



BRACHIONUS PLICATILIS
SPECIES COMPLEX AND ITS
PRESENCE IN EUROPEAN
HATCHERIES

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Rotifers and Aquaculture

- Important live feed for the larviculture of marine fish
- Improved culture methods have led to rotifer mass production
- Success of the industry depends on rotifer mass cultures (mainly belonging to *Brachionus*)
- Basic and applied research on rotifers are needed to improve mass culturing



Problems in Rotifer Cultures

Sudden + frequent crashes may be caused by:

- Water quality, feed type and quality, human error, competition
- Uncontrolled exchange of “unidentified” strains among hatcheries → mixed populations

Different species **REQUIRE** different culture conditions



Great confusion on the species status of *Brachionus*

(until 1990s)

MORPHOLOGY → 2 TYPES

L

B. plicatilis

S

B. rotundiformis

(during 1990s)

MORPHOLOGY → 3 TYPES

L

B. plicatilis

SM

B. ibericus

SS

B. rotundiformis

Great confusion on the species status of *Brachionus*

(2003) DNA ANALYSES → 9 BIOTYPES (SPECIES ?)

L

SM

SS

B. plicatilis

“Nevada”

“Austria”

“Manjavacas”

B. ibericus

“Tiscar”

“Almenara”

“Cayman”

B. rotundiformis



Great confusion on the species status of *Brachionus*

Unpublished molecular data: 13-15 different biotypes/species within *Brachionus plicatilis* complex

But ...Hatcheries usually identify rotifers only as ...

L (*B. plicatilis*)

S (*B. rotundiformis*)

Morphology alone cannot give accurate information

Molecular methods & Identification

- Overcome problems of morphological identity
- Require minute amounts of tissue
- Can be applied to microscopical species
- Identification of a single rotifer individual or egg
- After standardisation can provide fast results at a medium cost
- Admittedly, they require expertise and appropriate facilities





Aims of this Work

- How can genetic markers help in the identification of rotifers in European hatcheries ?
- What is the species status of rotifer strains used in European hatcheries ?
- Do morphological identification and DNA data agree ?
- Are hatchery strains monoclonal ?
- How can molecular markers monitor changes in rotifer composition during hatchery cultures ?

Sampling

ORIGIN	HATCHERIES	STRAINS
Norway	6	7
Portugal	1	2
Spain	2	3
France	2	2
Italy	2	8
Greece	3	5
TOTAL	16	27

Genetic Markers & Levels of Sensitivity

- RFLP analysis of COI mtDNA region with appropriate endonucleases
- SSCP analyses of 16S rDNA
- DGGE analysis of 16S rDNA
- Microsatellite analysis of 7 loci

All above techniques have been previously used for species identification purposes

