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SEM Study of Diversity in the Cyst Surface Topography of Nine Parthenogenetic *Artemia* (Crustacea: Anostraca) Populations from China

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KEY WORDS *Artemia*; scanning electron microscope; cyst shell; surface morphology

ABSTRACT The cysts of nine Chinese populations of parthenogenetic *Artemia* were studied by scanning electron microscope. In the 270 cysts examined, 15 different morphological patterns were recognized with most of them not recorded in previous studies and the "tubercled shell surface" being the most common pattern. Results also displayed high intrapopulation variability, with the maximum of 11 patterns (in 30 cysts) recorded from the Barkol population. No positive correlation between the diversity of cyst shell patterns and ploidy compositions was found. Principal components analysis suggests higher similarity among coastal populations than among inland populations, which may be attributed to the identity of physicochemical conditions among coastal salterns and dissimilarity among inland saline lakes. *Microsc. Res. Tech. 77:1005–1014, 2014.* © 2014 Wiley Periodicals, Inc.

INTRODUCTION

The cysts (resting eggs) of Branchiopoda are able to tolerate environmental stresses for long periods (Amarouayache et al., 2009; Amarouayache and Derbal, 2011; Brendonck et al., 1996; Plodsomboon et al., 2012). This physiological adaptation is the main reason that branchiopods can be dispersed to new habitats via water birds and wind (Brendonck and Riddoch, 1999; Figuerola and Green, 2002; Green and Figuerola, 2005; Green et al., 2005). Observations of branchiopod cysts have shown that the surface patterns were often species specific and have been used as traits to prepare preliminary taxonomical keys as well as to evaluate intraspecific variability (Belk, 1989; Brendonck, 1989; Brendonck and Coomans, 1994a, 1994b; Brendonck et al., 1992; Miličić and Petrov, 2009; Munuswamy et al., 1985; Mura et al., 1984; Mura, 1986, 2001; Mura and Thiery, 1986; Martin, 1989; Martin and Belk, 1989; Shepard and Hill, 2001; Rabet, 2010; Timms and Lindsay, 2011). For the genus *Artemia*, surface structures of cyst shells have been studied at first by optical microscopy (Morris and Afzelius, 1967; Mazzini, 1978) and more recently by scanning electron microscopy (SEM). The SEM studies have shown inter- and intrapopulation variability (Gilchrist, 1978; Mazzini, 1978; Mura, 1986; Munuswamy, 1988; Ramasubramanian and Munuswamy, 1993; Rosowski et al., 1997; Shepard and Hill, 2001; Spotte and Anderson, 1988; Sugumar and Munuswamy, 2006; Wang and Sun, 2007).

Parthenogenetic *Artemia* consist of populations with different ploidy degrees (di-, tri-, tetra-, pentaploid and also heteroploids) and populations of mixtures of different ploidies. Diploid parthenogens are characterized by automixis and therefore usually polyclonal, while polyploids are apomictic parthenogens with monoclonal generations (Abatzopoulos et al., 2002, 2003; Barigozzi, 1974; Browne and Bowen, 1991; Triantaphyllidis et al., 1998). To date, cyst shell morphology of parthenogenetic *Artemia* has been studied for only handful populations, including one population from Italy, four populations from India and three populations from China (Mura, 1986; Ramasubramanian and Munuswamy, 1993; Sugumar and Munuswamy, 2006; Sivagnanam et al., 2013; Vetriselvan and Munuswamy, 2011; Wang and Sun, 2007). In this paper the cyst shell surface of nine *Artemia* populations are studied by SEM. The aim of the study is to gain a better understanding of the variety of cyst shells of parthenogenetic *Artemia*.

MATERIALS AND METHODS

Parthenogenetic *Artemia* cysts were collected from nine sites in China, including all reported ploidy levels in parthenogenetic *Artemia*, i.e., di-, tri-, tetra-, and pentaploidy (Table 1; Fig. 1).

In each population, 30 undamaged and dry cysts were selected for observing the topography of shell surface. All specimens were coated with a conductive layer of gold-palladium about 5 nm in thickness and observed with a Hitachi S3400 Scanning Electron Microscope.

The frequencies of morphological patterns of cyst shells were used as traits for the grouping of populations with principal component analysis (PCA). The computer program SPSS 16 was performed for the statistical analysis.

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TABLE 1. List of the studied populations of parthenogenetic Artemia *from China and their summarized information*

