogenetic strains is very high (Triantaphyllidis et al., 1997b), which is reflected in the dendrogram. Except for the two strains from Greece, no parthenogenetic strain figures in other groups in the dendrogram.

The presence of the four strains from the Urmia Lake area in the "parthenogenetic" group merits more consideration. Firstly, the diversity among the four samples is high (the similarity between ARC 1505 and 1229 on one hand, and ARC 1444 and 1226 on the other, is only 62%). This may indicate that this area harbors distinct populations. There is growing evidence that the Urmia Lake and its adjacent lagoons and saltpans are inhabited by *A. urmiana* as well as by parthenogenetic strains (Van Stappen, 2002). The results presented here could reflect this situation. This would however imply that *A. urmiana* is closely related to parthenogenetic *Artemia* strains, as has been suggested by Beardmore and Abreu-Grobois (1983), Triantaphyllidis et al. (1997a) and Sun et al. (1999a,b). The possible syntopic occurrence of parthenogenetic strains and *A. urmiana* might result in cyst samples that contain these different species. In this study one sample (ARC 1230: described in literature as *A. urmiana*; Triantaphyllidis et al., 1997c) from the Urmia area was analysed that clearly displayed double restriction patterns with four enzymes. This finding suggests that this particular sample (ARC1230) contains at least two different *Artemia* types, one of which could be *A. urmiana*. That fact that by RFLP analysis this sample turns out to be a mixed sample, might be related to the fact that DNA in unhatchable cysts is analysed as well. Also the ARC1229 sample has been described in literature as containing *A. urmiana* (Table 1). The RFLP analysis in this study on this sample did not reveal indications for the mixed presence of strains or species. Hence this sample is presumably *A. urmiana*. The RFLP results in combination with literature data highlight that caution is needed when interpreting literature results published on material from Urmia Lake. In conclusion, further research on samples from the Urmia area is necessary in order to unravel the local complexity, preferentially making use of multidisciplinary approaches as advocated by Abatzopoulos et al. (2002) and Gajardo et al. (2002).

For this purpose, analysis should be performed on individuals (animals or cysts) rather than on a batch of cysts.

In summary RFLP analysis on the mitochondrial DNA fragment groups most of the parthenogenetic strains (except two Greek strains), which allows for their authentication. This could be confirmed through the correct identification of the parthenogenetic strains mentioned in Table 2. With the current resolution of the technique and the current status of reference samples being analysed, it is difficult to distinguish between parthenogenetic strains and putative *A. urmiana* strains. However, viewing the limited commercial importance of *A. urmiana*, the unability of the current procedure to distinguish them from parthenogenetic strains is at least for the time being less relevant. Because of the diversity in this group only one unique restriction fragment (generated by HpaII digest) was found, that could be used as a marker fragment.

All *A. sinica* samples (either with verified species status or named using circumstantial evidence, Table 1) were grouping together, indicating that identification of *sinica* samples is possible. Also for this group the presence of unique restriction fragments (1486 bp TaqI and 464 bp HinfI fragments) can facilitate the authentication. The situation for *A. salina* is very similar. In this case three marker fragments could be identified.

At least one *Artemia* site in Tibet is the habitat of the novel *A. tibetiana* species (Abatzopoulos et al., 2002). During the course of this study one Tibet sample (ARC 1347) was analysed that clearly contained more than one RFLP pattern, which again indicates that different populations might occur syntopically. This finding corroborates the observation by Van Stappen (2002) that salt lakes in that area might be inhabited by *A. tibetiana* as well as parthenogenetic strains. More insight needs to be generated in order to incorporate *A. tibetiana* samples into the reference database.

Diversity observed in samples collected from the same habitat (e.g. Karabogaz-Gol parthenogenetic *Artemia;* ARC1374; 1322; 1380; 1407; 1504, minimal similarity among them is 62%, Fig. 3) can be considerable. Further detailed research should confirm this interpopulation diversity. The fact that samples from the same location do not always display high similarities (for which a variety of reasons could be conceived, including spatial or temporal differences in population structure in one lake) prohibits at the moment to authenticate commercial samples below the species level. To achieve this below-species level of authentication, it will be necessary to study the inter-population and intra-population spatial and temporal differences in much more detail.

Although it is difficult to anticipate the future commercial availability of various *Artemia* strains and species on the world market, it is clear that using the RFLP methodology to discriminate between populations below the species level, may be potentially useful for some species but less relevant for others. The *A. franciscana* superspecies complex certainly warrants in-depth study. This is due to a variety of reasons, including: the high genetic diversity of its natural populations, which are spread widely over a vast area with a variety of biotopes (Van Stappen, 2002), its widespread use in aquaculture and the occurrence of newly established populations, as a result of deliberate inoculation, which are developing a genetic adaptation to their new environment (Clegg et al., 2000, 2001). From a commercial point of view, refinement and validation of the technique to further authenticate the conglomerate of parthenogenetic populations, especially those originating from continental Asia, is of importance. In spite of their

availability on the world market, the origin of commercial batches is commonly obscure. This is due to the complexity of local trade markets, the secrecy of the commercial actors involved, and/or the fact that batches from different origin may be mixed for processing and subsequent marketing.

During the composition of the reference database, samples have been analysed that displayed easily scorable double restriction patterns with four or more restriction enzymes. Such type of samples, which presumably contained a mixture of *Artemia* species or subspecies, cannot be authenticated using the currently described procedure (namely DNA extraction on 10 mg of cysts, followed by PCR amplification of mitochondrial rDNA and RFLP analysis). In such cases the analysis should be performed on a cyst-by-cyst basis, which would be of course more expensive and time-consuming. However, other techniques could be used to investigate a batch of cysts in a cost-effective way. The high diversity detected within the rDNA fragment certainly offers the possibility to develop methods, such as DGGE (denaturing gel gradient electrophoresis) analysis on PCR fragments, with which the presence of a mixture in commercial samples could be established. Upon detection of a mixture with the DGGE technique, authentication of the components of the commercial sample could be accomplished by performing the RFLP analysis on reamplificated isolated bands or on individual cysts.

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