UNIDENTIFIED STRAIN



SPECIES



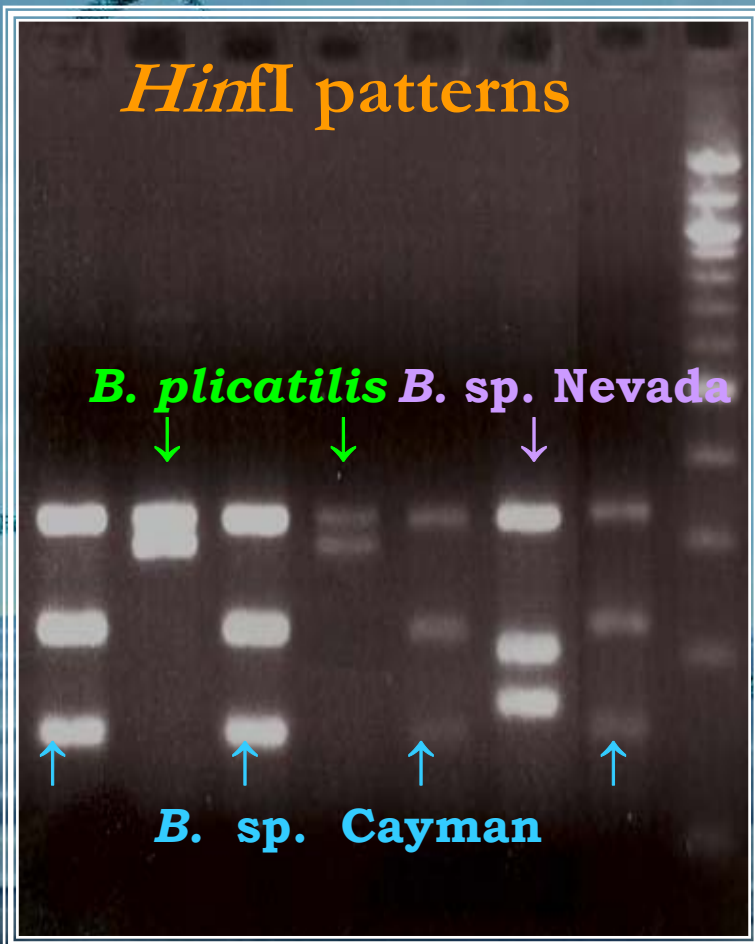
CLONES

INDIVIDUALS

Biotype/Species Identification

RFLP analysis

SSCP analysis

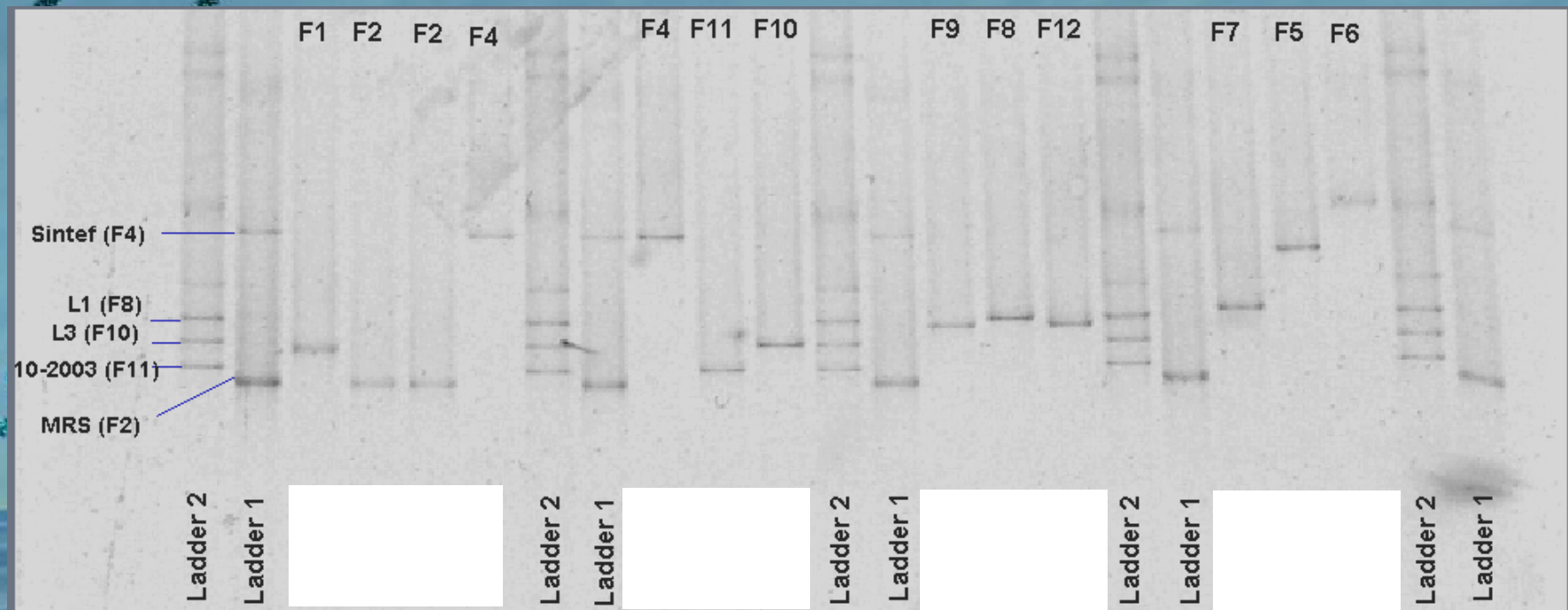


B. sp. Cayman

B. sp. Nevada

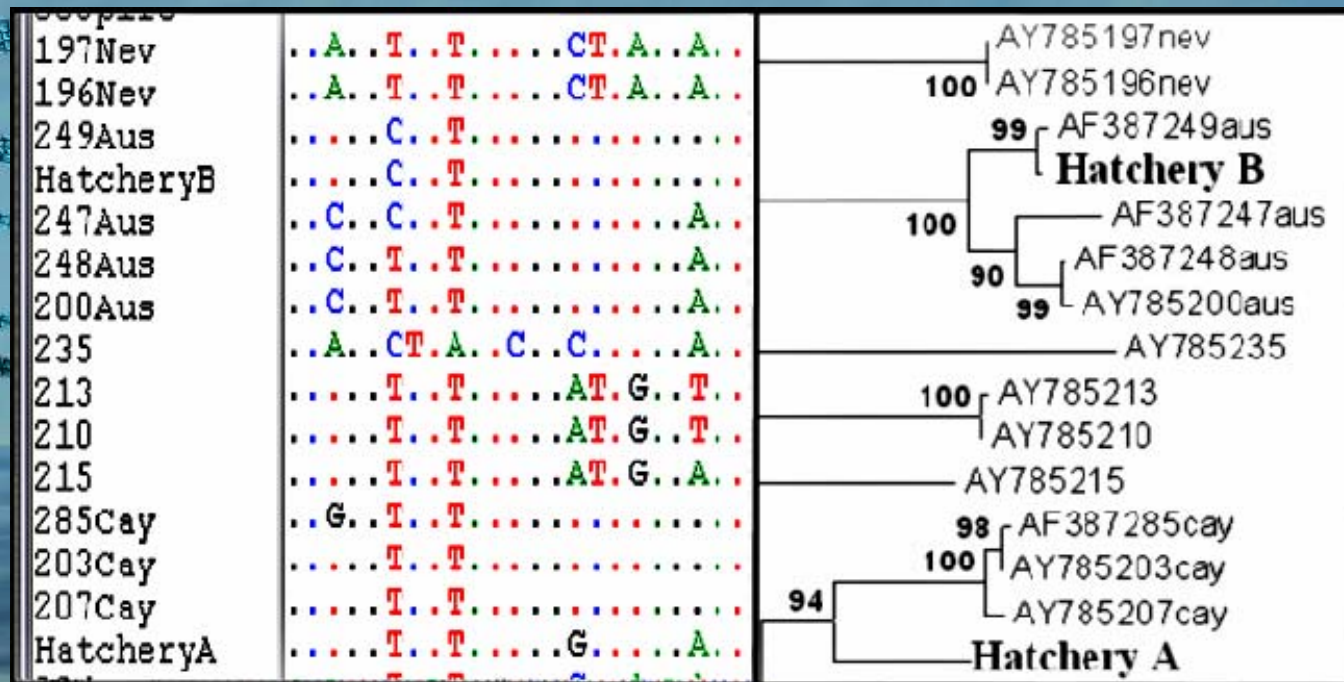
Biotypes/Species Identification

DGGE characterisation: 12 unique fingerprints



Biotypes/Species Identification

Confirmation of results was done with Sequencing of mitochondrial loci and comparison of results with more than 200 deposited DNA sequences in GENBANK



