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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.

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

Table 1
Rotifer samples from the period 1999–2001+ROTIGEN samples

	Sample Label	Sample Origin	COI Characterisation (Papakostas et al., 2006a,b)	16S DGGE result	16S sequencing haplotype-biotype (EMBL acc. n°)
Hatchery strains [C]	Hatchery1[R]	Portugal	*B. plicatilis* sp. Cayman	F2 (**D1**)	–
	Hatchery2[R]		–	F2 (**D1**)	–
	French hatchery[R]	France	–	F2	–
	Spanish hatchery strain PL[R]	Spain	*B. plicatilis* sp. Nevada	F4 (**D3**)	10-nev
	Spanish hatchery strain PS[R]		*B. plicatilis* sp. Cayman	F2 (**D1**)	2-cay
	Hatchery1[R]	Norway	*B. plicatilis* sp. Cayman	F2	2-cay
	Hatchery2[R]		*B. plicatilis* sp. Nevada	F4	10-nev
	Hatchery3[R]		*B. plicatilis* sp. Austria	F7	11-aus
	Hatchery4[R]		–	F7	11-aus
	Hatchery5[R]		–	F2 and F4	-
	MRS[R]	Italy	*B. plicatilis* sp. Cayman	**F2 (D1)**	2 (**AM040257**)-nev
	GBA[R]		*B. plicatilis* sp. Austria	F7 **(D2)**	11-aus
Laboratory strains	**Sintef**[R]	Norwegian University of Science and Technology, Norway	*B. plicatilis* sp. Nevada	**F4 (D3)**	10 (**AJ748694**)-nev
	A*		–	F4	10-nev
	G1*	Marine Science Institute, University of Texas, USA	–	F1	–
	G2*		–	F1	1 (**AJ748707**)-cay
	G3*		–	F1	1-cay
	Manila1*	SEAFDEC, South East Asian Fisheries Development Center, The Philippines	–	F1	1 (**AJ748697**)-cay
	Manila2*		–	F1	–
	Manila3*		–	F1	–
	Manila4*		–	F1	–
	Garcia1*	CIAD, Unidad Mazatlan, Mexico	–	F1	1 (**AJ748695**)-cay
	Garcia2*		–	F1	–
	Garcia3*		–	F1	–
	Garcia4*		–	F1	–
	Portugal	Fisheries Institute IPIMAR, Portugal	–	F2	2 (**AJ748698**)-cay
	F(ukuoka)	Nagasaki University, Japan	–	F2	–
	O(bama)		–	F4	10-nev
	N(otojima)		–	F4	–
	F*	Unité mixte de Nutrition des Poissons INRA-IFREMER, France	–	F2	2-cay
	E*	Oceanographic & Limnological Research Ltd. National Center for Mariculture, Israel	–	F2	2-cay
	B*	Institute of Marine Biology of Crete, Ambrakikos Bay, Greece	*B. plicatilis s.s.*	F10	6 (**AJ748704**)-pli
	Z-A*	Dept. Ichthyology & Fisheries Science, Rhodes University, South Africa	*B. plicatilis s.s.*	**F12**	9 (**AJ748702**)-pli
	Taiwan	Tungkang Marine Laboratory – TFRI, Taiwan	–	**F3** and F1	3 (**AM040260**) and –

(continued on next page)

Table 1 (*continued*)