Locality	Abbreviation	Geographic coordinates	Sampling year	Altitude (m)	Ploidy composition	References
Aibi Lake (Ebinur), Jinghe, Xinjiang	AB	44°53′N, 83°00′E	2000	194	$2n > 90\%$	Yang et al. (1995)
Barkol Lake (Balikun Lake), Barkol, Xinjiang	BRK	43°40′N, 92°44′E	1986	1617	$2n = 27.4\%$, $3n = 4.9\%$ $4n = 37.8\%$, $5n = 29.9\%$	Yang et al. (1995)
Dongfanghong Saltern, Laizhou, Shandong	DFH	37°20′N, 119°55′E	1985	~Sea level	$2n > 98\%$	Pan et al. (1991)
Gaodao Saltern, Wendeng, Shandong	GD	36°59′N, 122°03′E	1985	~Sea level	$2n = 72.9\%$, $4n = 6.8\%$ $5n = 20.8\%$	Pan et al. (1991)
Xiaotan Saltern, Haiyang, Shandong	XT	36°41′N, 121°08′E	1985	~Sea level	$2n > 92.4\%$	Pan et al. (1991)
Hoh Lake (Keke Salt Lake), Ulan, Qinghai	HOH	36°57′N, 98°17′E	1988	2995	$4n = 100\%$	Xu et al. (1993)
Xuwen Saltern, Xuwen, Guangdong	XW	20°15′N, 109°56′E	1987	~Sea level	Not analyzed	
Dongfang Saltern, Dongfang, Hainan	DF	19°11′N, 108°41′E	1988	~Sea level	$4n > 80\%$	Chang and Sun (pers. comm.)
Yinggehai Saltern, Ledong, Hainan	YGH	18°31′N, 108°44′E	1987	~Sea level	$5n > 97\%$	Xu et al. (1993)

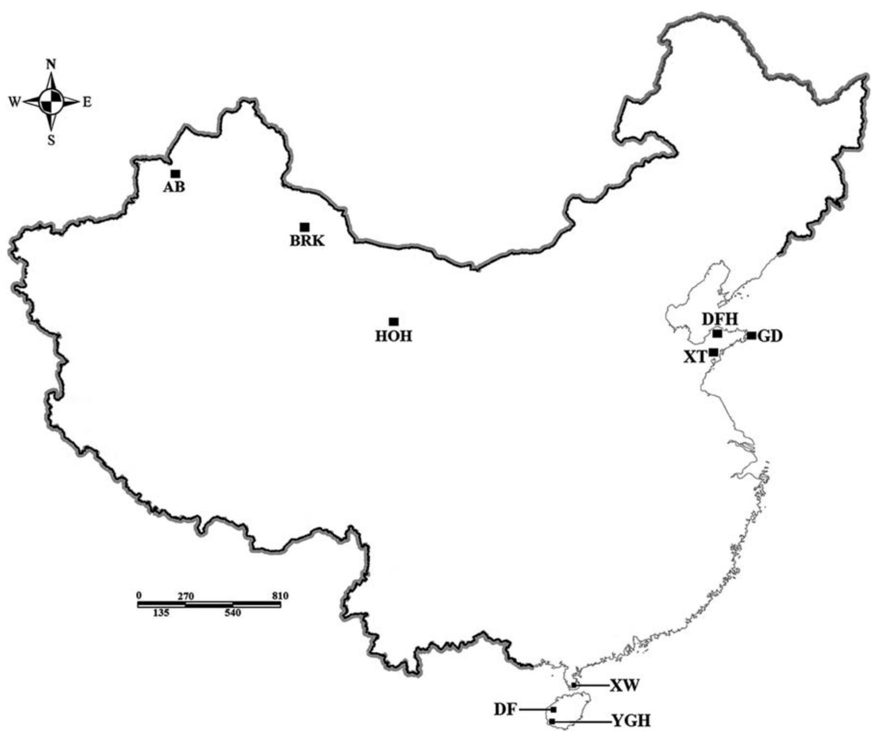


Fig. 1. Geographical distribution of the nine studied parthenogenetic *Artemia* populations from China.

RESULTS

The SEM observations of 270 cysts (30 cysts/population × 9 populations) showed that the decorations of shells could be classified into 15 types (Table 2; Fig. 2). The frequencies of the observed types are listed in Table 3. Type VI was observed in all populations and had the highest total frequency (91 cases). Type VI also had the highest frequency in all populations with the exception of DF, DFH, and AB. The highest frequencies in DF were affiliated to types VIII and XII (nine cases), as well as Type XIII in DFH (eight cases) and Type V in AB (seven cases). The most appearance of Type VI belonged to XW and YGH (17 cases). Three morphological Types were recorded only in one population (BRK: Types XI; AB: Types IV; and DFH: Type IX). BRK, AB, and DFH exhibited the highest

TABLE 2. *Description of the 15 types of shell surface topography of* Artemia *cysts shown in Figure 2*

Type	Description
I	Spongy surface
II	Surface with shallow pocks, which are either evenly distributed (Figs. 2A and 2B left) or intermittently distributed (surface with smooth areas and pocked areas; Figs. 2A and 2B right)
III	Surface with densely distributed large nipple-like knobs
IV	Rugged surface that is divided into various blocks by narrow creases
V	Surface with pore-like fossulae, which may be densely distributed (Figs. 2A and 2B left) or restricted to some areas of the shell surface (mosaic distribution; Figs. 2A and 2B right)
VI	Rugged surface with densely distributed tiny tubercles
VII	Wrinkled surface with sparsely distributed wart-like knobs
VIII	Surface approximately smooth, but with shallow depressions
IX	Rugged surface with tubercles and wide pocks
X	Surface with numerous tiny nipple-like knobs
XI	Surface with rugged swellings and pore-like fossulae
XII	Surface with densely distributed creases and shallow pocks
XIII	Surface with big lumps, which possess tiny tubercles
XIV	Surface with big lumps, which are covered with nipple-like tubercles
XV	Surface with densely distributed lumps, lumps covered with tiny tubercles