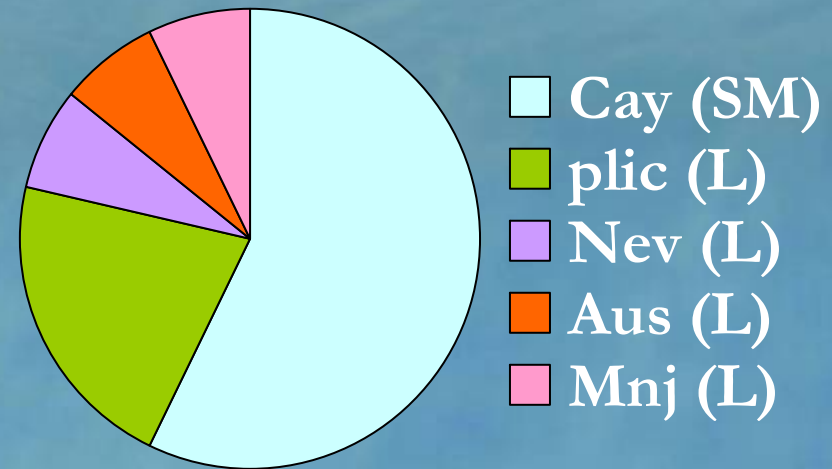
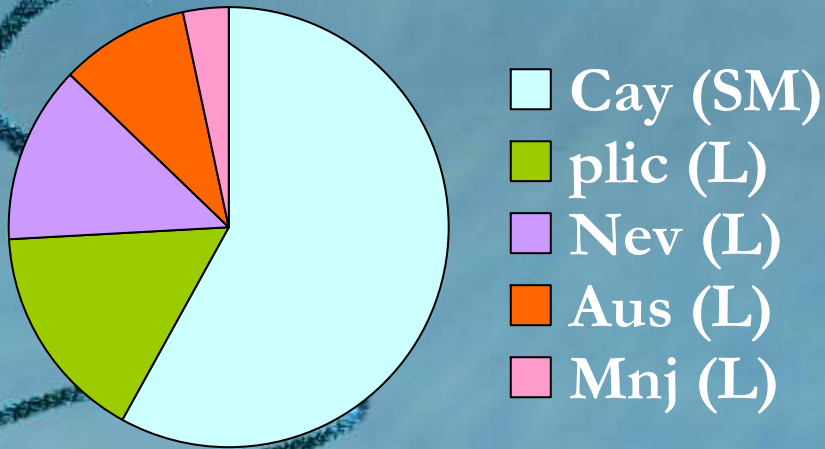
Genetic Identification of European Hatchery Strains

Hatchery	Strains	RFLP Biotypes	SSCP Haplotypes	DGGE Haplotypes
Norway1	Strain 1 Strain 2	Nev Cay	S4 -	F4 -
Norway2	Strain 1	Cay	S1	F2
Norway3	Strain 1	Nev	S4	F4
Norway4	Strain 1	Aus	S7	F7
Norway5	Strain 1	Aus	S7	F7
Norway6	Strain 1	Cay/Nev		F2/F4
Portugal1	Strains 1,2	Cay	S1	

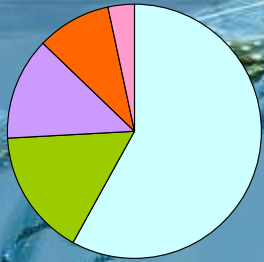
Genetic Identification of European Hatchery Strains

Hatchery	Strains	RFLP Biotypes	SSCP Haplotypes	DGGE Haplotypes
Spain1	Strain 1 Strain 2	Cay Nev	S1 S4	F2 F4
Spain2	Strain 1	Plic (20) Mnj (3) Cay (1)		
France1	Strain 1	Plic		
France2	Strain 2	Cay		
Italy1	Strains 1-6 Strain 7	(6) Cay (1) Aus	S1 S7	F2 F7
Italy2	Strain 1	Cay	S1	F2
Greece1	Strain 1 Strain 2	Cay (28)/Plic (4) Cay (23)/Plic (9)		
Greece2	Strain 1 Strain 2	Cay Plic		
Greece3	Strain1	Cay		

Overall Representation of *Brachionus* Biotypes in 16 European Hatcheries & 14 Laboratories*



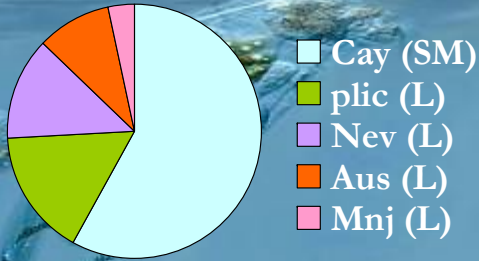
* Canada, Ecuador, France, Greece, Israel, Japan, Mexico, Philippines, Portugal, Spain, Taiwan, UK, USA, Vietnam



□ Cay (SM)
■ plic (L)
■ Nev (L)
■ Aus (L)
■ Mnj (L)

