



# The “MetaCopepod” project: Designing an integrated DNA metabarcoding and image analysis approach to study and monitor biodiversity of zooplanktonic copepods.

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Με τη συγχρηματοδότηση της Ελλάδας και της Ευρωπαϊκής Ένωσης



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# The “MetaCopepod” project

**Aim:** to develop a novel methodology, based on the combination of **DNA metabarcoding** and **image analysis**, to assess and monitor the diversity of marine zooplanktonic copepods (and cladocera), in the Mediterranean and the Black Sea, in a high-throughput, cost-effective, accurate and quantitative way.

**Coordinator:** Dr. Panagiotis Kasapidis, Hellenic Centre for Marine Research (HCMR), GREECE

**Study area:** Mediterranean and the Black Sea

**Duration:** Feb. 2014 – Oct. 2015

**Budget:** 180,000 euros

**Funding:** European Social Fund (ESF) and National Funds through the National Strategic Reference Framework (NSRF) 2007-2013, Operational Programme "Education and Life-Long Learning", Action "ARISTEIA II", Greek Ministry of Education and Religious Affairs, General Secretary of Research and Technology.



# Studying zooplankton diversity: limitations of traditional approaches

- Quite laborious (sorting, identification under stereoscope) → bottleneck in sample processing.
- Requires local taxonomic expertise
- Difficult to identify immature stages
- Misidentifications
- Cryptic species





# Image analysis



## + Pros

- High throughput
- Quantitative results (abundance, size spectra, biomass)

## - Cons

- Low resolution → can recognize taxa similar to the ability of a trained taxonomists to identify a taxon under stereoscope at a glance
- “train” image analysis software separately for different types of zooplankton communities (not once for all).



# DNA metabarcoding (NGS analysis) for studying marine biodiversity

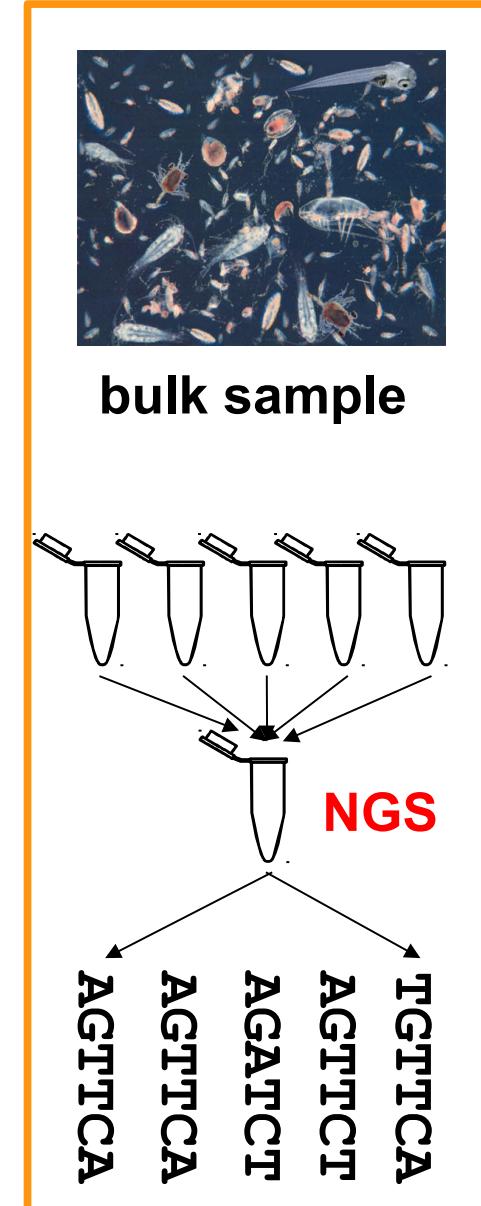
## + Pros:

- Faster processing of the samples
- Potentially higher accuracy in species' identification (even for difficult taxa, immature stages, cryptic species)
- No need of taxonomy expert but requires a well curated and complete reference genetic database

## - Cons:

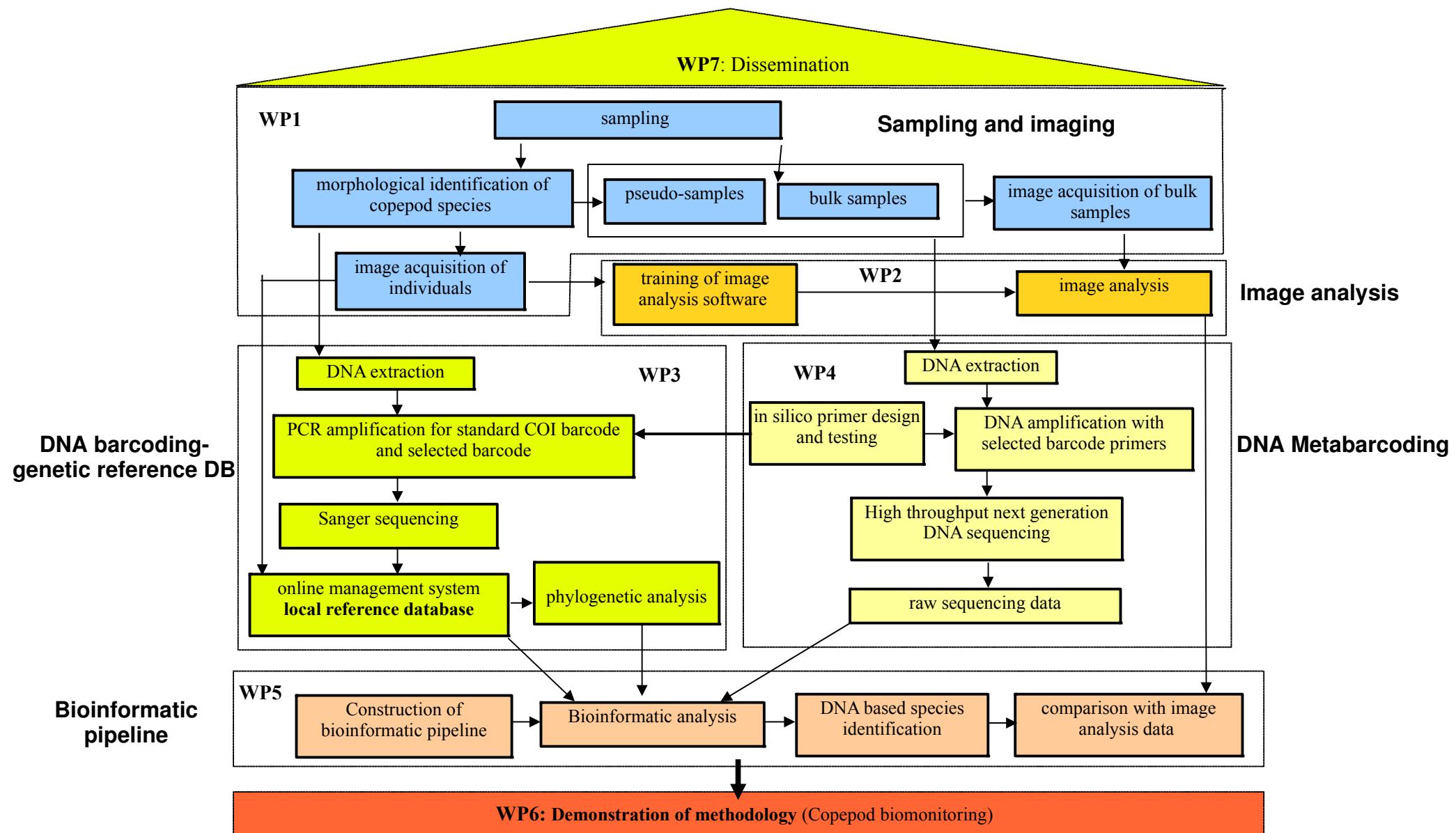
- Results are semiquantitative due to the PCR
- Biases in species' relative abundance mostly due to PCR amplification bias

The “MetaCopepod” project aims to combine the Pros of both methods in order to increase accuracy in assessing zooplankton diversity.