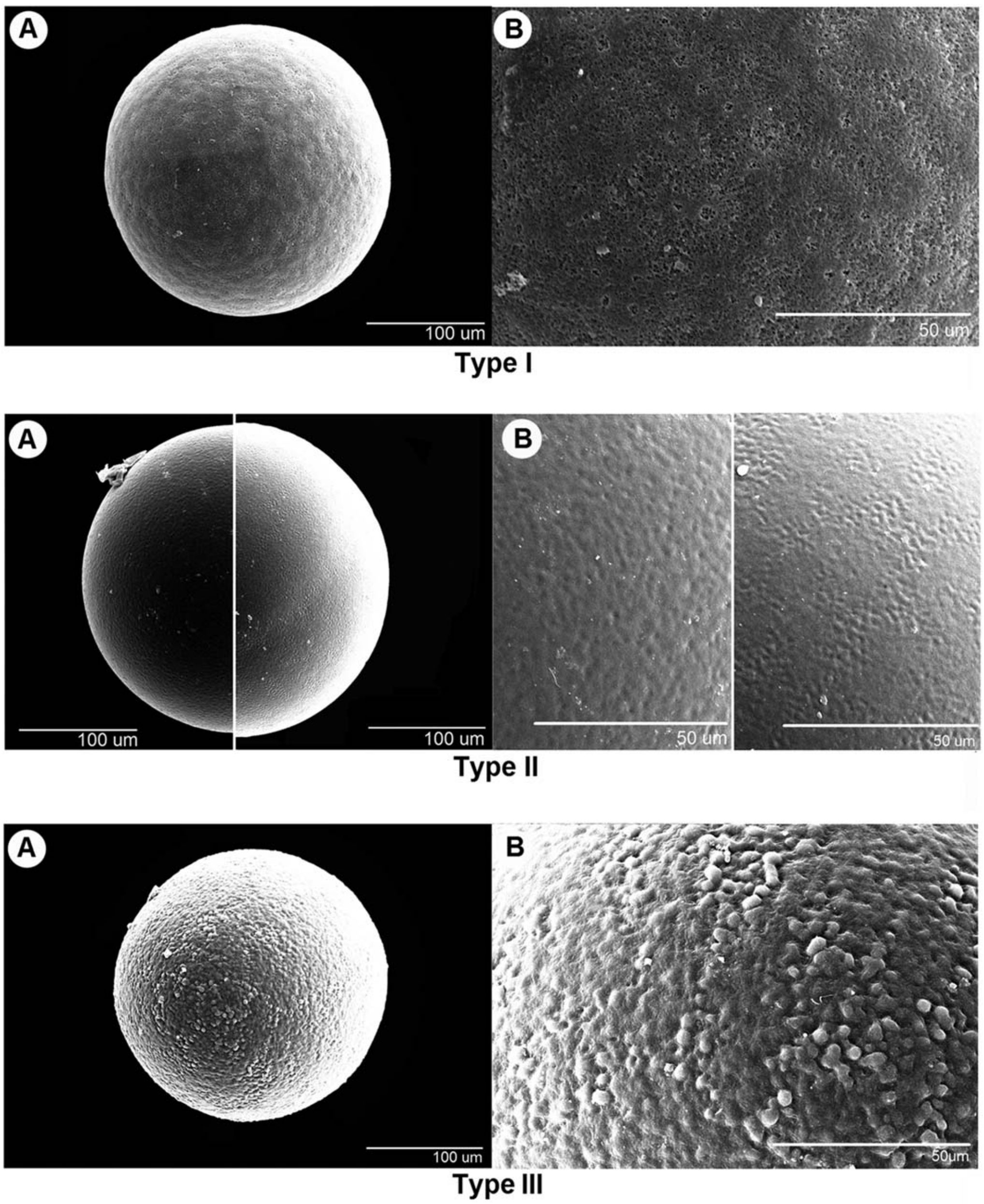
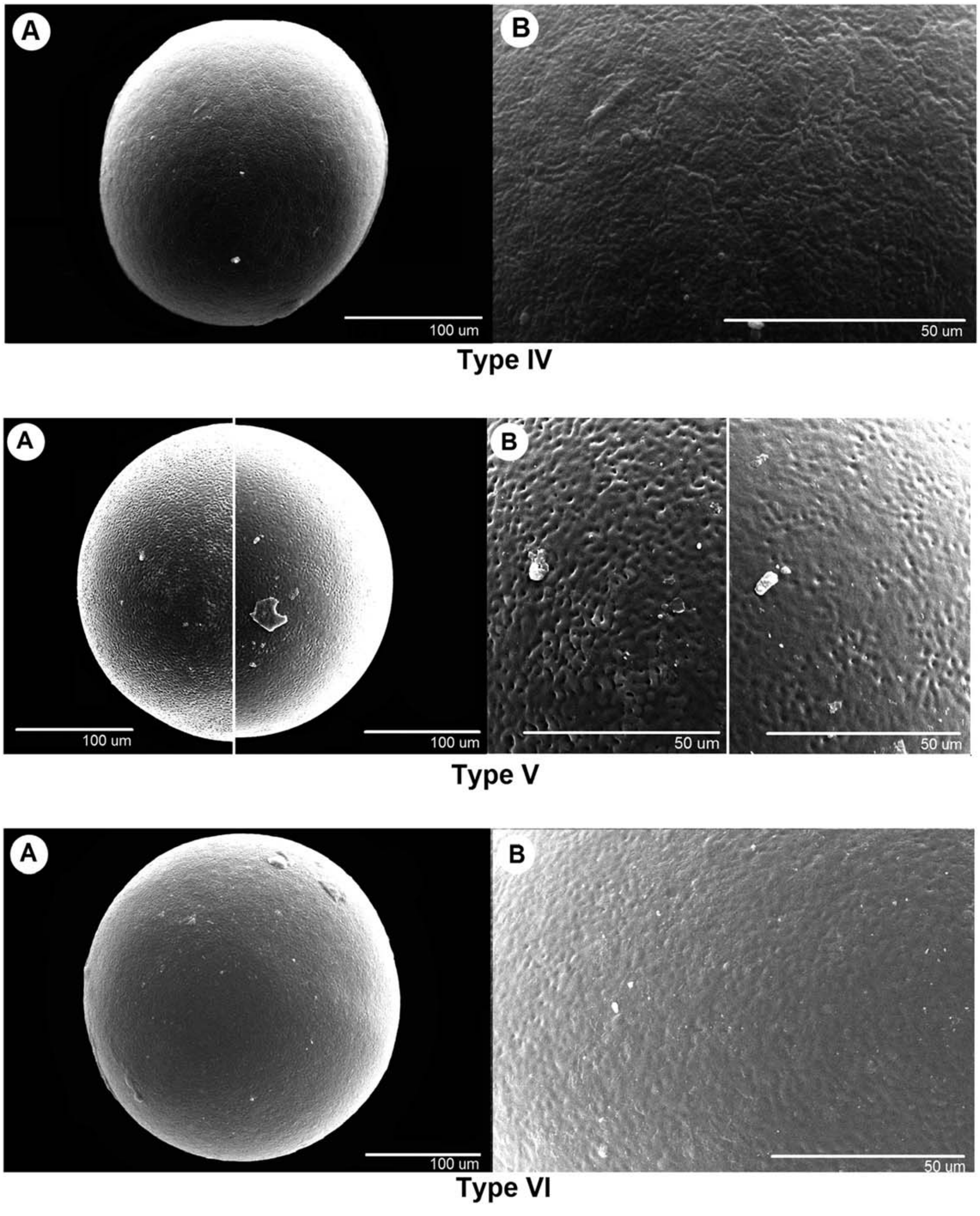