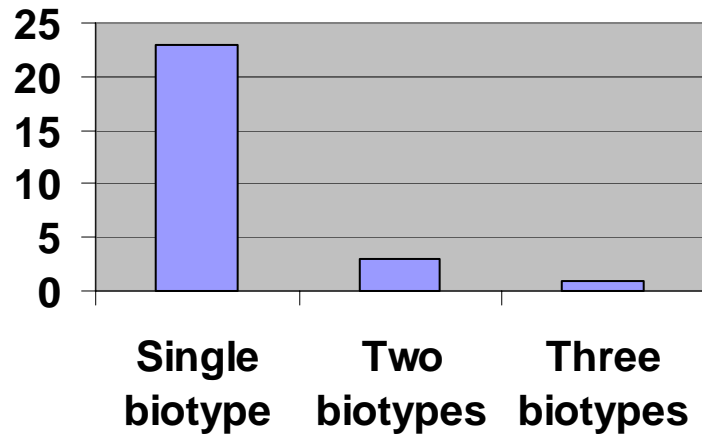
Species identification in hatcheries (*B. sp* Cayman-type SM)

- *B. sp.* Cayman was the dominant strain (58%)
- How, when, why was it imported in European hatcheries? *Brachionus sp.* Cayman has been found in American natural basins but never in Europe
- No studies exist on its reproduction and morphological characteristics
- The only studies on its growth characteristics have been done during the course of the project



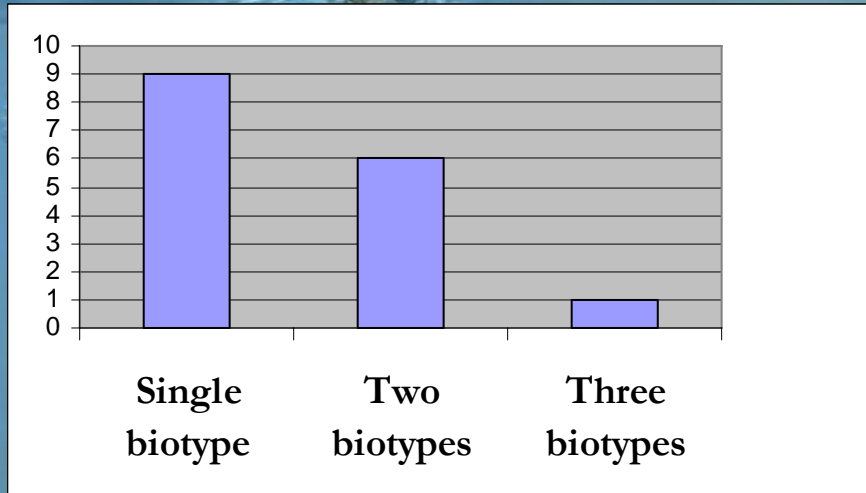
Species identification in hatcheries (L type and SS type rotifers)

- Regarding Type L rotifers, *B. plicatilis* s.s. was found in 16% of the strains
- However, the name *B. plicatilis* is frequently used to denote *Brachionus* strains
- All other Type L *Brachionus* sp. biotypes (Nev, Aus, Mnj) were found in higher abundance (26%)
- The L type rotifers are morphologically similar – Easy to confuse them when exchanging samples
- *B. rotundiformis* (Type SS) was never found
- There is an obvious problem in morphological identification of rotifers