# Workflow of the “MetaCopepod” project





# Sampling



**Partner Network and sampling stations.**  
Monthly sampling (★), non-regular sampling (☆)



# Samples

- 108 zooplankton samples collected and preserved in 95% EtOH
- 80 zooplankton samples were scanned for image analysis and then used for DNA metabarcoding
- 97 copepod species were morphologically identified ( >350 specimen collections from different zooplankton samples)

## For standardizing the methodology:

- Five of the zooplankton samples were taxonomically identified by taxonomist
- Six pseudosamples (mock samples) created by mixing taxonomically identified specimens at known numbers (6-16 species, 1-3 individuals per species)



# Image analysis

## (Epson scanner similar to Zooscan and Zoolimage software)

For “training” the image analysis software we used:

- scanned images of taxonomically identified taxa (“gold” standards)
- taxonomically identified taxa from scanned zooplankton images (“silver” standards)



The image analysis software was evaluated by:

- self-evaluation
- using pseudosamples and taxonomically identified samples

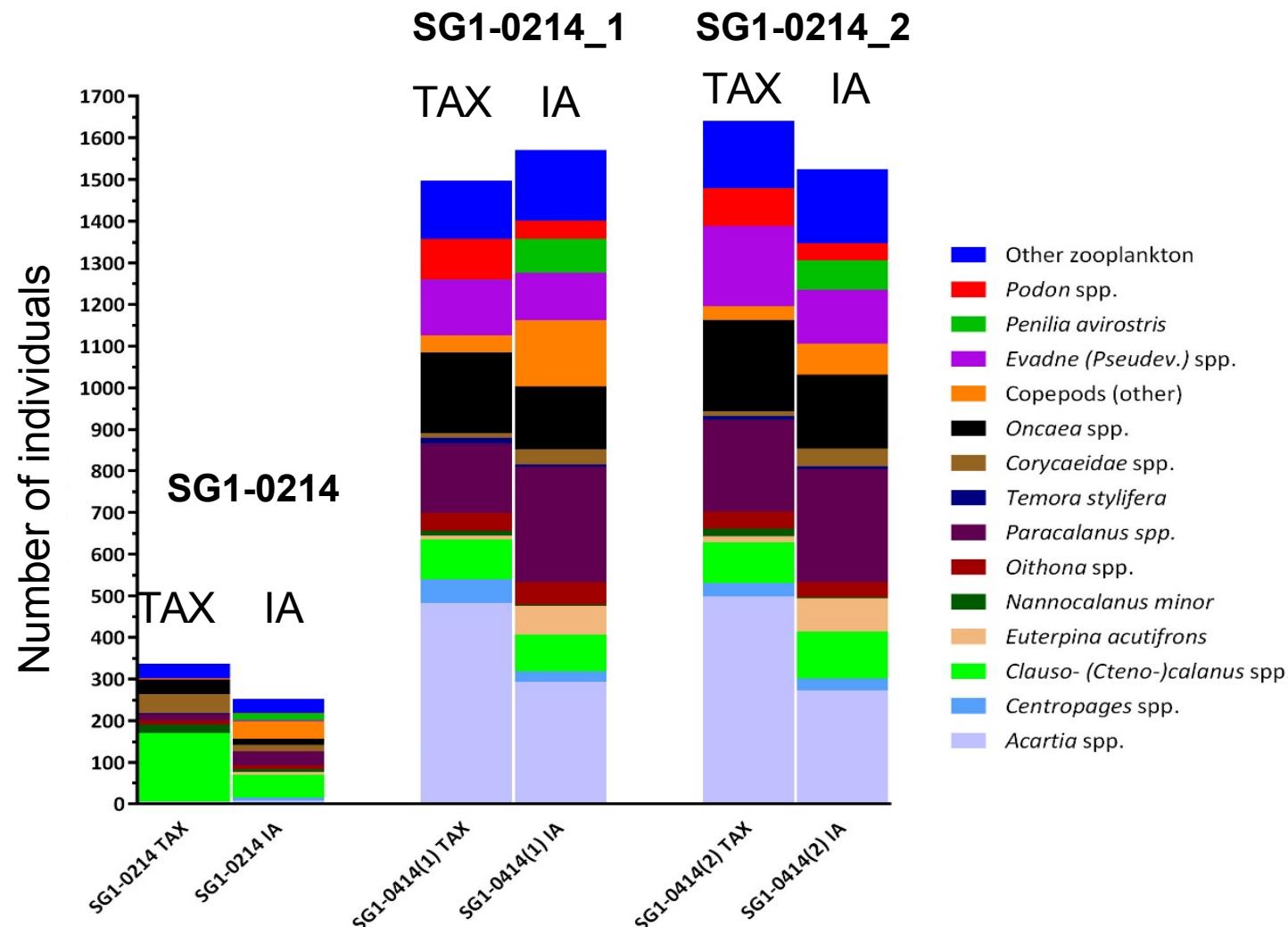


# Self evaluation of the Image Analysis software

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41						
Acartia clausi	1	86	0	0	0	0	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Corycaeus sp.	2	0	74	0	0	11	6	0	5	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Calanus helgolandicus	3	0	0	94	1	0	0	0	0	0	0	3	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Candacia sp.	4	0	0	0	98	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Centropages sp.	5	0	5	0	0	80	0	2	3	0	0	0	0	0	0	0	0	0	0	1	7	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Clausocalanus furcatus	6	1	1	0	0	0	39	0	3	0	5	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Clausocalanus jobei	7	5	0	0	0	4	14	65	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Clausocalanus lividus	8	0	5	0	0	0	0	0	1	91	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Ctenocalanus vanus	9	1	1	0	0	1	14	1	0	77	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Euchirella rostrata	10	0	0	4	0	0	0	0	0	0	88	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Euterpea acutifrons	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Farranula rostrata	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Nannocalanus minor	13	0	0	0	4	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Neocalanus gracilis	14	0	0	5	0	0	0	0	0	0	10	0	0	0	2	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oithona sp.	15	3	0	0	0	0	0	0	0	0	0	3	7	0	0	50	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Oncaea media	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Oncaeida scotticarloii	17	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Paracalanus namus	18	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Paracalanus parvus	19	0	0	0	0	0	4	0	0	2	0	0	0	0	0	0	4	0	0	91	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Pleuromamma sp.	20	0	0	4	0	1	0	0	2	0	0	0	1	0	0	0	0	0	0	66	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Temora stylifera	21	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	1	96	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Evadne (Pseudoevadne) sp.	22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	92	0	1	0	0	0	0	0	3	0	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	
Penilia avirostris	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	92	3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Podon intermedius	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	96	0	0	0	0	0	0	4	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0		
Bad focus or multiple	25	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	
Multiple	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0	0	0	6	0	2	0	0	0	0	0	0	0
Appendicularia	27	1	0	0	0	4	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Chaetognatha	28	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Euphausiacea larvae	29	5	0	0	0	9	0	0	5	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	
Gastropoda larva	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Macro-Brachyura larvae	31	3	3	3	5	5	0	0	5	0	3	0	0	0	0	0	0	0	0	3	8	5	3	0	3	0	8	8	3	5	3	24	0	0	0	0	0	0	0	0	0	0	0				
Ostracoda	32	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	4	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
Pteropoda	33	3	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	5	3	3	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	
Salpida	34	0	0	0	0	5	0	0	0	0	2	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Siphonophora	35	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Bubbles	36	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Debris (marine snow)	37	2	0	0</																																											