Fig. 2. SEM photographs of the cyst shell surface of parthenogenetic *Artemia* from nine Chinese sites, showing the morphological difference of 15 types of shell surface topology (A: overall view; B: close up view). To be continued.



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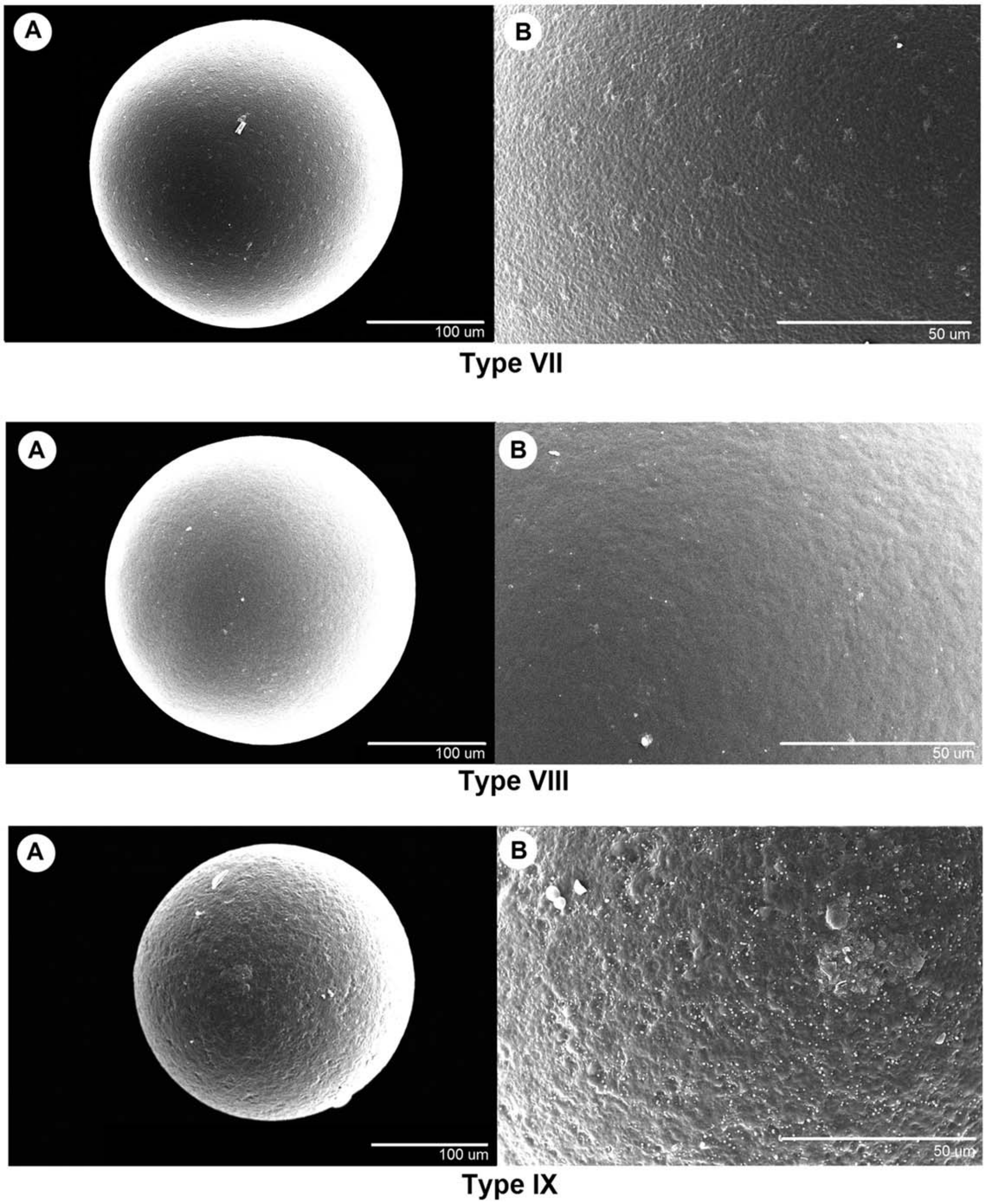
diversity, while XW and YGH had the lowest morphological variation (Table 3).