	Sample Label	Sample Origin	COI Characterisation (Papakostas et al., 2006a,b)	16S DGGE result	16S sequencing haplotype-biotype (EMBL acc. n°)
	Ds*	Strain established from resting eggs from Florida Aqua Farms, now used in Israel.	*B. plicatilis* sp. Austria	**F7**	11 (**AJ748701**)-aus
	Dm*		–	F7	–
	Brown	Ocean Sciences Centre, Memorial University of Newfoundland (Canada)	–	F9	5-pli
	China	Salt Research Institute, Tanggu	–	F7	11-aus
	S-1	Instituto de Ciencias Marinas de Andalucia (CSIC), Spain	–	F11	7-pli
	Le	Island Scallops Ltd. (Canada)	*B. plicatilis s.s.*	**F9**	5 (**AJ748703**)-pli
	Am		*B. plicatilis s.s.*	F9	5-pli
	GB	Seasalter Shellfish (Whitstable) Ltd., Kent (UK)	–	F2	2-cay
	#76*	Florida Aqua Farms (USA)	–	**F13**	8 (**AM180755**)-pli
	#79*		–	F13	8-pli
	Amat	Wild strain from Petrola Lagoon introduced in the laboratory, Instituto de Acuicultura de Torre de la Sal (Spain)	–	F5 and F6	–
Laboratory clones (collection 2001)	1, 4, 6 – 9	CIAD, Unidad Mazatlan (Mexico)	–	F1	–
	2		–	F1	–
	3		–	F1	1-cay
	5		*B. plicatilis* sp. Cayman	F1	1-cay
	10-2001		–	F1	1-cay
	10-2003	???	*B. plicatilis s.s.*	**F11**	7 (**AM180754**) -pli
	16	CIAD, Unidad Mazatlan (Mexico)	–	F1	1-cay
	20		–	F1	1-cay
	11 – 15, 17 – 19		–	F1	–
	21	Kyushu University (Japan)	–	F1	–
	22, 23		–	F1	-, 1-cay
	24		*B. plicatilis* sp. Cayman	**F1**	1(**AM180756**)-cay
	25, 26 and 27		–	F1	1,1-cay
	28 and 29		–	F1	–
	30		–	F1	1-cay
	31	Can Tho University (Vietnam)	–	F2	–
	32		–	F2	2-cay
	33, 34, 38		–	F2	2,-,2-cay
	61	Instituto de Ciencias Marinas de Andalucia (CSIC, Spain)	–	F11	7 (**AM040261**)-pli
	65		–	F11	7-pli
	67		–	F11	–
	68		–	F11	7-pli
	69		–	F11	–
	72	Wild strain from Petrola Lagoon introduced in the laboratory, Instituto de Acuicultura de Torre de la Sal (Spain)	*B. plicatilis* sp. Manjavacas	**F6**	13 (**AJ748707**)-mnj
	73		*B. plicatilis* sp. Manjavacas	**F5**	12 (**AJ748699**)-mnj
	74		–	F6	13-mnj
	75		–	F5	12-mnj
	76		–	F5	12-mnj
	78		–	F6	–
	80		–	F6	13-mnj

Table 1 (*continued*)

Sample Label	Sample Origin	COI Characterisation (Papakostas et al., 2006a,b)	16S DGGE result	16S sequencing haplotype-biotype (EMBL acc. n°)
81	Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM, Ecuador)	–	F1	–
84		–	F1	–
85		–	F1	1-cay
88		–	F1	1-cay
L1	University of Valencia (M. Serra)	*B. plicatilis s.s.* (Goméz et al., 2002b)	**F8 (D4)**	4 (**AM040258**)-pli
L3			**F10 (D5)**	6 (**AM040259**)-pli
HON-SS		*B. rotundiformis* (Goméz et al., 2002b)	**F14a**	14a (**AM180762**)-rot
SS2			**F14b**	14b-rot
SM2		*B. ibericus* (Goméz et al., 2002b)	**F15**	15 (**AM180761**)-ibe
SM5				15-ibe
SM20		*B. plicatilis* sp. Tiscar	**F20**	20-tis
SM28				
7C29		*B. plicatilis* sp. Almenara	**F21**	–

*Lyophilised samples; [R]Samples from the EU-project ROTIGEN; [C]For confidentiality reasons within the scope of the EU-project ROTIGEN, all hatcheries are labelled anonymously. Abbreviations: *B. plicatilis s.s.* (plic), *B. rotundiformis* (rot), *B. ibericus* (ibe), *B.* sp. Nevada (nev), *B.* sp. Cayman (cay), *B.* sp. Manjavacas (mnj), *B.* sp. Austria (aus), *B.* sp. Tiscar (tis), *B.* sp. Almenara (alm). Sample labels in bold are the reference samples for which a sequence was deposited in GenBank (except for sample 7C29) and for which the DGGE fingerprint was stored in the DGGE database. In the column '16S DGGE result' the 16S DGGE fingerprint from the preliminary study (Papakostas et al., 2006b) is displayed between brackets and in bold for nine samples.

geographical origin of the rotifer strains is lacking. Therefore, any correlation between geographical location of the collection sites of the rotifer samples and the genetic diversity detected is impossible.

Ten living rotifer samples did not survive the shipment, therefore clonal rotifer strains were started from the other seven surviving samples (Table 1): Ten individual females from each sample, carrying at least one amictic egg, were isolated and each individual was put into a falcon tube with 20 ml of autoclaved 25 g/l seawater. Clonal rotifer cultures were established with a success ratio of 65%. These clonal cultures were fed daily with *Chlorella* sp. at a final concentration of 10^5 cells/ml. While feeding the *Chlorella*, the volume was gradually increased to 35–40 ml. The tubes were mounted on a rotor (4 rpm) and placed in front of cool white tube lights with a constant light intensity of 2000 Lux and at a room temperature of 25–28 °C. Stock cultures were restarted every week. During intensive maintenance of this rotifer collection, some rotifer clones crashed and got lost. Nevertheless, an extensive collection of 54 clonal cultures was maintained for 2 years at ARC laboratory. For DNA extraction, the discarded rotifers were rinsed with distilled and autoclaved water, and stored at −80 °C.

Within the scope of a EU-supported research project, ROTIGEN (Q5RS-2002-01302, http://www.aquaculture.ugent.be//rend/rotigen/index.html), additionally twelve hatchery strains were characterised (Table 1).