Number of *Brachionus* Biotypes per Strain

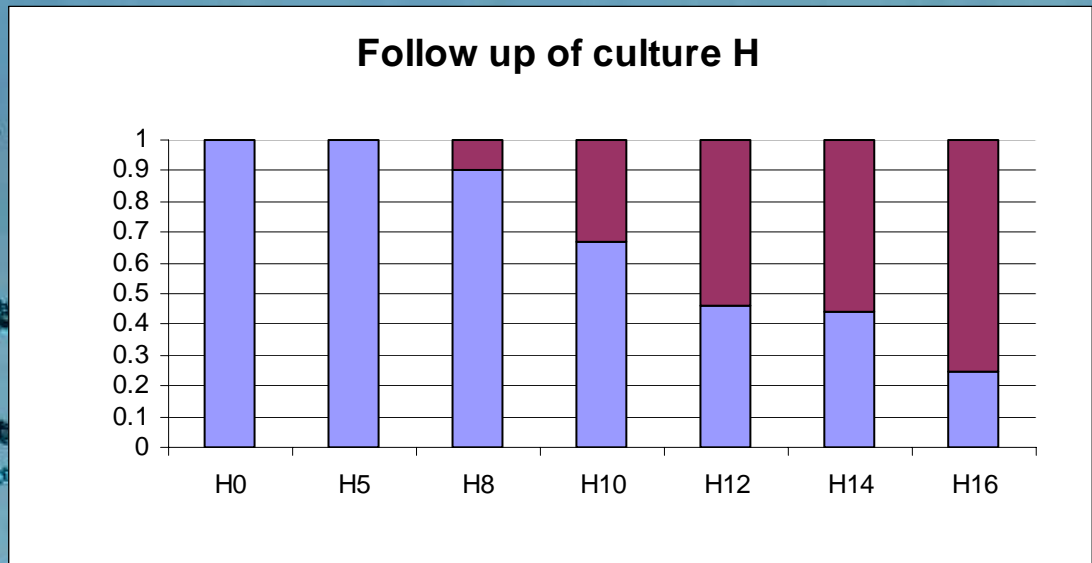
- In 23/27 strains examined only one biotype/species was present
- Could this be due to culture conditions & management?
- Why were there mixes of different biotypes/species in the rest (4) strains?
- Due to contamination problems or were these mixtures better suited to the culture conditions of the specific hatchery?



Number of *Brachionus* Biotypes per Hatchery

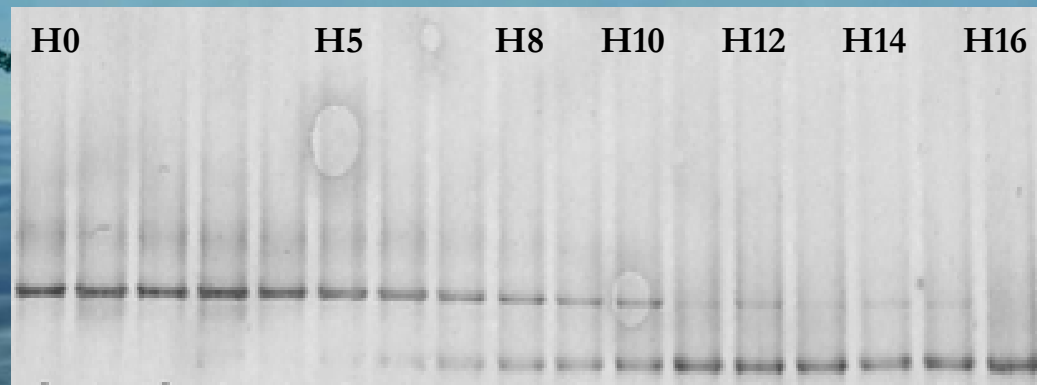
- In 7/16 hatcheries presence of at least 2 different biotypes/species was recorded
- Are different strains in the same hatchery cultured differently?
- Caution is needed to avoid contamination within hatcheries

Molecular techniques can give important answers to hatcheries on contamination and/or presence of mixtures in *Brachionus* cultures