# Evaluation of the Image Analysis software with taxonomically identified samples



- Three samples from Saronikos Gulf analyzed both by a taxonomist and by IA
- IA software performs well both for taxa recognition and abundance estimation



## Categories identified with accuracy by Image analysis (standardized for Saronikos Gulf)

Larger categories	Categories for Image Analysis	Taxa in each category
Big Calanoida	<i>Calanus helgolandicus</i>	<i>Calanus helgolandicus</i>
	<i>Candacia-Paracandacia</i>	<i>Candacia</i> <i>Paracandacia simplex</i>
	<i>Euchirella</i>	<i>Euchirella</i>
	<i>Nannocalanus minor</i>	<i>Nannocalanus minor</i>
	<i>Neocalanus</i>	<i>Neocalanus</i>
	<i>Pleuromamma</i>	<i>Pleuromamma</i>
Medium Calanoida	<i>Acartiidae</i>	<i>Acartia</i> <i>Paracartia grani</i>
	<i>Centropagidae</i>	<i>Centropages</i>
	<i>Temora</i>	<i>Temora</i> <i>Aetidus</i> <i>Lucicutia</i>
Small Calanoida		<i>Calocalanus</i> <i>Clausocalanus</i> <i>Paracalanus</i> <i>Ctenocalanus vanus</i> <i>Phaenna spinifera</i> <i>Scolecithricella &amp; Scolecithrix</i> <i>Isias clavipes</i> <i>Mecynocera clausi</i>
	<i>Small Calanoida</i>	
Cyclopoida	<i>Oithona</i>	<i>Oithona</i>
Harpacticoida	<i>Euterpina acutifrons</i>	<i>Euterpina acutifrons</i>
	<i>Clytemnestra</i>	<i>Clytemnestra</i> other Harpacticoida n.d.
Poecilostomatoida	<i>Micro &amp; Macrosettela</i>	<i>Micro &amp; Macrosettela</i>
	<i>Coryceaidae</i>	<i>Coryceaus</i> <i>Farranula rostrata</i>
	<i>Oncaeidae</i>	<i>Oncaeaa</i> <i>Triconia</i>
Ctenopoda	<i>Penilia avirostris</i>	<i>Penilia avirostris</i>
Onychopoda		
	<i>Onychopoda</i>	<i>Evadne</i> <i>Pseudevadne</i> <i>Podon</i> <i>Pleopis polyphemoides</i>



# Critical factors for DNA metabarcoding

- **Primers**
  - designed on conserved regions across target taxa → reduce amplification bias
  - amplify a variable region → high resolution, ideally at species' level
  - amplify a short region → easier PCR amplification even for degraded samples
- **Genetic reference database**
  - well curated (no errors or misidentifications)
  - as complete as possible



# Primer design and reference database

**Primers** were designed (ecoPrimer software), which amplify a region of ~150 bp of the mitochondrial 16S rRNA gene.

**Reference database** (GenBank largely incomplete for 16S rRNA)

Out of the 97 taxa taxonomically identified

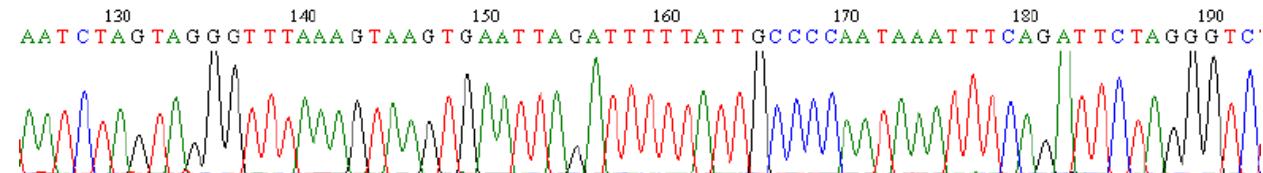
- 93 lacked 16S sequences and 36 lacked COI sequences in GenBank

We performed DNA barcoding for both COI and 16S genes

- 50 new additions of 16S barcode (55 species sequenced in total)
- 8 new additions for standard COI barcode (48 species sequenced in total)

