Trying to resolve the taxonomic confusion of *Paracalanus parvus* species complex (Copepoda, Calanoida) in the Mediterranean and Black Seas through a combined analysis of morphology, molecular taxonomy and DNA metabarcoding

OR

Looking for tiny hair on the ladies' legs!

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Overview

1) The *P. parvus* species complex and its taxonomic difficulties

2) DNA barcoding (COI) to resolve taxonomy and geographic distribution

3) Additional data from DNA metabarcoding (16S rRNA)

4) Combined morphological and DNA barcoding analysis

5) Conclusions
The *Paracalanus parvus* species complex

- Species of *P. parvus* complex abundant in many marine ecosystems from temperate to tropical regions
- Consists of 7 morphological species
  - *P. parvus*
  - *P. indicus*
  - *P. quasimodo*
  - *P. nanus*
  - *P. intermedius*
  - *P. tropicus*
  - *P. serrulus*

Taxonomic difficulties in distinguish among three, mainly between *indicus* and *quasimodo* (inconspicuous characters) (Bowman, 1971; Bradford, 1978; Kang, 1996; Ounissi and Khelifi-Touhami, 1999; Khelifi et al., 2007)
Morphological comparison of *P. parvus*, *P. indicus* and *P. quasimodo* (modified from Bradford, 1978)

<table>
<thead>
<tr>
<th>Species</th>
<th>Outer distal edge of Ex3 serrated</th>
<th>B1 of P2-P4 with many posterior surface spinules</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. parvus</em> ♀</td>
<td>− − −</td>
<td>−</td>
<td>1,2,3</td>
</tr>
<tr>
<td><em>P. parvus</em> ♂</td>
<td>? ? ?</td>
<td>−</td>
<td>2</td>
</tr>
<tr>
<td><em>P. indicus</em> ♀</td>
<td>+ + −</td>
<td>+</td>
<td>1,2,3,4</td>
</tr>
<tr>
<td><em>P. indicus</em> ♂</td>
<td>+ + +</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td><em>P. quasimodo</em> ♀</td>
<td>+ + +</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td><em>P. quasimodo</em> ♂</td>
<td>+ + +</td>
<td>+</td>
<td>2</td>
</tr>
</tbody>
</table>


Image from G. Harding

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ICES/PICES 6th Zooplankton Production Symposium, 9-13 May 2016, Bergen
## Additional criteria (female) according to Bowman (1971)

<table>
<thead>
<tr>
<th>Species</th>
<th><em>P. parvus</em></th>
<th><em>P. indicus</em></th>
<th><em>P. quasimodo</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body</strong></td>
<td>Slender</td>
<td>Slender-Shorter</td>
<td>Stocky</td>
</tr>
<tr>
<td></td>
<td>0,85-0,90 mm</td>
<td>0,9 mm</td>
<td>1,0 mm</td>
</tr>
<tr>
<td><strong>Dorsal hump</strong></td>
<td>no</td>
<td>Slightly developed</td>
<td>Developed</td>
</tr>
<tr>
<td><strong>Prosom/Urosome</strong></td>
<td>3,3-3,4/1</td>
<td>3,2/1</td>
<td>3/1</td>
</tr>
<tr>
<td><strong>Gnsgm with posterior dorsal spinules</strong></td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Spermatheca</strong></td>
<td></td>
<td>Subelliptical</td>
<td>Obovate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not narrowing distally</td>
<td>Narrowing distally</td>
</tr>
<tr>
<td><strong>A1 reaches:</strong></td>
<td>Midlenght of</td>
<td>Anal segment</td>
<td>Posterior margin of</td>
</tr>
<tr>
<td></td>
<td>caudal ramus</td>
<td></td>
<td>caudal ramus</td>
</tr>
</tbody>
</table>
What was believed to be the distribution of *P. parvus* complex in the Mediterranean

- In the Mediterranean Sea, until recently, *P. parvus* was considered the dominant species.

- Bowman (1991) demonstrated that some specimens from several regions identified as *P. parvus* (Claus, 1863) were actually *P. indicus* Wolfenden, 1905 (Mediterranean, Atlantic and Indian Ocean) or *P. quasimodo* Bowman, 1971 (West Atlantic).