The results of PCA were shown in Table 4 and Figure 3. The first and second factors demonstrated 28.5% and 21. 4% of the variance, respectively, and on the whole the two components involved 49.4% of separation (Table 4). Types V (0.885) and VII (0.866) were effective characters in the first component as well as Type XI and XIV (0.978) in the second one. The least competent characters related to Types XIII (0.022) in the first component and Type X (−0.002) in the second one (Table 4). Principal components analysis categorized the nine *Artemia* populations into five separate groups. The first one included XT, GD, and DF, the second contained DFH, YGH, and XW, and the other three groups comprised a single population of BRK, AB, and HOH, separately (Fig. 3).

DISCUSSION

The first observation on the surface morphology of *Artemia* cysts dates back to Morris and Afzelius



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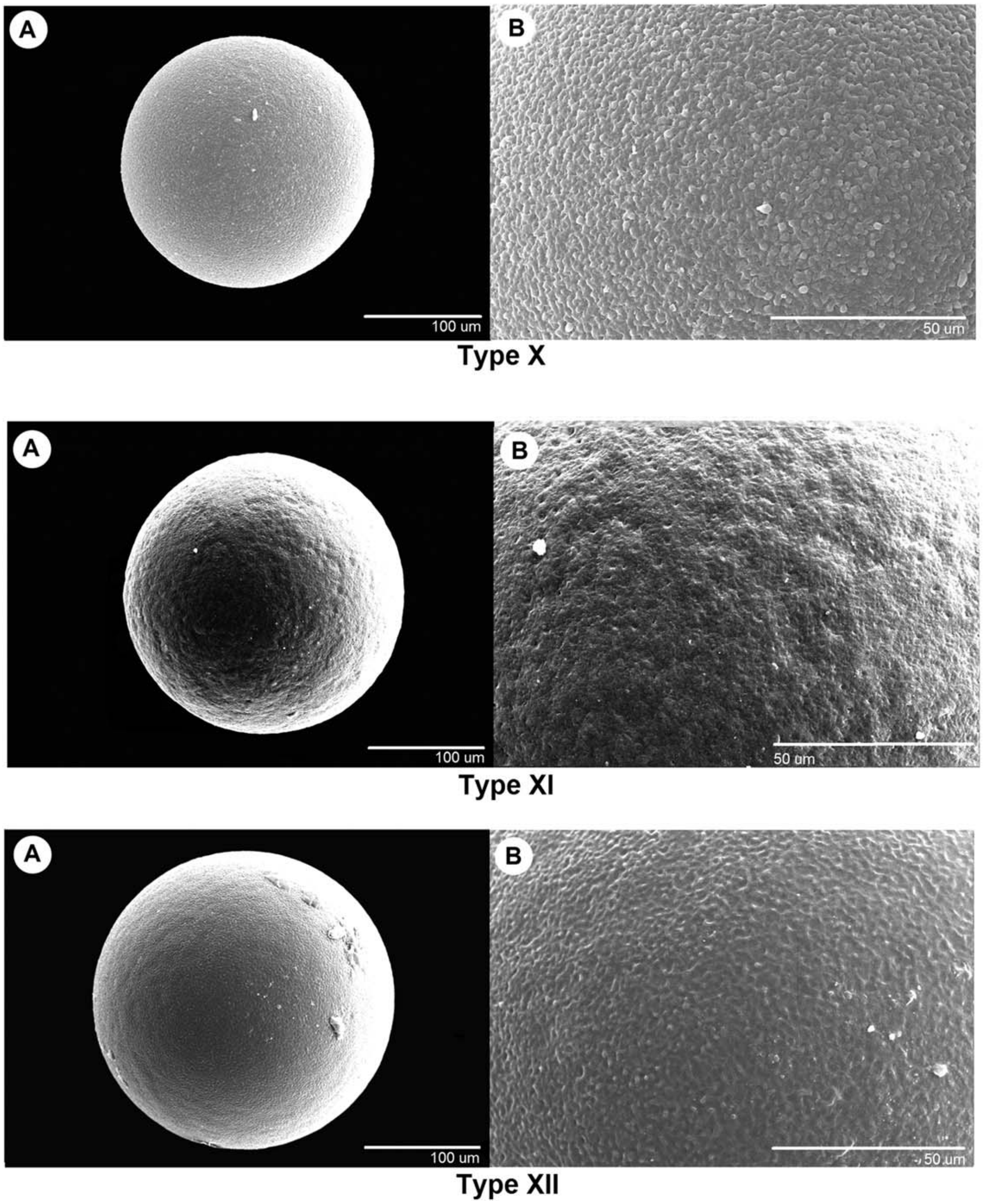
(1967), who reported that their commercial cysts (from North Carolina) had a grainy surface under dark field optics. Mazzini (1978) recounted that cysts from San Francisco were also grainy under light microscopy, but SEM observations showed that cysts from San Francisco possessed a smooth surface (Gilchrist, 1978; Mazzini, 