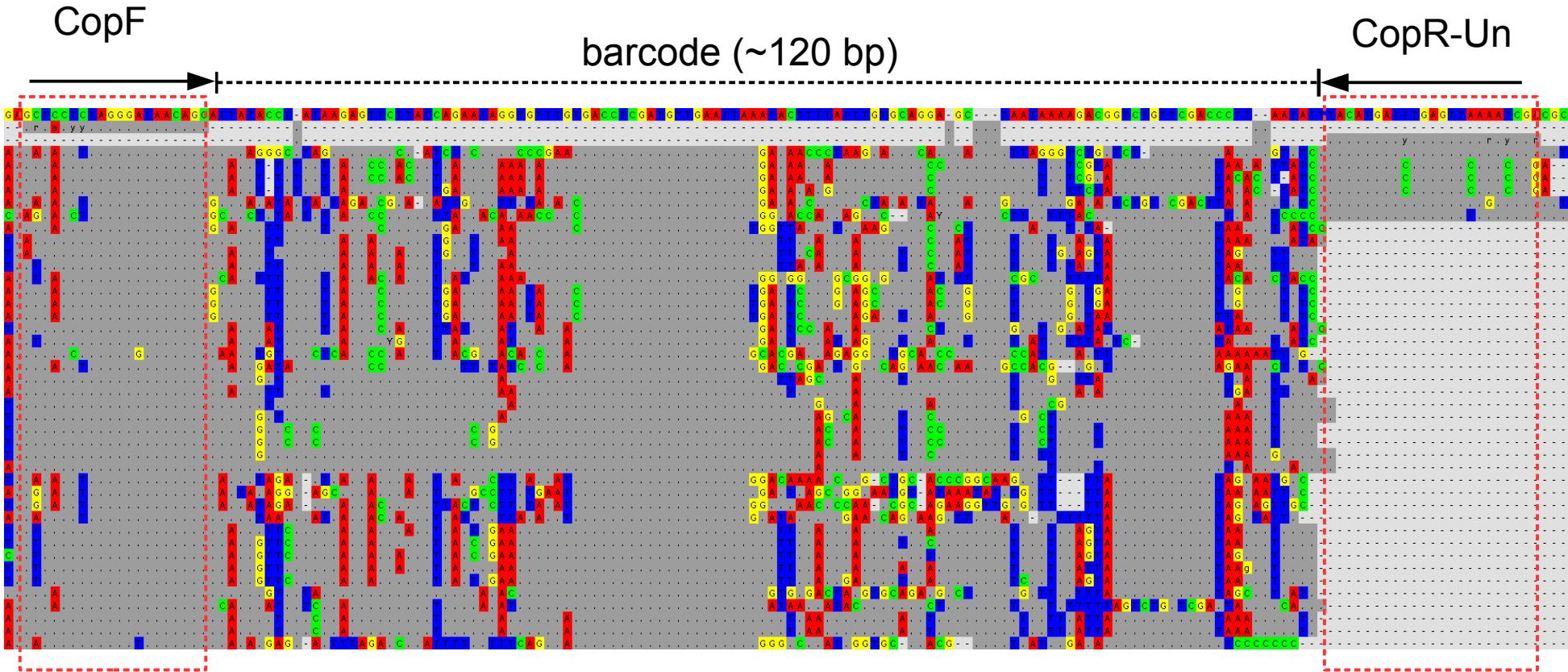


*Sapphirina opalina*

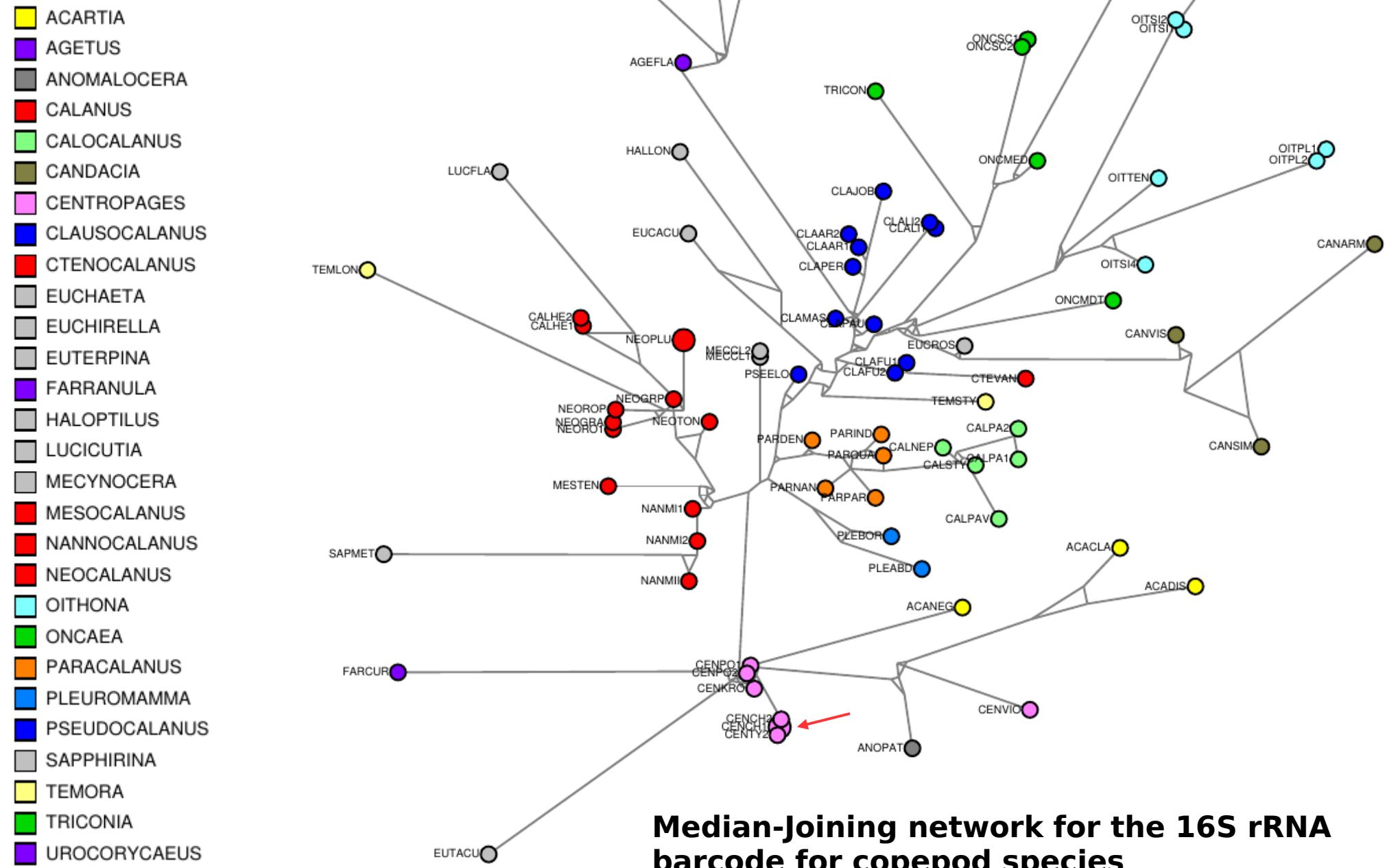




# Evaluation of the 16S barcode to discriminate species



Alignment of 16S copepod sequences from NCBI and from MetaCopepod  
(produced with universal primers)



# Median-Joining network for the 16S rRNA barcode for copepod species



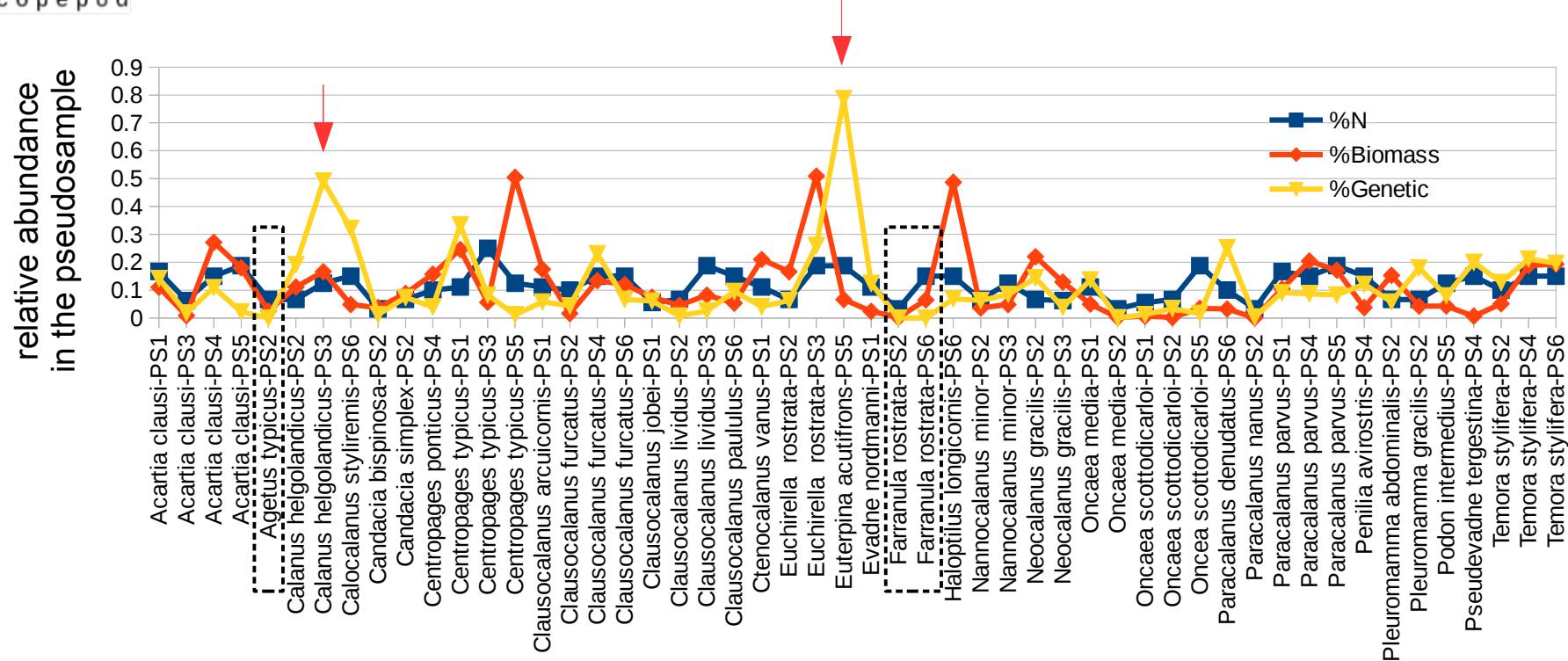
# DNA metabarcoding of zooplankton samples