Two stock cultures (*i.e.* MRS and GBA) of the Italian hatchery MRS (Maricoltura di Rosignano Solvay, Livorno, Italy) were genetically identified (Table 1). Additionally a series of 112 samples from the Italian rotifer mass culture (production season 2003–2004) was collected. Starting from the end of October 2003 and continuing until May 2004, rotifer cultures were performed at a commercial scale (1000 l tanks). Every culture ranged between 4 and 28 cycles (divided in batch cultures of 3 days). At the end of each subsequent run, rotifers were taken and stored for analysis. The subsequent samples of the batches were analysed as a first case study of a follow-up of a commercial rotifer production experiment with the DGGE technique.

Nine clones (HON-SS, SS2, SM2, SM5, SM20, SM28, 7C29, L1 and L3) from the collection of Prof. Manuel Serra, University of Valencia (Spain), were used as reference material (Table 1).

At the occasion of the Larvi 2005 — 4th Fish and Shellfish Larviculture Symposium (University of Ghent, September 5th–8th 2005) twenty-nine rotifer samples were added to this study (Table 2). These samples were used to test the DGGE-technique as a tool for molecular characterisation.

DNA extraction for all specimens was done with the WIZARD® GENOMIC DNA PURIFICATION KIT (Promega, The Netherlands).

2.2. PCR and DGGE analysis

As double-stranded PCR fragments migrate through the denaturing gradient, they will melt and hence

Table 2
Rotifers from the second sample gathering campaign (Larvi 2005 — 4th Fish and Shellfish Larviculture Symposium)

	Sample Label	Sample Origin	16S DGGE result	16S sequencing	16S based characterisation result
Hatchery strains	Hatchery1[C]	Turkey	F2	–	Cayman
	Hatchery2[C]	Greece	F2	–	Cayman
	Hatchery3[C]	France	F2	–	Cayman
	Hatchery4*,[C]	Scotland	F2 and F4	–	Cayman and Nevada
Laboratory strains	SEAFDEC	South East Asian Fisheries Development Center, The Philippines	F1	–	Cayman
	IPIMAR	Fisheries Institute, Portugal	F2	–	Cayman
	CCMar*	Aquagroup CCMar Algarve University, Portugal	F2	–	Cayman
	Israel 1*	Oceanographic & Limnological Research Ltd. National Center for Mariculture (Israel)	F1	1	Cayman
	Israel 2*		F1	–	Cayman
	Israel 3*		F1	–	Cayman
	Israel 4*		**F16**	16 (**AM180759**)	*B. rotundiformis*
	Israel 5*		**F17**	17 (**AM180757**)	Cayman
	Israel 6*		**F18**	18 (**AM180758**)	*B. plicatilis s.s.*
	Israel 7*		F9	–	*B. plicatilis s.s.*
	Israel 8*		F4	–	Nevada
	Israel 9*		F2	–	Cayman
	Israel 10*		F1	–	Cayman
	Israel 11*		F9	–	*B. plicatilis s.s.*
	Israel 12*		F2	–	Cayman
	TAFI*	Tasmanian Aquaculture & Fisheries Institute, Australia	F7	11	Austria
	Australia (resting eggs)	Northern Fisheries Center Cairns Australia	**F19**	19 (**AM180760**)	*B. rotundiformis.*
	Peru	Marine Research Institute of Peru	F13	8	*B. plicatilis s.s.*
	Vietnam	Fisheries University, Nha Trang, Vietnam	F3 and F1	–	Taiwan-like
	China L	Salt Research Institute, Tanggu, China	F7	11	Austria
	China S		F10	–	*B. plicatilis s.s.*
	Libia	Marine Biology Research Center, Libia	F2	–	Cayman
	MAP-B	Marine Laboratory, Florida, USA	F1 and F11	–	Cayman *B. plicatilis s.s.*
	MAP		F1	–	Cayman
	USA-ML		F13	8	*B. plicatilis s.s.*

*Lyophilised samples; [C]For confidentiality reasons all hatcheries are labelled anonymously. Sample labels in bold are the reference samples for which a sequence was deposited in GenBank and for which the DGGE fingerprint was stored in the DGGE database.

changing mobility drastically. To prevent complete strand dissociation, a GC-rich fragment (GC-clamp) was introduced during fragment amplification with *Brachionus*-specific primers: the reverse primer Brach16S_R (5′-ATAGGATCCGATAATCCAACATCGAGGTAGTAG-3′) and the forward primer Brach16S_F_GC (5′-CCGGGGCCCGGGGGCCCCCGGGCCGGGCCCGGG-AGATGGATCCAAAGTATCTTCTGCCCACT-3′). Both primer sequences were based on the *Brachionus*-specific partial sequence of the 16S rRNA gene published in EMBL Nucleotide Sequence Database (accession number: AF108106; Garey et al., 1998). PCR reactions were performed in 20 μl final volume containing 1 μl template DNA (5 ng), 10 μl JumpStart™REDTaq™ReadyMix™

