GENETIC ANALYSIS OF *Brachionus plicatilis* STRAINS USED FOR AQUACULTURE: PRELIMINARY RESULTS

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INTRODUCTION

Rotifers are considered an invaluable live feed and nowadays rotifer mass culturing comprises a critical branch of the global marine fish culture industry.

Most attention has been focused on *Brachionus plicatilis* (class Monogononta), a cosmopolitan cyclical parthenogen typically occurring in inland salt lakes and coastal lagoons. A series of studies, initiated in the mid 90s, have given a thorough insight into the taxonomic status and population structure of this commercially important zooplankter [1]. The employment of modern molecular tools (microsatellites, DNA sequencing) on adults as well as resting eggs, has unraveled patterns of ecological diversification and adaptation previously undetected. As a result, *Brachionus plicatilis* is considered a cryptic species complex comprised of a number of relatively undescribed species.

Increased rotifer production and culture standardization are highly dependent on determination of mixtures of species with possibly divergent culture requirements and ecological optima. It is therefore obvious that more population genetic approaches are needed which will also greatly benefit applied research on the field. In this regard and within the framework of an EU multi-partner research project we have undertaken the genetic characterization of *Brachionus* laboratory clones, hatchery samples as well as wild populations through microsatellite genotyping and DNA sequencing. This preliminary survey will be subsequently used for establishing mixtures of genetically identified clones in order to evaluate possible genetic impoverishment related to culture crashes.

MATERIALS AND METHODS

Microsatellite genotyping

- Laboratory clones

Four different clones, namely 10, L1, L3, L7, sent to us from Laboratory of Aquaculture & ARC (Ghent University, Belgium) were investigated. Sample size for each clone was 139, 95, 93 and 34 individuals, respectively. DNA extractions were performed using the Chelex protocol [2]. PCR amplification for a battery of 7 microsatellite loci (Bp1b, Bp2, Bp3, Bp4a, Bp5d, Bp6b and Bp3c) followed conditions described in [3]. Samples were genotyped (Fig. 1) on a Li-COR 4200 DNA Analyzer using the Saga^{GT} software.

- Wild population

Sixty individuals from Koronia Lake (Thessaloniki, Greece) were also analyzed for 2 microsatellite loci (Bp5d, Bp3c). Results were treated with Genepop and Genetix computer programs.

Sequencing of the Cytochrome Oxidase I (COI) mitochondrial region

A total of 14 clones, originating from 6 laboratories (Mexico, Belgium, Japan, SpainA, SpainB, Vietnam) and 2 hatcheries (Norway, Italy), all raised in ARC, were analyzed through sequencing of a 713 bp region of the mitochondrial COI gene. DNA extractions and PCR amplification followed the protocols outlined in [1]. The PCR product was purified with the QIAGEN gel purification kit and subsequently used for the sequencing reactions, performed with EXCELL II DNA sequencing kit (EPICENTRE) on a Li-COR 4200 DNA Analyzer. The sequences obtained were analyzed and aligned with the GENBANK sequences of [1] by using the Bioedit computer program. A UPGMA tree (Fig. 2) based on pairwise distances between sequences was also constructed with the PHYLIP software.

TABLE I. Genotyping results of clones 10, L1, L3 & L7

	Microsatellite locus						
	Bp1b	**Bp2**	**Bp3**	**Bp4a**	**Bp5d**	**Bp6b**	**Bp3c**
Clone							
10	247/247	136/154	139/139	218/227	231/240	119/119	202/208
L1	238/238	136/136	218/218	218/218	231/231	119/125	211/211
L3	232/238	136/136	142/151	203/227	231/234	119/119	199/199
L7	247/247	145/145	151/151	180/180	240/240	125/125	205/205

TABLE II. Genetic variability estimates at two microsatellite loci (Bp5d, Bp3c) in Koronia (Greece) *Brachionus* sp. population

Locus	**Sample size**	**Number of alleles per locus**	**Ho**	**He**	**P**
Bp5d	60	2	0.417	0.493	0.293
Bp3c	60	7	0.633	0.591	0.022*
Mean	60	4.5	0.525	0.542	

P: probability of conformity to H-W proportions (exact test), *: significant at the 0.05 level

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RESULTS - DISCUSSION

Microsatellite genotyping

- Laboratory clones

All clones were successfully genotyped at all loci (Table I, Fig. 1). Certain microsatellite loci can be used to distinguish different mixtures of *Brachionus* clones. For example, locus Bp3c serves as a marker for all possible combinations of clonal mixtures (due to the presence of clone-specific alleles) whereas Bp6b can only discriminate a limited number of mixtures. However, use of microsatellite loci combinations can best achieve confident clone designation as well as evaluation of overall mixture variability. The same approach could also provide critical information on mixture susceptibility to crashes.

- Koronia Lake population

Genetic variability estimates for the two microsatellite loci analyzed are shown in Table II. Genetic variability levels in the Koronia Lake population are similar to those of other wild populations reported in the literature [4]. However, we report on the presence of two novel alleles, namely 208 and 217, at the Bp3c locus albeit in low frequencies (Table III). Absence of Hardy-Weinberg (H-W) equilibrium (based on a single locus, see Table II) cannot be safely evaluated before more loci on additional samples are tested.

Sequencing of the COI mitochondrial region

According to the recent work of [1] there are at least nine genetically divergent lineages within the *B. plicatilis* species complex. Our results (Fig. 2) show that the majority of the laboratory clones analyzed, do not belong to *B. plicatilis* or *B. rotundiformis* but to the 'cayman' species. This species was found in 10 out of the 14 clonal samples investigated. Only one clone from Mexico was found to be *B. plicatilis* sensu stricto. The other 'species' found were 'manjavacas' (one of the two SpainB clones) and 'nevada' (both Norwegian clones). These facts raise important questions about the supposed species status of the strains used in hatcheries around the world and the management and culture conditions that should be used to avoid crashes. Future analyses will include sequencing of more clones as well as identification of clones with restriction enzymes that could ventilate species-specific patterns.

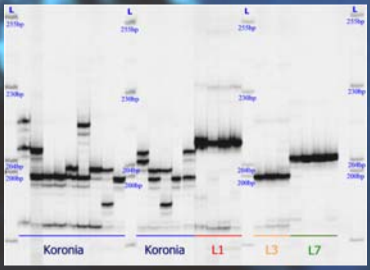

Figure 1. Example of genotyping (Bp3c locus).

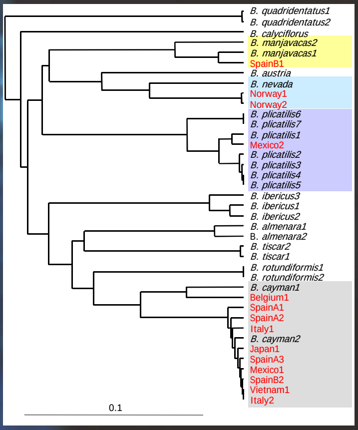

Figure 2. UPGMA dendrogram based on mtDNA COI sequences. Red font indicates samples analyzed in this work.

TABLE III. Allele frequencies for two microsatellite loci in Koronia (Greece) *Brachionus* sp. population

Locus	**Allele**	**Frequency**
Bp5d	236	0.442
	239	0.558
Bp3 c	190	0.050
	199	0.600
	202	0.192
	205	0.008
	208	**0.075**
	211	0.017
	217	**0.058**

Values in **bold** indicate novel alleles

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