- Unsorted zooplankton samples, taxonomically identified samples and pseudosamples → DNA extraction, PCR and sequencing on a MiSeq Illumina platform.
- Raw sequencing data analyzed using a bioinformatic pipeline constructed for the project.
- Sequences assigned to taxa using the reference database





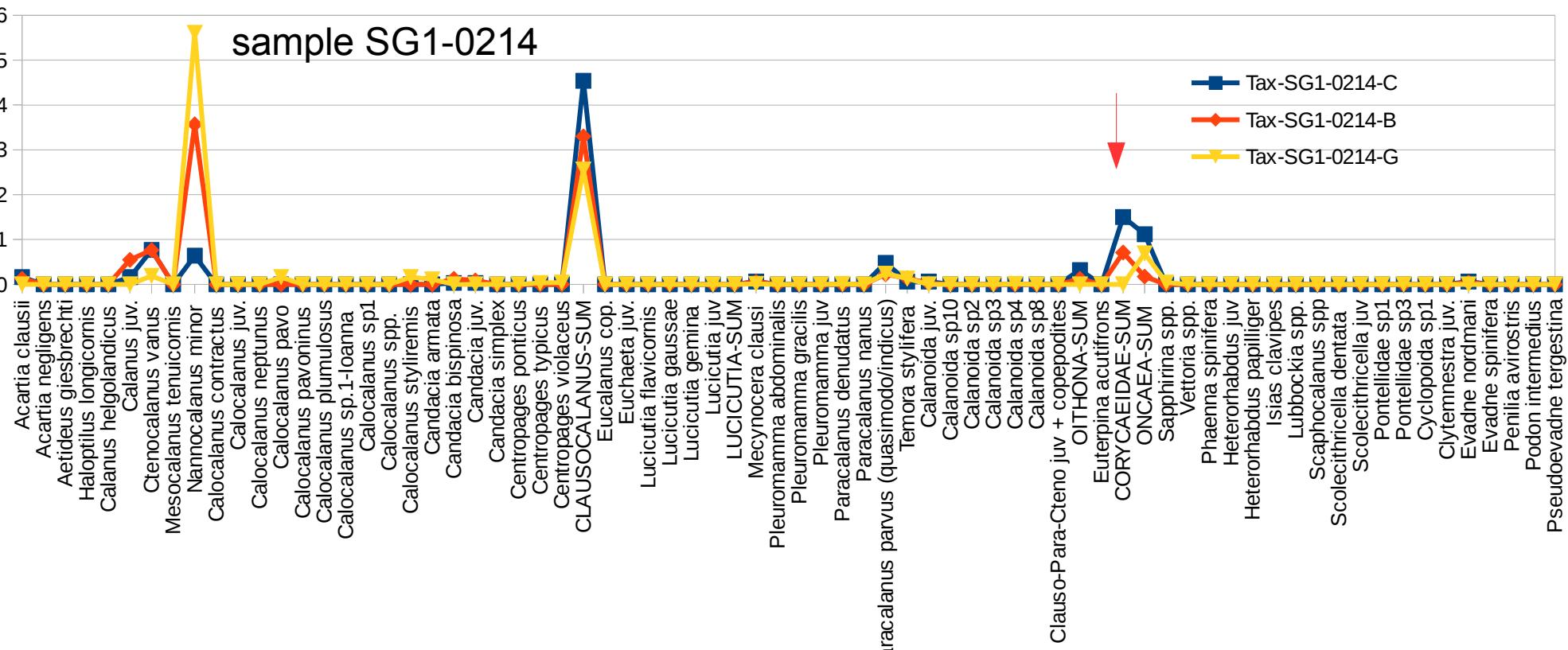
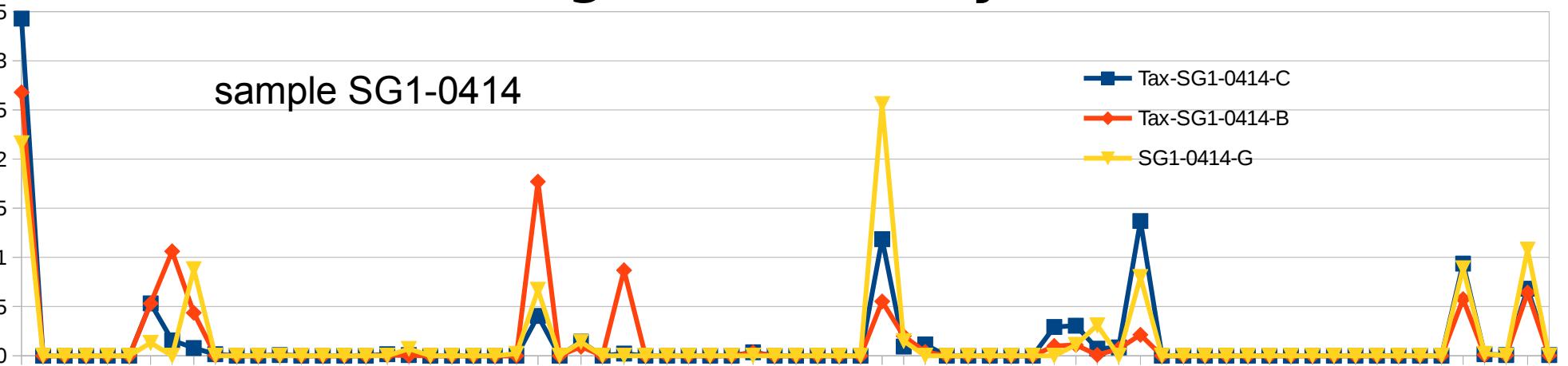
# Pseudosamples: comparison between genetic data, % abundance (%N) and % biomass



- Pseudosamples contained 6-16 species of 1-3 individuals each.
- All species detected by genetic analysis (even as low as 0.12% of the total sample biomass), except for the Corycaeidae [*Farranula rostrata* (max 6.5% biomass) and *Agetus typicus* (2.5% biomass)].
- For few species (e.g. *Calanus helgolandicus* and *Euterpina acutifrons*), large discrepancies between genetic and actual data. Systematic? Due to technical handling? → more checks necessary.