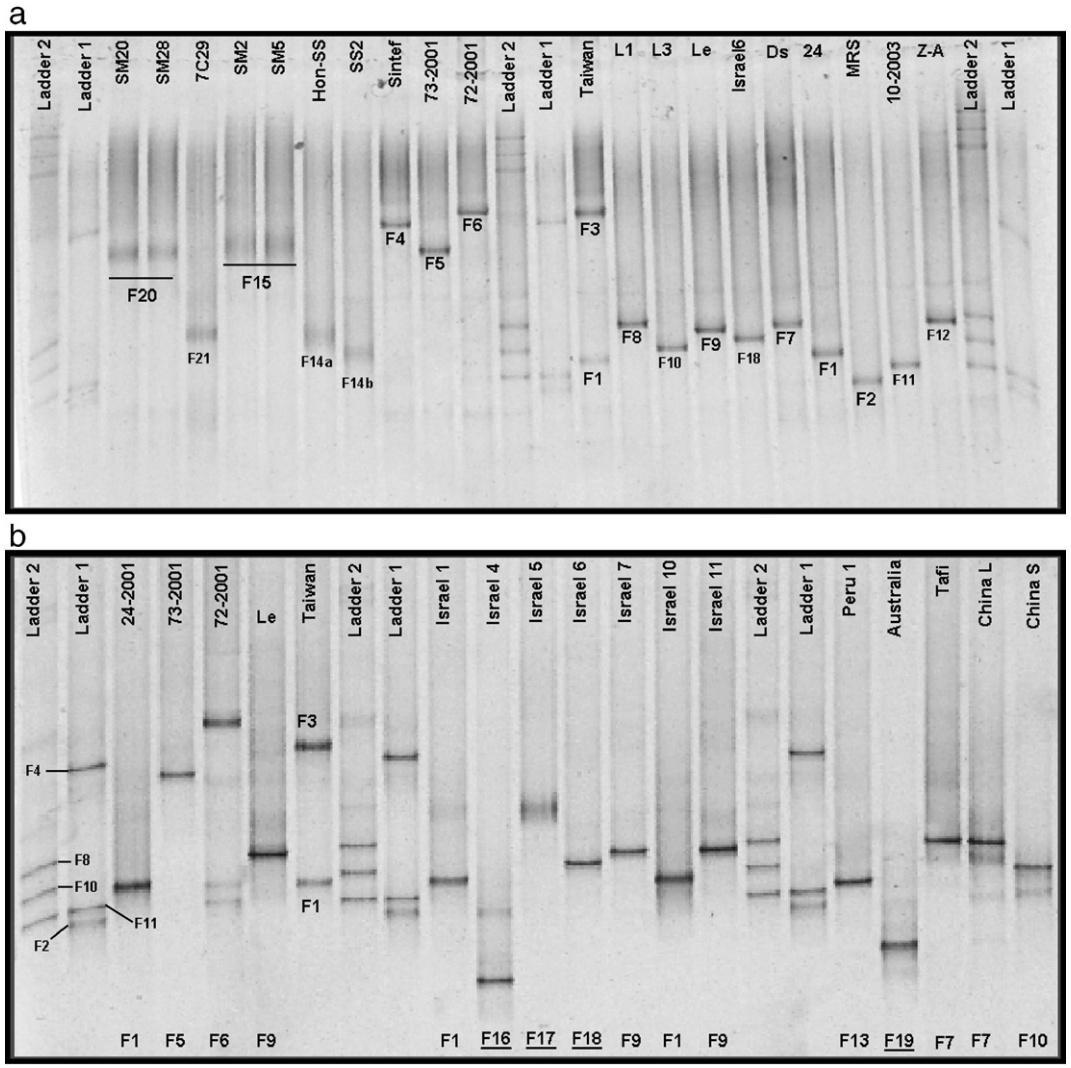

Fig. 1. a and 1b: Twenty-two (18 in Fig. 1a and 4 in Fig. 1b) PCR–DGGE fingerprints are illustrated. Labels of the rotifer samples are given on top of the lanes, while DGGE fingerprint numbers are given at the bottom of the lanes or are located near the DGGE fingerprint.

PCR Reaction Mix (Sigma Aldrich, Belgium) and 2 μl of each primer (10 pmol/μl). Amplification reactions are performed under the following cycling conditions: an initial denaturation step of 2 min at 94 °C; subsequently 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C; and finally 4 min at 72 °C.