1978). Although in these works the species were reported as *Artemia salina* (Linnaeus, 1758), the Californian *Artemia* from San Francisco is *Artemia franciscana* Kellogg, 1906 (Van Stappen, 2002). Further studies confirmed the smooth surface of *A. franciscana* cysts (Rosowski et al., 1997; Spotte and Anderson, 1988; Shepard and Hill, 2001). The other Californian species, *Artemia monica* Verrill, 1869 is quite distinct from *A. franciscana* in having a smooth to rugose cyst surface covered by button-like projections (Shepard and Hill, 2001). Two Italian *Artemia* populations from Margherita di Savoia (mixture of di- and tetraploid parthenogens) and Simbirizzi (*Artemia* sp.) displayed smooth surface lacking pores (Mura, 1986). Since only small numbers of cysts were likely checked in these studies



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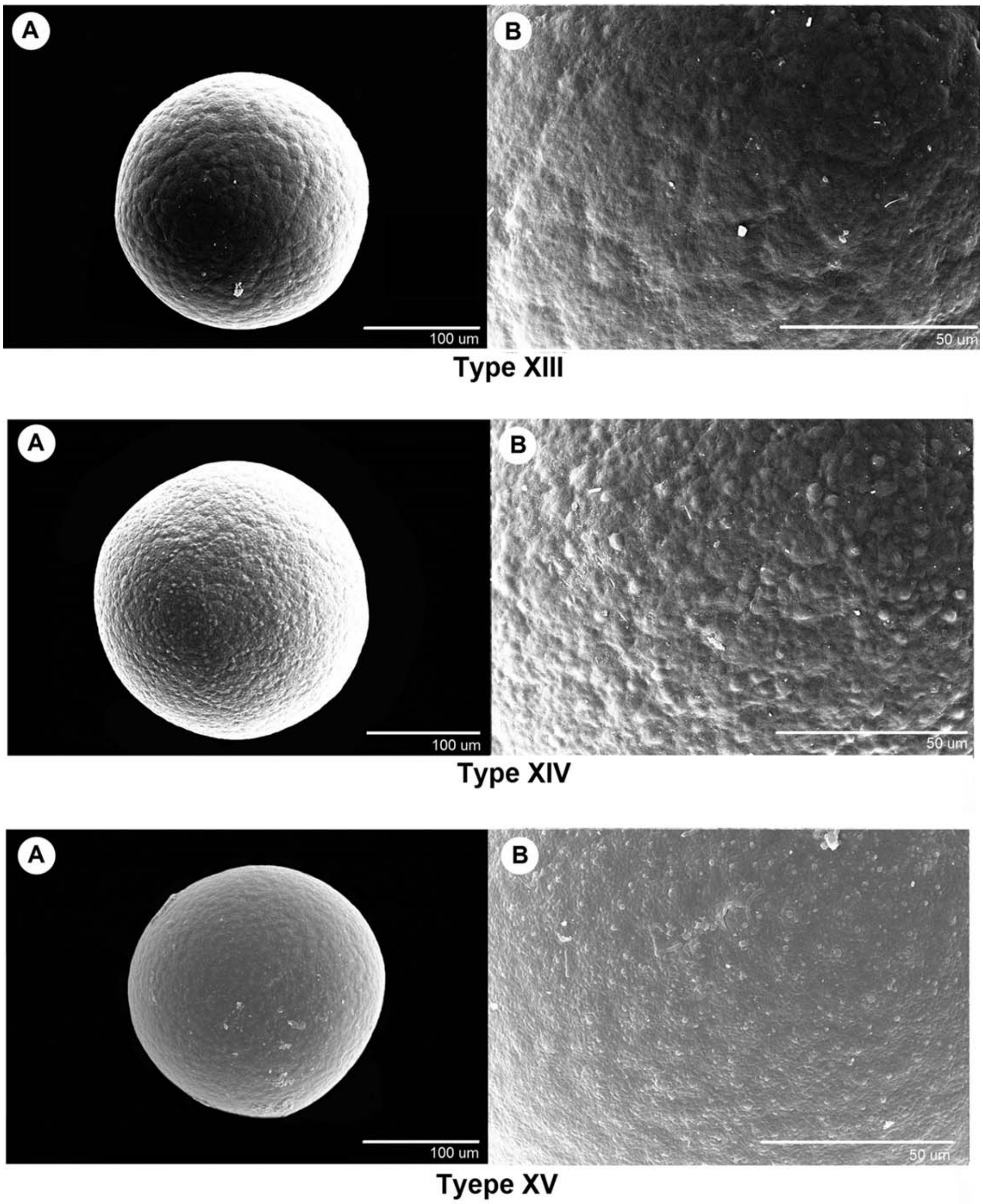
(sample size not mentioned), the intra species/population variations in cyst shell morphology might have been underestimated. For instance, Munuswamy (1988) reported that the Indian population (near to Tuticorin) had a smooth cyst shell with granular structure, while the same population was described with very smooth shell surface by a later work (Sugumar and Munuswamy, 2006).