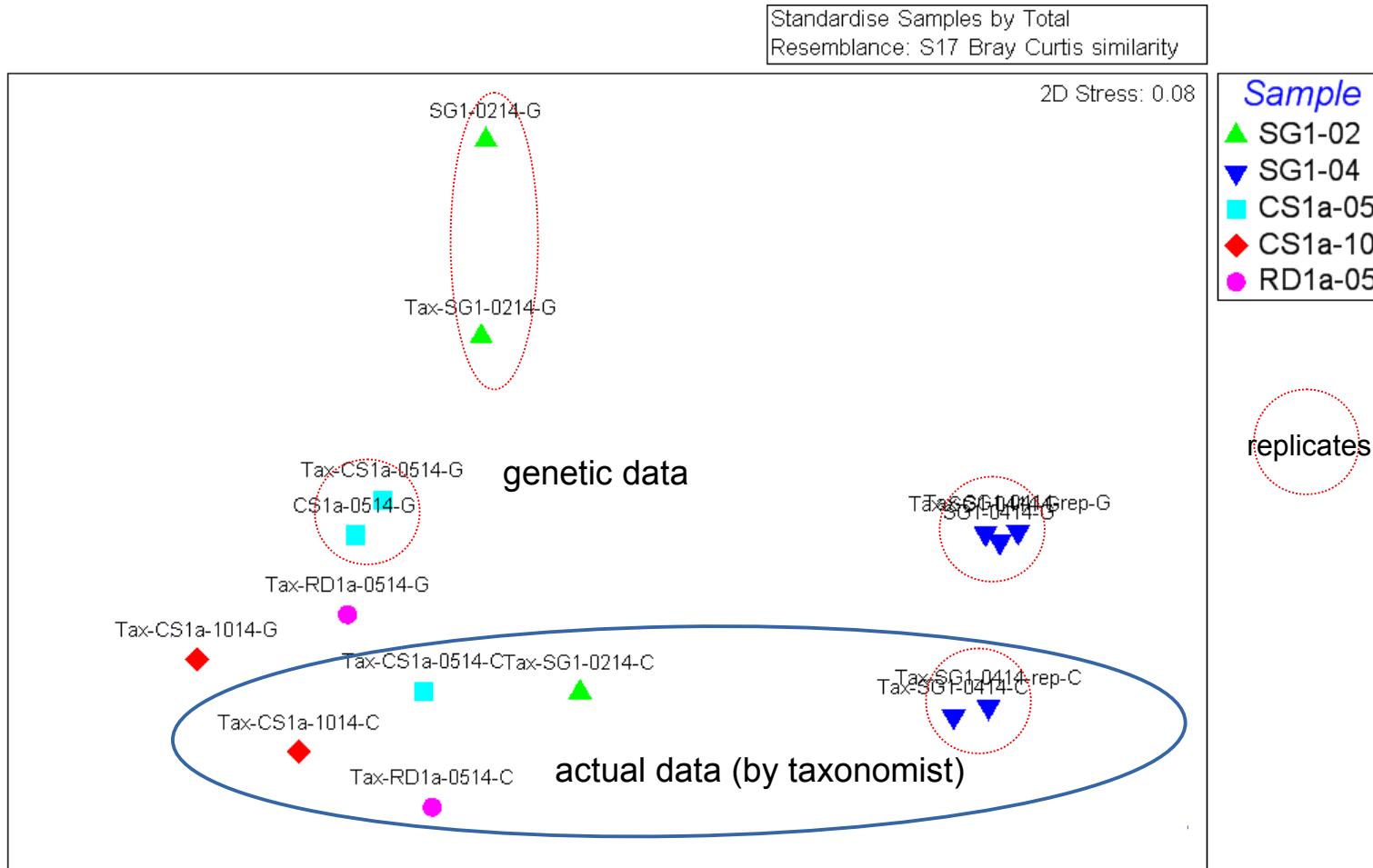


# Taxonomically identified samples: Comparison between actual counts (blue line) and genetic data (yellow line)





# MDS for taxonomically identified samples: Comparison between genetic data (-G) and actual counts (-C)



The observed shift between the two data sets is mainly due to the inability to match exactly some taxa categories (e.g. *Calocalanus* juv. and *Calocalanus* sp. identified by the taxonomist to which of the 7 *Calocalanus* species identified by DNA metabarcoding correspond to ?)



# Conclusions from standardization of DNA metabarcoding

High repeatability (sub-samples of the same sample give very similar results)

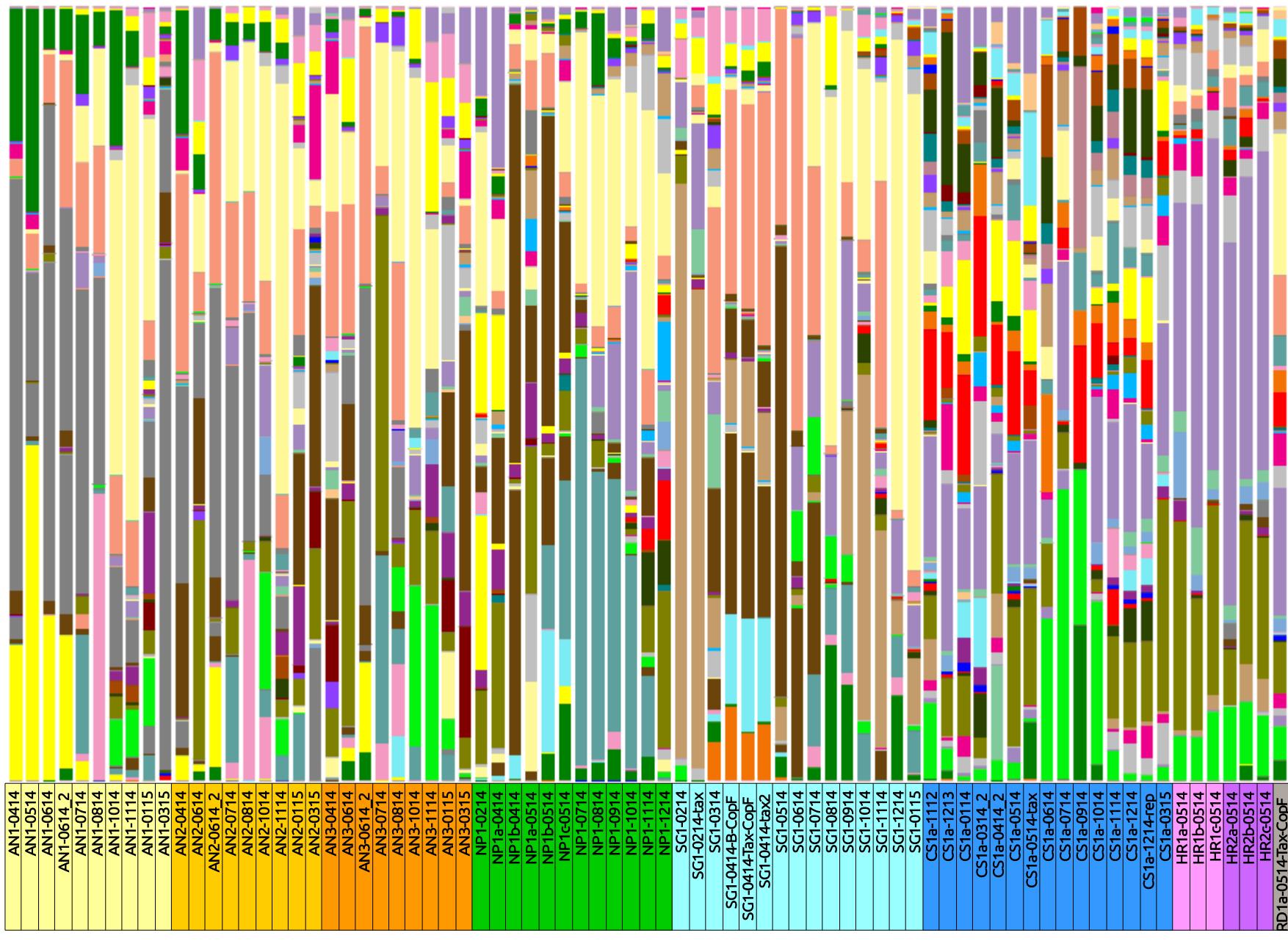
Generally good concordance between morphological identification and NGS analysis  
but, *Corycaeidae* almost absent in DNA metabarcoding analysis (both in pseudo  
and taxonomically identified samples), although some species present in the  
reference DB and are individually amplified in PCR with the 16S primers.  
redesign primers? problems in DNA extraction?