The PCR product was analysed by DGGE using the Ingeny PhorU (Ingeny, The Netherlands) apparatus according to manufacturer's instructions. Samples were loaded on a 9% polyacrylamide gel in 1× TAE (Tris–Acetate–EDTA) buffer. An optimal separation was achieved with a parallel and linear denaturing gradient ranging from 15 to 35% 7M urea–40% (V/V) formamide. A 9% polyacrylamide stacking gel was poured to create solid slots for efficient loading of 50 ng of the amplicons, to prevent difficulties caused by the elevated urea concentration. Gels were run overnight at 57 °C and 120 V, stained with SYBR® Gold (Invitrogen, Belgium) for 30 min at room temperature and photographed under an UV transilluminator.

To make the interpretation of the DGGE gels easier, two ladders were constructed in-house and loaded on each gel. One ladder contains a 1:1 mix of 2 DGGE–PCR fragments from the samples named SINTEF and MRS (Ladder 1), some batches of Ladder 1 also contained the DGGE-amplicon of sample 10-2003 (1:1:1). The other ladder is constructed with DGGE–PCR fragments from the samples L1, L3 and 10-2003, also in a 1:1:1 ratio

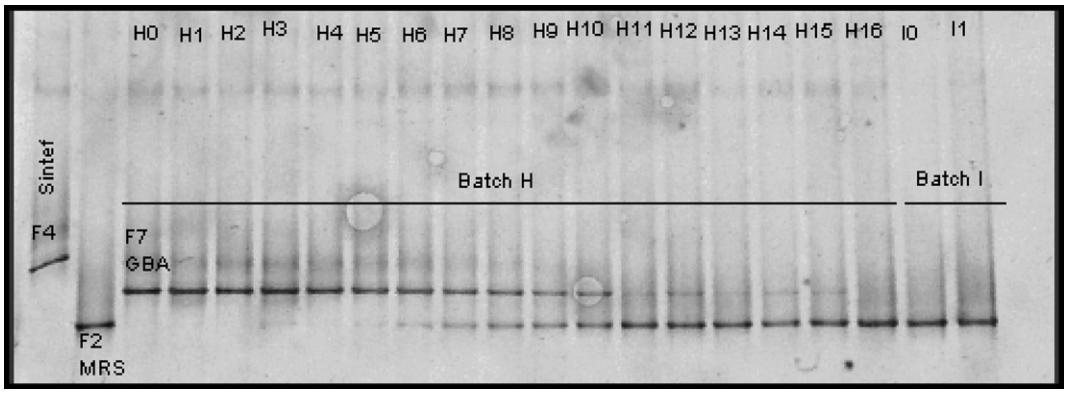

Fig. 2. DGGE analysis of samples from batches H and I from MRS (Italy), production season (2003–2004). The number of culture cycle is indicated on top of each lane.

(Ladder 2). These references represent five out of twenty-two detected DGGE fingerprints (Fig. 1a and b).

Twenty-two DGGE fingerprints (Fig. 1a and b) from samples (Tables 1 and 2), which generated DGGE-amplicons with different melting temperatures, were stored in a database using the GelComparII software (Applied Maths NV, Belgium).

2.3. Sequence analysis

Amplicons from samples with different melting temperatures were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Belgium). The purified PCR fragments were sent to the internationally acting genomics company Agowa GmbH (Berlin, Germany) for sequence analysis. To ensure proper sequence confirmation sequencing was performed in both directions for 66 amplicons. For management and analyses of these data (multiple alignments and construction of a library of all 16S haplotypes) the sequence analysis software Kodon (Applied Maths NV, Belgium) was used. Forward and reverse sequences were aligned and checked for quality. Ambiguous base pairs were visually checked on the chromatograms.

A reference sequence of each sequence type was deposited in GenBank (accession numbers AJ748693–AJ748707, AM040257–AM040261, AM180754–AM180762).

Because for five samples (*i.e.* #76, Israel4, Israel5, Israel6, Australia) a different DGGE fingerprint was obtained, which could not be allocated to any fingerprint in the existing sample collection and because no COI-based identification was available, the 16S amplicons were sequenced by Agowa GmbH (Berlin, Germany). The obtained 16S sequences were submitted to a BLAST analysis (http://www.ebi.ac.uk) for identification of the rotifer biotype.

Melting temperatures (T_m's) for the lowest melting domain of all the reference haplotype sequences were calculated with Primo Melt 3.4 (PCR Primer Design for DGGE and TGGE) software (http://www.changbioscience.com/primo/primomel.html). In addition the relative migration position in the gel (starting from the wells) from the respective DGGE amplicons was calculated with GelComparII software (Applied Maths NV, Belgium).

2.4. Problems encountered and solutions

Two problems of the PCR–DGGE technique were (i) the very close position of amplicons in the DGGE-gel (*i.e.* F7, F8, F9, F10, F12 and F18) and (ii) the fuzziness of some bands. Both problems reduced the discriminatory power: exact positioning of the fingerprints and thus identification was hindered. In the present study reloading the in-house constructed ladders on a new DGGE-gel next to the ambiguous samples solved this problem in most cases. If results were not satisfying the 16S amplicons were sequenced.