RFLP

DGGE



aus →

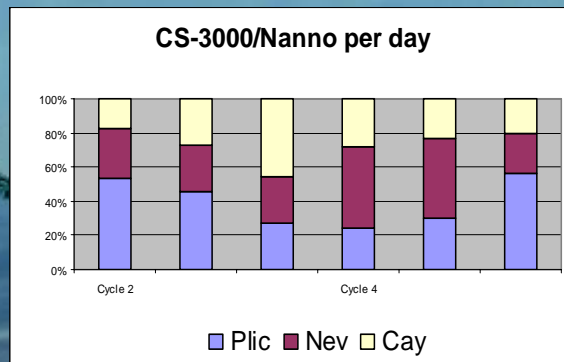
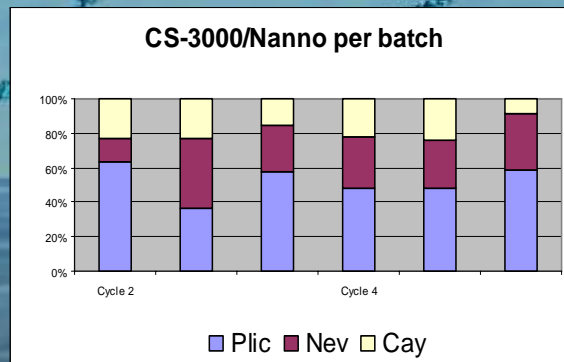
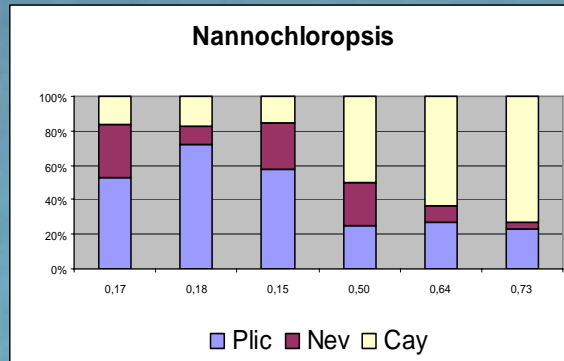
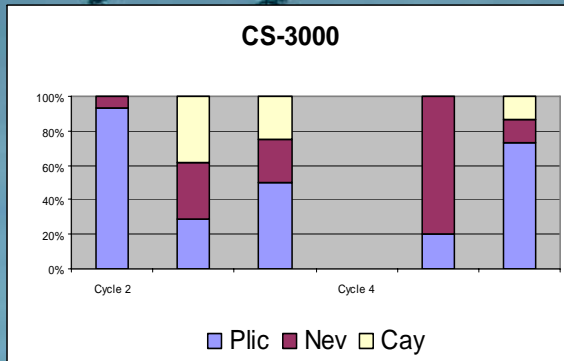
← cay

How to avoid contamination in STOCK cultures

- Always keep stock cultures well closed
- Do not open rotifer stock cultures in a place where other clones are present
- Disinfect hands between the handling of 2 different clones (or change gloves)
- Do not leave the bottle with feed open while the rotifer stock culture is open
- Use filter micropipette tips
- Work under laminar flow



Molecular techniques can follow up progress of cultures and changes in their clonal composition, i.e. monitor the effect of different feeds, probiotic bacteria, different culture methods, etc...



Cycle 2 → Cycle 4

CS-3000

R1: plic → plic
 R2: all → nev
 R3: plic → plic

Nanno

R1: plic → cay
 R2: plic → cay
 R3: plic → cay

Batch

R1: plic → plic
 R2: nev → plic
 R3: plic → plic

Day

R1: plic → nev
 R2: plic → nev
 R3: cay → plic

Variability within Biotypes/Species

- Microsatellite analysis is the most informative technique at individual level
- Primers only available for *B. plicatilis* s.s.
- New microsatellite markers are needed for all *Brachionus* species

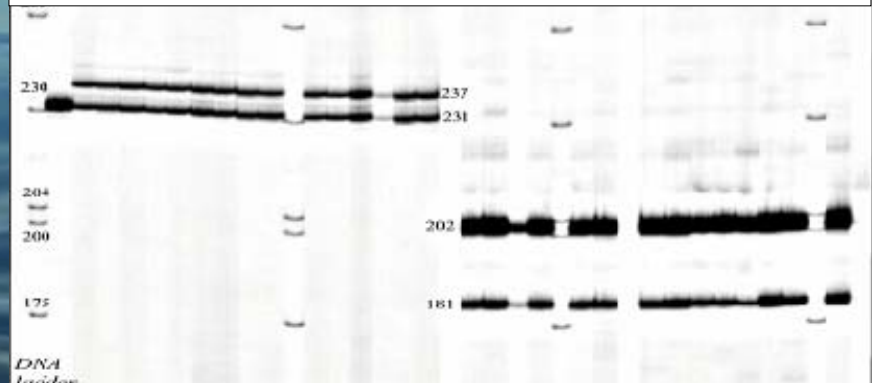
Variability within Biotypes/Species

- Microsatellite data on 2 *B. plicatilis* s.s. hatchery strains have shown that individuals of each strain share the same genotype, i.e. that strains are monoclonal
- These two strains from two different hatcheries were 90% genetically similar
- Does this mean that hatcheries share and use the same strains?

