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Denaturing Gradient Gel Electrophoresis (DGGE) as a tool for the characterisation of *Brachionus* sp. strains

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Abstract

Many zooplanktonic organisms, like the cyclic parthenogenetic rotifer *Brachionus plicatilis* (Rotifera: Monogononta), are actually a complex of species and biotypes with a high degree of morphological similarity (*i.e.* cryptic species). Various phylogenetic studies with molecular markers (*e.g.* ITS1 and COI) on wild *Brachionus* populations described the presence of at least nine genetically divergent *Brachionus* species and biotypes. Because different studies found evidence that these cryptic species and biotypes differ significantly in ecological preferences and thus probably behave differently in response to rearing conditions in the hatchery, questions rise on the actual identity of the rotifer strains used in aquaculture, where *Brachionus* discrimination is still based on morphology. This study is a part of an investigation of the genetic make-up of strains used in hatcheries, aquaculture research institutes and laboratories, and describes the rapid and sensitive PCR–DGGE method for the detection of *Brachionus* species and biotypes based on nucleotide sequence variation within the mitochondrial 16S rRNA gene. Considerable genetic diversity was found, albeit smaller within hatcheries than within laboratories and aquaculture research institutes. All 16S haplotypes produced an unambiguous DGGE fingerprint out of which a database was constructed.
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Keywords: Rotifers; *Brachionus plicatilis*; Fingerprinting; 16S rRNA gene; Mitochondrial DNA; Culture crashes

1. Introduction

Today, survival of the fish larvae in most aquaculture systems is highly dependent upon rotifer availability, which results in a major interest in the cultivation of these organisms. However, the unpredictability in rotifer production is still a bottleneck in the industrialisation of the larviculture process. Frequent problems, such as

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reduced reproduction or total mortality (crashes), are causing high losses in larviculture and may have major economical impact (Snell et al., 1987; Candreva et al., 1996). Until now, most research projects have focussed on mismanagement of the culture conditions or bacterial contaminations (Dehasque et al., 1995; Verdonck et al., 1997) to explain these crashes, while genetic causes are rarely considered due to limited scientific documentation or lack of experience. Hatchery identification is still based on morphological criteria. These criteria are also the basis for the exchange of rotifers when a hatchery suffers from a crash or when the hatchery is in need of huge quantities of rotifers during the high production season. Studies on wild cryptic rotifer species revealed that they tend to differ in ecological preferences and life cycle parameters and therefore, probably, behave differently in response to growth and rearing conditions in the hatchery (Serra et al., 1998; Ortells et al., 2003; Xi et al., 2005). Therefore it could be possible that the exchanged rotifers are not able to adapt to the local hatchery conditions, resulting in poor production levels. Thus, discrimination between distinct sympatric species lineages is important for the aquaculture industry.

The phylogenetic and taxonomic status of the *B. plicatilis* species complex remains controversial. Recently, several population genetic studies on *Brachionus plicatilis* were performed (Gómez et al., 2002a,b; Ortells et al., 2003; Papakostas et al., 2005). It has become clear that currently the existing biological diversity is not fully described and that *B. plicatilis* is actually a species complex possibly comprised of more than nine genetically different lineages.

In this study we developed a rapid, sensitive and easy-to-use molecular characterisation method for commercial rotifer strains. Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR–DGGE) is a sensitive technique based on differential melting and separation of similar-sized PCR-amplicons in a linear urea-formamide gradient (Myers et al., 1987). Although DGGE was originally developed for the detection of limited numbers of single-base mutations in disease studies (*e.g.* Valero et al., 1994) and later for the study of bacterial communities (*e.g.* Muyzer and Smalla, 1998), it can also be applied for population analysis of genetic markers with substantial sequence variation (Miller et al., 1999). In rotifers, the studies of Gómez et al. (2002b) and Ortells et al. (2003) on wild *Brachionus* populations were based on both mitochondrial (COI) and nuclear (ITS1) genes. However, when analysing communal samples from commercial hatcheries, the COI gene fragment did not have the desired features as marker fragment for the identification of aquaculture rotifer strains, because PCR-based COI-amplification very often generated co-amplification of COI from algal origin. Such co-amplification was not a problem with *Brachionus* samples cultured in the laboratory, on which analyses were done on rotifer individuals (Papakostas et al., 2006a,b). Because of this experimental difficulty and because different studies already explored the use of a fragment of the 16S rRNA gene as molecular marker (Lavery et al., 2004; Therriault et al., 2004; Govindarajan et al., 2005; Vences et al., 2005), even within rotifers (Papakostas et al., 2005), it was decided to explore the possibility of using a 388 bp fragment of this 16S rRNA gene for PCR–DGGE-based identification of commercial rotifer cultures. Combining a short DNA amplicon with the DGGE technique created the opportunity for the development of a method, which has enabled a large-scale screening of commercial rotifer populations.

Because the scientific literature is not consistent in the use of terms as rotifer strains, clones, lineages and biotypes, following definitions were made. The rotifer population of a hatchery or a research institute is called a **strain**, a term commonly used by fish farmers. As a consequence, a sample from a strain may contain more than one 16S haplotype. The definition is contradictory to the definition of *e.g.* a bacterial strain, which is a pure culture. If only one 16S haplotype is present, the population is called a **clone or clonal rotifer strain.** The clonal rotifer cultures were established from a single amictic female (see also Materials and methods — Sample collection). A **lineage** or **biotype** is a group of rotifer strains clustering together in a phylogenetic tree. So far nine different lineages have been described based on two molecular markers ITS1 (ribosomal internal transcribed spacer 1) and COI (Cytochrome c oxidase subunit I) (Gómez et al., 2002b): *B. plicatilis s.s.*, *B. ibericus*, *B. rotundiformis*, *B.* sp. Cayman, *B.* sp. Nevada, *B.* sp. Austria, *B.* sp. Manjavacas, *B.* sp. Tiscar, *B.* sp. Almenara. Among these lineages three *Brachionus* **species** have been described, *i.e. B. plicatilis s.s.*, *B. rotundiformis* (Segers, 1995) and *B. ibericus* (Ciros-Pérez et al., 2001).

2. Materials and methods

2.1. Sample collection and DNA extraction

During the period 1999 until 2001 thirty-seven (20 lyophilised and 17 alive) rotifer samples from several marine aquatic research institutes and hatcheries around the world were collected (Table 1). Because these were mainly the result of trading, information on the original

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