3. Results

3.1. 16S rDNA sequence variation and DGGE analysis

A total of twenty-two 16S haplotypes was obtained and stored in a database. Screening of the samples of Table 1 resulted in the separation of DGGE amplicons at 18 different melting temperatures (*i.e.* F1 to F13, F14a, F14b, F15, F20 and F21). Fig. 1a shows 17 of the

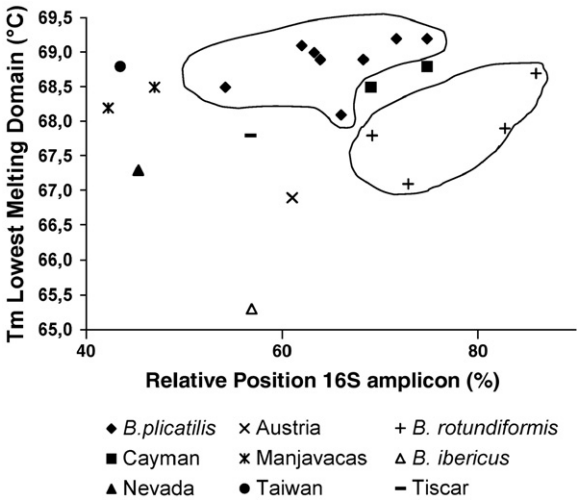

Fig. 3. Relative migration position of DGGE amplicons to their T_m for the lowest melting domain of the DGGE amplicon.

DGGE fingerprints (one fingerprint in Fig. 1a was from a sample of Table 2). Fingerprint F13 is located between F1 and F11 (*e.g.* sample Peru1 on Fig. 1b). After the screening of the rotifer samples mentioned in Table 2 another four melting temperatures were found (F16 to F19, Fig. 1b). This resulted in a total amount of twenty-two different DGGE fingerprints. Hence every 16S haplotype displayed a different DGGE migration pattern.

A difference was noticed in the number of unique fingerprints in hatcheries vs. laboratory samples. Three fingerprints (F2, F4 and F7) were found within the 16 hatchery samples compared to 22 fingerprints (F1 to F21) found within the 114 laboratory samples. However, when normalized there were 1.9 unique fingerprints per 10 samples in both cases.

Seven migration patterns were located very close in the DGGE-gel under the electrophoresis conditions used in this study. Consequently, in some cases DGGE fingerprinting could not identify the 16S haplotype explicitly, possibly due to unidentified gel-related problems. Therefore, the 16S amplicon was sequenced and compared to the database of 16S haplotypes.

DGGE analysis of the MRS series of samples revealed that the technique is suitable for the follow-up of rotifer mass cultures. The hatchery has been using one particular rotifer strain (MRS strain) for many years in their mass cultures. DGGE analysis, resulting in fingerprint F2, identified this stock culture as *B.* sp. Cayman (Table 1). During the 2003–2004 production season a new rotifer strain (GBA strain, imported from a hatchery in the USA, Portsmouth) was introduced in the rotifer mass production system of the hatchery, which was genetically identified as *B.* sp. Austria, represented by DGGE fingerprint F7 (Table 1). First screening of the samples was done on a restricted amount of samples: from each cultured rotifer batch two samples (the first and the last) were analyzed (results not shown). A shift from fingerprint F7 to fingerprint F2, *i.e.* the GBA strain to the MRS strain, was noticed in batches H and I. In consequence, all samples from batches H and I were analyzed. A gradual changing over a period of 16 three-day culture cycles from *B.* sp. Austria to *B.* sp. Cayman was noticed (Fig. 2).

3.2. Congruence between 16S-DGGE and 16S-sequencing

Data from DGGE and 16S rDNA sequencing were linked to previously published COI-RFLP analyses results (Table 1). Fingerprints F8, F9, F10, F11 and F12 correspond to five *B. plicatilis s.s.* strains (respectively samples L1, Le, L3, 10-2003 and Z-A). Also fingerprints F13 (sample #76) and F18 (sample Israel6) correspond to *B. plicatilis s.s.* strains according to a BLAST search, because 99% sequence identity was found with strains Le and B and with clones Bp1 and Bp3 (Papakostas et al., 2005). Two fingerprints were found for *B.* sp. Manjavacas (F5 and F6) and three for *B.* sp. Cayman (F1, F2 and F17, which had a BLAST-result of 99% sequence identity with clone 24 and MRS strain). Only one fingerprint was found for *B.* sp. Nevada (F4) and *B.* sp. Austria (F7). Four fingerprints were obtained for *B. rotundiformis*, F14a, F14b, F16 (BLAST-result: 94% sequence identity with SS2 clone) and F19 (BLAST-result: 93% sequence identity with SS2 clone), while *B. ibericus* was only represented by one fingerprint (F15) (Fig. 1a and b). Also, *B.* sp. Tiscar and *B.* sp. Almenara were represented by only one fingerprint, F20 and F21 respectively (Fig. 1a).