Genotyping of *B. plicatilis* s.s. hatchery individuals at 2 loci

Bp 5 locus

Bp 7 locus





Variability within Biotypes/Species

- It seems there is absence of polymorphism within cultures i.e. most strains are monoclonal or oligoclonal
- Has most of the variability within strains been lost during maintenance of rotifer stocks?
- Or is it lost during repeated rotifer mass cultures?
- Is the absence of polymorphism true for most hatcheries?



Variability within Biotypes/Species

- Molecular markers used so far cannot effectively detect all levels of polymorphism
- Rotifers are cultured in huge quantities
- Methods are required to obtain results at quantitative and/or qualitative level
- DGGE and Real Time PCR analyses are possible candidates



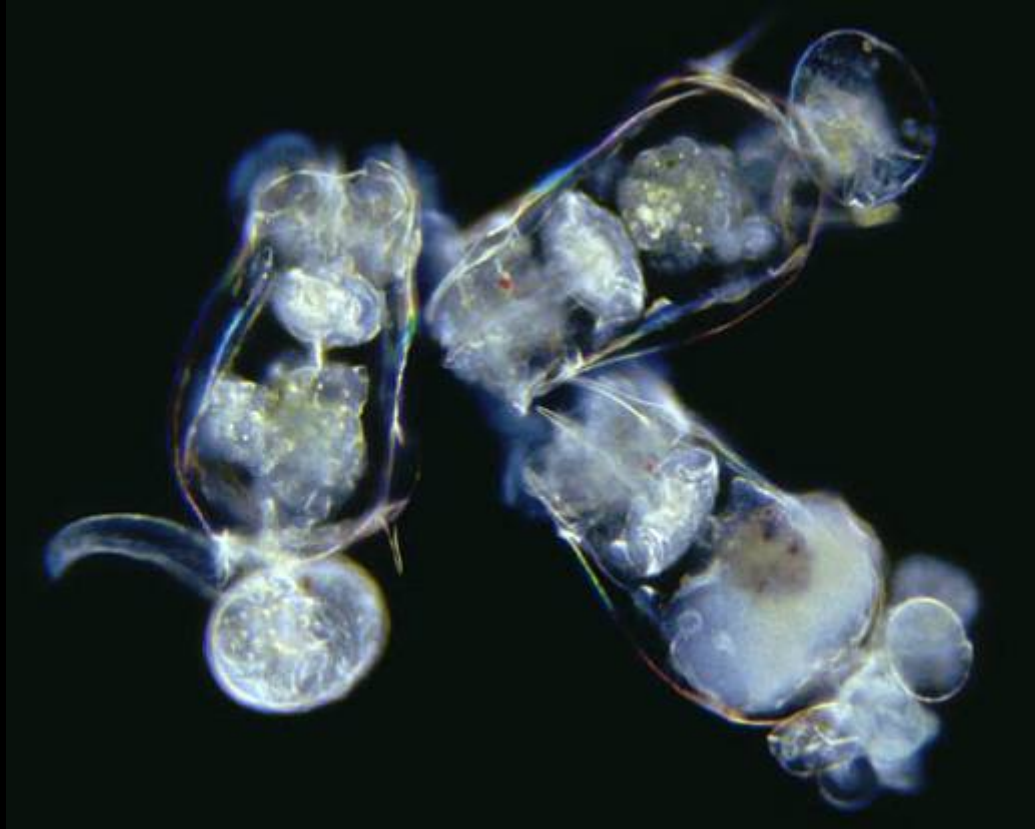
Conclusions

- Lack of knowledge regarding biotype/ species status of hatchery strains is evident
- Morphologically identical samples may be genetically different!
- Caution is needed regarding frequent exchange of samples among hatcheries
- Caution is needed also to avoid contamination within hatcheries
- DNA markers **CAN HELP** identify biotypes, detect contamination, follow-up of cultures



Conclusions

- Studies on *Brachionus* biotypes/species have shown that each one has different temperature and salinity optima
- Future analyses will provide data on the different strains and on the advantages of using particular *Brachionus* biotypes according to the needs of each hatchery
- Feedback from hatcheries is ESSENTIAL
- These data could be used in order to make rotifer cultures more efficient, more predictable and less affected by human errors



Thank you