The first extensive study of the cyst shell morphology of Chinese bisexual and parthenogenetic *Artemia* was done by Wang and Sun (2007). They examined 30 cysts for each of the seven populations studied by SEM, and recorded six different ornamental patterns for shell surface. Population specific surface patterns were found for cysts of two parthenogenetic populations. Ga Hai cysts were characterized by a surface with wart-like ornaments that are composed of packed minute tubercles, while "rugged surface" was only recognized in Chengkou cysts. This study also found that smooth surface was a common pattern in *Artemia tibetiana* Abatzopoulos et al., 1998 (three populations), *Artemia sinica* Cai, 1989 (one population) and in the



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parthenogenetic Aqqikkol Lake population. Among the present 15 patterns of cyst surface topography, Type VI (Fig. 2; Table 2) was similar to the “rugged surface, with minute tubercles densely arranged but not piling up to form larger humps” of Wang and Sun (2007: Fig. 1D); Type V (Fig. 2; Table 2) was similar to the “surface with numerous, densely spaced pore-like fossulae” of Wang and Sun (2007: Fig. 1F); Type XV (Fig. 2; Table 2) was similar to the “surface with wart-like structures, which are densely arranged and composed of packed minute tubercles” of Wang and Sun (2007: Fig. 1C). Although the smooth surface has been reported for cysts of both bisexual and parthenogenetic *Artemia* (Gilchrist, 1978; Mazzini, 1978; Mura, 1986; Spotte and Anderson, 1988; Ramasubramanian and Munuswamy, 1993; Rosowski et al., 1997; Shepard and Hill, 2001; Sugumar and Munuswamy, 2006; Vetriselvan and Munuswamy, 2011; Wang and Sun, 2007), no completely smooth shell surface was found in the present work (Table 2; Fig. 2). Additionally, Indian parthenogenetic populations collected from saltpans of Kelambakkam, Thamaraikulam, and Vedaranyam revealed smooth surface and

TABLE 3. The frequency of the 15 types of cyst shells in nine parthenogenetic Artemia *populations (data shown as number of cysts observed)*

Type	Population									Total	Population frequency[a]
	BRK	AB	XW	XT	DF	DFH	YGH	GD	HOH		
I	1	0	0	0	0	0	0	0	4	5	2
II	2	4	0	0	0	1	0	0	2	9	4
III	2	0	0	1	0	0	0	0	0	3	2
IV	0	1	0	0	0	0	0	0	0	1	1
V	4	7	0	1	0	2	0	1	7	22	6
VI	7	6	17	11	3	7	17	11	12	91	9
VII	0	1	1	0	0	0	0	0	2	4	3
VIII	1	1	0	0	9	1	8	0	2	22	6
IX	0	0	0	0	0	1	0	0	0	1	1
X	3	0	8	6	3	6	0	7	0	33	6
XI	1	0	0	0	0	0	0	0	0	1	1
XII	0	1	0	10	9	3	4	3	0	30	6
XIII	2	4	4	0	2	8	1	2	1	24	8
XIV	3	0	0	0	0	0	0	0	0	3	1
XV	4	5	0	1	4	1	0	6	0	21	6
Total	30	30	30	30	30	30	30	30	30	270	
Type frequency[b]	11	9	4	6	6	9	4	6	7		62

[a]Population frequency = number of populations observed for a certain type of cyst shell.
[b]Type frequency = number of types observed in a certain population.