Some taxa are systematically over or under-represented in DNA metabarcoding  
analysis → image analysis can greatly assist reducing the bias

# Relative abundance (species level) of copepoda & cladocera in monitoring stations of the Mediterranean (genetic data)



- Sapphirina sp1*
- Sapphirina opalina*
- Sapphirina metallina*
- Oncaea sp9*
- Oncaea sp8*
- Oncaea sp7*
- Oncaea sp6*
- Oncaea sp5*
- Oncaea sp4*
- Oncaea sp3*
- Oncaea sp2*
- Oncaea sp1*
- Oncaea scotticarloi*
- Oncaea mediterranea*
- Oncaea media*
- Agetus limbatus*
- Euterpnosia acutifrons*
- Oithona tenuis*
- Oithona sp8*
- Oithona sp7*
- Oithona sp6*
- Oithona sp5*
- Oithona sp4*
- Oithona sp3*
- Oithona sp2*
- Oithona sp1*
- Oithona similis sp3*
- Oithona similis sp2*
- Oithona similis sp1*
- Oithona similis*
- Oithona plumifera*
- Temora stylifera*
- Paracalanus sp4*
- Paracalanus sp3*
- Paracalanus sp2*
- Paracalanus sp1*
- Paracalanus quasimodo*
- Paracalanus parvus*
- Paracalanus nanus*
- Paracalanus indicus*
- Paracalanus denudatus*
- Other*
- Pleuromamma sp1*
- Pleuromamma gracilis*
- Pleuromamma borealis*
- Pleuromamma abdominalis*
- Mecynocera sp2*
- Mecynocera clausi*
- Lucicutia flavicornis*
- Euchaeta acuta*
- Other*
- Pseudocalanus sp2*
- Pseudocalanus sp1*
- Clausocalanus sp3*
- Clausocalanus sp2*
- Clausocalanus sp1*
- Clausocalanus pergens*
- Clausocalanus paululus*
- Clausocalanus mastigophorus*
- Clausocalanus lividus*
- Clausocalanus jobei*
- Clausocalanus furcatus*
- Clausocalanus arcuicornis*
- Centropages violaceus sp1*
- Centropages violaceus*
- Centropages sp1*
- Centropages ponticus*
- Centropages kroyeri*
- Centropages chierchiae*
- Candacia sp1*
- Candacia simplex*
- Candacia bispinosa*
- Candacia armata*
- Calocalanus styloremis*
- Calocalanus sp3*
- Calocalanus sp2*
- Calocalanus sp1*
- Calocalanus pavoninus*
- Calocalanus pavo sp2*
- Calocalanus pavo*
- Calocalanus neptunus*
- Neocalanus gracilis*
- Nannocalanus sp2*
- Nannocalanus sp1*
- Nannocalanus minor Type II*
- Nannocalanus minor Type I*
- Mesocalanus tenuicornis*
- Mesocalanus sp1*
- Ctenocalanus vanus*
- Calanus helgolandicus*
- Euchirella longicornis*
- Euchirella rostrata*
- Acartia sp1*
- Acartia negligens*
- Acartia discoidata*
- Acartia clausii*
- Penilia avirostris*
- Pseudevadne tergestina*
- Podon intermedius*
- Pleopis polyphemoides*
- Other*
- Evdaina spinifera*
- Evdaina nordmanni*
- Daphnia magna*



Annaba-Algeria (1,2,3)