3.3. Congruence between T_m, position in DGGE gel and characterisation

The relationship between those two variables is shown in Fig. 3. A cluster of *B. plicatilis s.s.* and a cluster of *B. rotundiformis* T_m's were clearly present. Gelposition–T_m relationships for haplotypes belonging to other species (*e.g. B. ibericus*) were outside these clusters. The Taiwan sample and both haplotypes from the Manjavacas biotype were also located outside this cluster. This was also the case for the haplotypes for the Nevada, Austria and Tiscar biotypes. The Cayman

Table 3
Comparison of characterisation results of 6 Israeli rotifer samples

Sample label	Sample label in Boehm et al. (2000) study	Sample origin	Microsatellite characterisation according to Boehm et al. (2000)	16S-based characterisation
Israel 1	Fiji	Fiji	*B. rotundiformis*	*B.* sp. Cayman (16S haplotype 1)
Israel 2	Tahiti	Tahiti	*B. rotundiformis*	*B.* sp. Cayman (16S haplotype 1)
Israel 4	Saipan	The Philippines	*B. rotundiformis*	*B. rotundiformis* (16S haplotype 16)
Israel 5	Thai	Thailand	*B. rotundiformis*	*B.* sp. Cayman (16S haplotype 17)
Israel 6	AT	Atlit-Israel	*B. plicatilis s.s.*	*B. plicatilis s.s.* (16S haplotype 18)
Israel 7	26	Eilat-Israel	*B. plicatilis s.s.*	*B. plicatilis s.s.* S haplotype 9)

biotype was found on the boundaries of the *B. plicatilis s.s.* cluster and the *B. rotundiformis* cluster.

4. Discussion

The DGGE technique as an identification tool for rotifers was preliminarily described in the study of Papakostas et al. (2006b) comparing four different molecular characterization techniques (*i.e.* RFLP on the COI gene, SSCP (Single Strand Conformation Polymorphism) and DGGE on the 16S rRNA gene and microsatellite analysis). In that study only five 16S haplotypes and their respective DGGE fingerprints (D1–D5) were described (Table 1). In the present study another seventeen 16S haplotypes were found and all of them were represented by one unique DGGE fingerprint. Previous studies to identify *Brachionus* species in European hatcheries (Papakostas et al., 2006a,b) revealed that *Brachionus* sp. Cayman is the dominant biotype. These results were corroborated by the present study that is based on a more extensive worldwide sample collection. Seven sequence types of *B. plicatilis s.s.* strains and four *B. rotundiformis* strains were found within the rotifer sample collection. None of these haplotypes were found among the hatchery samples. In comparison, the *Brachionus* sp. Cayman biotype was found in most (73.33%) of the hatchery samples.

The PCR–DGGE technique also allowed detection of several *Brachionus* biotypes within one sample. At the beginning of this research project it was assumed that a fair amount of genetic diversity would be present in commercial rotifer strains. However, as the study progressed it became clear that almost all rotifer strains were monoclonal. Only six samples were comprised of two different rotifer strains (Tables 1 and 2). This could be explained by the intensive exchange of rotifer starter cultures between hatcheries and institutes all over the world. Starting from the 1990s busy rotifer traffic was going on between laboratories and hatcheries. Alternatively, it is conceivable that only a few *Brachionus* biotypes can adapt to the hatchery conditions, which might contribute to a low genetic diversity between hatcheries. However, the latter statement would need to be verified experimentally.

PCR–DGGE can be used to follow up potential changes in the clonal composition of mass cultures or laboratory cultures during the course of time, which was clearly demonstrated with the small case study of the Italian hatchery. It was reported by Papakostas et al. (2006b) that accidental mixes of different strains or clones cultured within one hatchery or research institute are highly probable. This was noticed at the ARC laboratory as well. Characterisation results for strains that were kept at the ARC laboratory for several years differed significantly over time: *e.g.* clone 10-2001 was identified as *B.* sp. Cayman while clone 10-2003 was identified as *B. plicatilis s.s.* (Table 1). Clones 61, 65, 67 and 70 were reported to be *B.* sp. Cayman by Papakostas et al. (2006a), while these clones were originally (samples dating from 2001) identified as *B. plicatilis s.s.* The high risk of contamination of rotifer cultures pose serious threats to the predictable growth of cultures, because of ecological preferences and life cycle parameters of cryptic species (Serra et al., 1998; Ortells et al., 2003). Also the exchange of rotifer cultures between hatcheries during high production season or when they suffer from crashes is not recommended until more biotype-specific knowledge on the growing and rearing characteristics of the *Brachionus* strains has been reported. Further studies on the nutritional value of the different *Brachionus* rotifer strains may show they are indispensable for the fish larvae industry. A certain strain might be well performing concerning temperature and salinity conditions, but of poor nutritional value to the fish larvae.