TABLE 4. Rotated component matrix of principal component analysis for the variables of cyst shell patterns in all nine populations

Variable	Component				
	1	2	3	4	5
I	0.799	0.128	−0.351	−0.145	−0.152
II	0.749	0.150	0.610	0.135	0.022
III	−0.069	0.929	0.029	−0.163	0.093
IV	0.380	−0.257	0.820	−0.017	0.081
V	0.885	0.126	0.385	0.027	0.014
VI	0.067	−0.270	−0.679	−0.342	0.368
VII	0.866	−0.319	−0.125	−0.156	0.155
VIII	−0.207	−0.155	−0.090	−0.123	−0.885
IX	−0.089	−0.062	−0.106	0.964	0.011
X	−0.616	−0.002	−0.214	0.211	0.661
XI	0.098	0.978	0.076	−0.021	0.041
XII	−0.698	−0.180	0.036	−0.168	−0.437
XIII	0.022	−0.147	0.163	0.919	0.241
XIV	0.098	0.978	0.076	−0.021	0.041
XV	−0.208	0.218	0.825	−0.121	0.066
Eigenvalues	4.27	3.21	2.53	2.14	1.26
% of variance	28.46[a]	21.43[a]	16.86	14.25	8.37

[a]First two factor produced by principal component analysis explain a cumulative 49.89% of total variance.

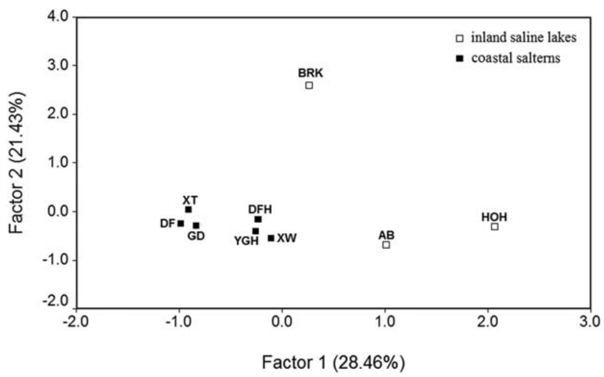

Fig. 3. Scatterplot of principal component analysis based on morphological patterns of cyst shells for nine parthenogenetic *Artemia* populations from China.

Puthalam population represented rugose pattern (Ramasubramanian and Munuswamy, 1993; Sugumar and Munuswamy, 2006; Vetriselvan and Munuswamy, 2011).

Our results showed higher intrapopulation variation in cyst surface ornamentation compared to all previous studies, with the highest variation occurring in BRK (11 patterns) and the lowest in YGH and XW (4 patterns; Table 3). Though the diploid populations like AB ($2n > 90\%$) and DFH ($2n > 98\%$) appeared to have more patterns than the polyploid populations, e.g., YGH (four patterns; $5n > 97\%$), a positive correlation could not be established between the diversity of cyst shell pattern and ploidy composition, e.g., the tetraploid HOH population ($4n = 100\%$) possesses higher variation (eight patterns) than the mostly diploid populations XT and GD (Tables 1 and 3). Hypothetically, the cyst shell patterns might be determined by genotypes, environmental conditions and/or physiological status of mothers, or both genetic feature and postnatal factors (i.e., the phenotypic plasticity). For a parthenogenetic *Artemia* population, genetic diversity might be through mutation (for both diploid and polyploidy stains), automixis (for diploid strains; Abatzopoulos et al., 2003), and/or mixture of strains with genetic differences. However, existing data do not provide any positive explanations for the determination of cyst shell patterns. Future comparative studies on the cysts of cloned laboratory populations may help to understand the roles of genetic and postnatal factors in the determination of cyst shell patterns.

PCA showed that the grouping of populations by cyst morphological patterns is not in accordance with ploidy compositions. For example, two tetraploid populations, HOH ($4n = 100\%$) and DF ($4n > 84\%$), are located in opposite side of factor 1 axis; XT ($2n > 92\%$) and DF ($4n > 84\%$) are collected in same group, while YGH ($5n > 97\%$) and DFH ($2n > 98\%$) in the other one (Fig. 3). The grouping of populations (Fig. 3) is also incongruous with the PCA results on biometrical data of cysts (Asem and Sun, 2014: Fig. 3). However, all the six coastal populations are located on the left and the three salt lake populations on the right, and the latter three populations are well separated in the PCA scatterplot (Fig. 3). This suggests that the cyst morphology of inland populations seems to have higher dissimilarity than the coastal saltern populations, which may be related to the higher divergence in the chemical and physical conditions among different inland hypersaline waters.

In conclusion, cyst shells of parthenogenetic *Artemia*, even with interpopulation variation, displayed high intrapopulation variability. Tubercled shell surface is the most common in cysts of the nine studied *Artemia* populations. The use of cyst shell morphology as a taxonomical criterion should be approached with caution as large sample sizes for all species from different populations need to be carefully examined. Comparative studies on cysts of cloned laboratory populations are suggested to understand the roles of genetic feature and environmental factors in determining the variation in cyst shell patterns.

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