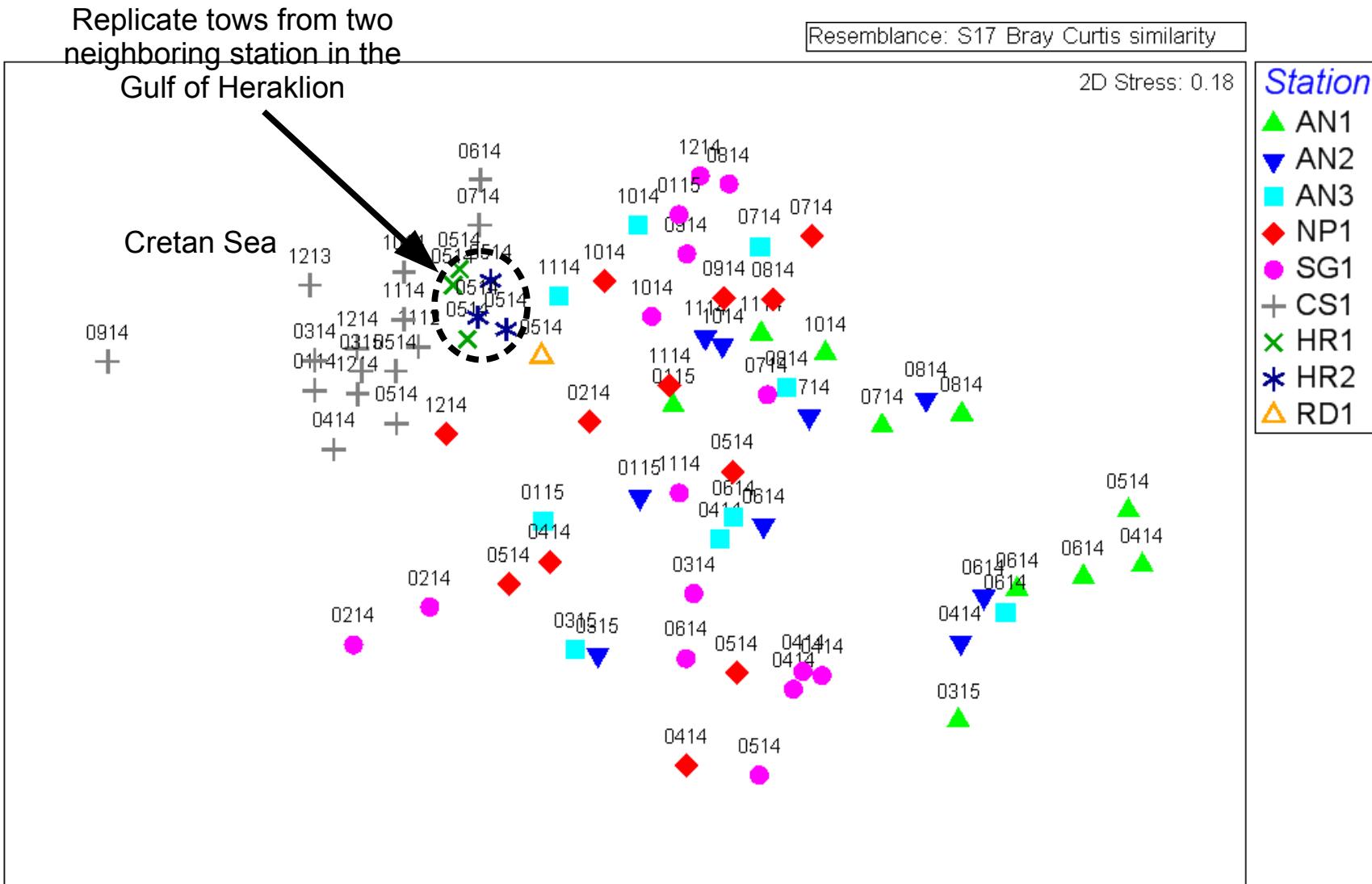
Napoli

Saronikos

Cretan Sea



# MDS plot based on genetic data for all samples

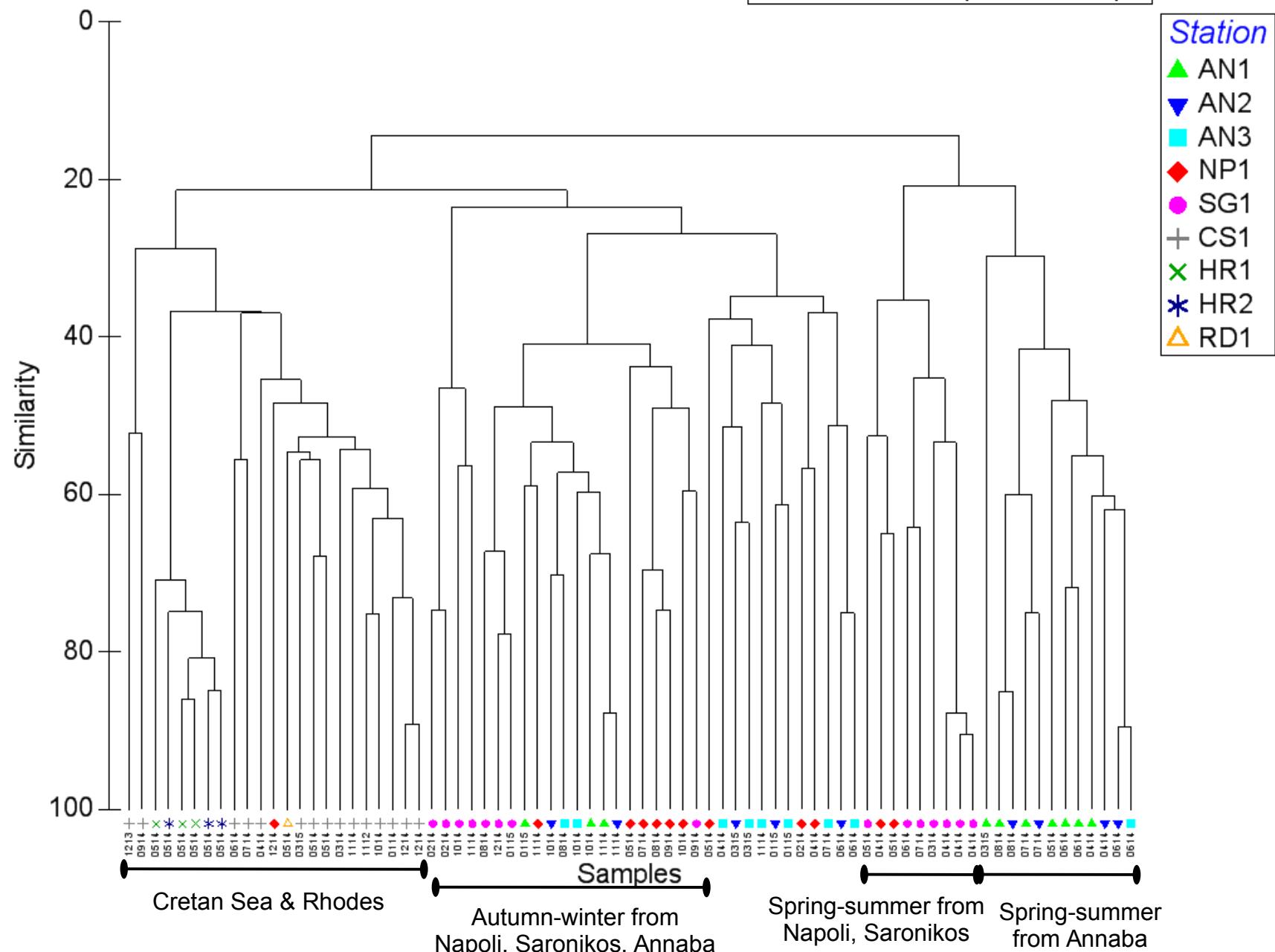




# Similarity dendrogram (Bray Curtis) of zooplankton samples (genetic data)

Group average

Resemblance: S17 Bray Curtis similarity





# Integration between image analysis and DNA metabarcoding analysis (under way)

A script is developed to combine automatically the output of image analysis and genetic analysis.

IA categories	IA %	DNA metabarcoding categories	Genetic data %	Final combined (%)
Calanus helgolandicus	20	Calanus helgolandicus	40	20
		Subtotal	40	20
Acartiidae	35	Acartia clausii	4	14
		Acartia negligens	6	21
		Subtotal	10	35
Small Calanoida	30	Clausocalanus arcuicornis	10	15
		Clausocalanus furcatus	2	3
		Clausocalanus jobei	5	7.5
		Ctenocalanus vanus	2	3
		Mecynocera clausi	1	1.5
		Subtotal	20	30
Oithona	15	Oithona similis	8	4
		Oithona nana	12	6
		Oithona sp.	10	5
		Subtotal	30	15
Total	100		100	100

Transformed to abundances and biomass.



# What next

- Results are currently reanalyzed and the methodology is optimized for higher integration between image analysis and NGS analysis
- The 16S primers designed perform quite well and have high resolution (also amplify quite well the other components of zooplankton but not evaluated yet for taxa other than copepods and cladocera). Redisign to amplify Corycaeidae.
- Genetic reference DB needs further improvement (few important species still missing, taxonomic issues for certain taxa)
- Image analysis software should be “trained” for the oligotrophic regions of Eastern Mediterranean and other regions (e.g Algeria)



# Partners of the "MetaCopepod" project

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# The project's webpage: <http://metacopepod.hcmr.gr/>

The screenshot shows the metacopepod website with a dark red header. The header features a logo with a red copepod on a barcode, the text "metacopepod", and the subtitle "DNA metabarcoding and image analysis of copepods". On the right side of the header are "Log in", a search bar, and buttons for "SEARCH", "All", and "Taxa". Below the header is a navigation bar with links: HOME, THE PROJECT, PARTNERS, PHOTOS, EVENTS, LINKS, MEMBER AREA, TAXA (which is highlighted in white), and LITERATURE.

The main content area shows the taxon page for *Acartia clausi*. The left sidebar displays a hierarchical tree of copepod taxa under the heading "COPEPODA". The tree includes Copepoda (2), Neocopepoda (2), Gymnoplea (1), Calanoida (32), Acartiidae (3), and Acartia (16). The species *Acartia clausi* is listed under this hierarchy. The right main area contains the taxon details for *Acartia clausi*, Giesbrecht, 1889. It includes tabs for Overview, Descriptions, Photos, Literature, Maps, Specimens, NCBI data, and Personal observations. Below these tabs, sections for Nomenclature and Media are shown. The Nomenclature section lists Family: Acartiidae, Genus: *Acartia*, Species: *Acartia clausi* Giesbrecht, 1889, and Usage: valid. The Media section shows a grayscale image of a copepod specimen.

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***Thank you for your attention!***