However, the relationship between nucleotide sequence, phylogenetic affiliation and the melting temperature is not well established for closely related organisms (Kisand and Wikner, 2003), our current database of DGGE fingerprints, revealed indications for certain relatedness of gel position (*i.e.* melting temperatures, T_m) and phylogenetic identification. These indications are only of qualitative nature. Although a tendency of

belonging to one or another haplotype was detected, especially for extreme gel positions, it was not possible to allocate a new 16S DGGE fingerprint unambiguously to one or another *Brachionus* biotype or species. Within one biotype more than one 16S haplotype was detected. The relevance of the exact knowledge of the 16S haplotype must be considered. Because this study is strongly oriented towards aquaculture industry, it is more useful to optimize DGGE conditions, allowing identification of the F7 haplotype, the only one belonging to the Austria biotype. Only when significant differences in growth characteristics of relevance to the aquaculture industry are documented for each strain, it will be necessary to improve the resolution of this technique. However, for ecological research it is of interest to improve methodology even more to obtain rapid and unquestionable DGGE-identification of all the different strains.

An interesting point regarding the identification of strains mentioned in Table 2 is the genetic characterisation of six of the Israeli samples (Israel1–Israel7), which were also used in a different study (Table 3). That study was initiated to search for species-specific and strain-specific satellite DNA sequences (Boehm et al., 2000). Our 16S DGGE characterisation confirmed the former microsatellite characterisation results in only 3 cases: Israel4 sample was confirmed by 16S analysis to be a *B. rotundiformis* strain. Samples Israel6 and Israel7 both were confirmed to be *B. plicatilis s.s.* The use of the 16S marker even revealed some intra-species (*B. plicatilis s.s.*) genetic divergence between these two samples, as Israel6 belongs to a different 16S haplotype than Israel7 (Table 3). As for the Israel1, 2, 3 and 5 samples, identification with the 16S marker inferred that these strains belong to the Cayman biotype. Within the microsatellite study, these strains were described as *B. rotundiformis* strains. The study of Boehm et al. (2000) was published before extended knowledge on the *Brachionus plicatilis* species complex became available (Gómez et al., 2002b). This may explain the different characterisation results. Microsatellite sequences published until present do not cover the complete biodiversity within the *B. plicatilis* species complex.

When screening the Larvi'05 samples four new 16S DGGE fingerprints (F16, F17, F18 and F19) were revealed. All these new fingerprints were obtained from samples of laboratory origin. No new fingerprints were obtained from the hatchery samples, which indicates that chances are small to encounter unidentified 16S haplotypes when screening randomly collected hatchery samples — at least in European hatcheries.

The ideal pattern of one sharp band was not found for the following samples, SM20, SM28, 7C29, SM2, SM5, Hon-SS, SS2 and Israel5. This was possibly due to inconsistencies in the melting behaviour along the sequence of their 16S marker fragment (Wu et al., 1998). Those DGGE amplicons showed fuzzy bands that may impede or even prevent the detection of a mutation. Gille et al. (1998) described the use of one psoralen clamp at each end of the PCR product. Within TGGE (Temperature Gradient Gel Electrophoresis) assays they showed that bipolar clamping may transform blurred bands into sharp ones and may visualize mutations that could not be detected by conventional single-sided clamping.

5. Conclusions

Hatcheries as well as marine research institutes are uncertain of the species status of the rotifer strains they use. The present study contributes with significant knowledge about the genetic identity of the aquaculture related *Brachionus* strains. The development of a simple and inexpensive technique as PCR–DGGE fingerprinting offers a quick and accurate molecular characterization tool for commercial and laboratory use.

Although the twenty-two known 16S amplicons can be separated, the retardation of the amplicon in the gel matrix may not properly indicate phylogenetic relatedness at high resolution. Nevertheless, in this study the mitochondrial 16S marker exhibited 100% success in the identification of the previously described *Brachionus* sp. lineages (Gómez et al., 2002b). Even within lineages the marker preserves its discriminatory power (*e.g. B.* sp. Cayman, *B.* sp. Manjavacas, *B. plicatilis s.s.* and *B. rotundiformis*). Papakostas et al. (2006a,b) described the use of the SSCP fingerprinting technique for the analysis of communal rotifer samples (*i.e.* the case for commercial hatchery samples). However, when using the SSCP technique it was not possible to distinguish between all *Brachionus* biotypes. This study resulted in the conclusion that the DGGE technique is the preferred technique to use in aquaculture applications, because the complete polymorphism within the *Brachionus plicatilis* species complex was unambiguously detected. The encountered problems did not affect the analyses of this study's samples but in the future stronger denaturing conditions during electrophoresis or the attachment of different clamps during PCR could expose the 16S rDNA polymorphism more explicitly and possibly remedy the problems.

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