- In SW Mediterranean (Algeria, Tunisia) the identity of the copepod previously known as *P. parvus* was clarified and reassigned to *P. indicus* based on morphological analysis (Khelifi-Touhami et al., 2007).
  - *P. parvus* (Claus, 1863): Alboran Sea, SW Mediterranean (Baleares, Algeria), Tyrrhenian Sea, Gulf of Naples, Adriatic Sea, Ionian Sea, Malta, Aegean Sea, Rhodes, Levantine Basin), Black Sea.
  - *P. indicus* Wolfenden, 1905: Alboran Sea, SW Mediterranean (Algeria), Gulf of Naples
  - *P. quasimodo* Bowman, 1971. Absent
Doubts about the taxonomic status of *P. parvus* in the Mediterranean Sea

- The community of zooplanktonologists in North Mediterranean had indications from the morphology that what was considered as *P. parvus* may not be a single species.

- In 2008-9, *Paracalanus* samples were collected in the frame of the FP-6 EU project “SESAME” and genetically analyzed in HCMR, as a small side project.
First indications that *P. parvus* in the Mediterranean is not a single species

- **Clade A1**
  - Esp2 19
  - Esp2 32
  - Esp2 30
  - AF474111 Ppar
  - IT4 14
  - IT7 7
  - IT2 4
  - IT2 6
  - Esp2 2
  - Gr1 29
  - IT7 10-GR1 14
  - Esp2 17-18-23
  - Gr1 22
  - Esp2 22
  - Gr1 4

- **Clade A2**
  - Esp2(1)Gr1(14)Gr2(1)IT4-6(19)IT7(5)

- **Clade B1**
  - EU856807 P.aculeatus
  - EU856802 Ppar
  - EU599545 Ppar
  - EU599546 Ppar
  - EU856803 Ppar
  - AF474110 Ppar

- **Clade B2**
  - EU856804 Ppar
  - EU856802 Ppar
  - EU599545 Ppar
  - EU599546 Ppar
  - EU856803 Ppar
  - AF474110 Ppar
  - AF513643 C.pergens

- **Pacific**

- **Specimens collected during SESAME project**
- **132 specimens from 6 localities sequenced for cytochrome c oxidase I (COI) mtDNA gene**
Evidence of cryptic and pseudocryptic speciation in the *Paracalanus parvus* species complex (Crustacea, Copepoda, Calanoida)

Astrid Cornils* and Christoph Held
Main conclusions:

- **P.parvus** not a single species but 10-12 putative species with differing geographic distribution
- **P.parvus s.s.** only in NE Atlantic (type locality)
- **P.quasimodo** in Atlantic and W. Mediterranean (includes specimens identified by others as **indicus**)
- **P.indicus** has global distribution (including specimens from Pacific identified by others as **parvus**)

\[\text{Cornils & Held, 2014}\]
Distribution and relative abundance of species of *P. parvus* complex in Mediterranean and Black Sea (specimens from SESAME reanalyzed and assigned to species according to Cornils & Held (2014))

- **P. indicus**
- **P. quasimodo**
- **P. parvus**
- *Paracalanus* sp. F

- **P. parvus** present in the Black Sea (the only *Paracalanus* species there), in N. Adriatic and in NW Mediterranean (off shore) [not only in NE Atlantic]
- **P. quasimodo** dominant in Naples and Aegean Sea, present in all Mediterranean sites)
- **P. indicus** present in all Mediterranean, but usually not abundant
- **P. sp.F** found in NW Mediterranean (off shore) [it is also found in Gulf of Maine and N. Zealand]
DNA metabarcoding analysis of bulk zooplankton samples

- “MetaCopepod” project: Develop a combined DNA metabarcoding and image analysis approach, to assess and monitor the biodiversity of marine planktonic copepods in a high-throughput, reliable and quantitative way.

- Methodology based on sequencing a portion of 16S rRNA mitochondrial gene in Miseq (Illumina).

- A genetic reference database for the 16S rRNA was also produced for the main copepod species of the Mediterranean and the Black Sea for assigning MOTUs (all Paracalanus species are distinguished well).

- Several bulk zooplankton samples were processed, collected mostly from 4 monitoring areas (Algeria, Gulf of Naples, Gulf of Saronikos and Cretan Sea) at a monthly base.
DNA metabarcoding results (16S rRNA) for the *P. parvus* complex in the Mediterranean Sea.
Distribution and relative abundance of *P. parvus* complex based on COI sequences (Sanger) and DNA metabarcoding data (16S rRNA)

- **P. indicus**
- **P. quasimodo**
- **P. parvus**
- Paracalanus sp. F

**P. quasimodo** dominant species in the coastal areas of the Mediterranean

★ DNA metabarcoding data
Combined morphological and genetic analysis of *Paracalanus* samples from the Mediterranean Sea

- *Paracalanus* specimens collected from Algeria (6), Aegean Sea (Saronikos) (20), Napoli (6), N. Adriatic (26) and Black Sea (10)
- Identified under microscope; whole animal, legs P3 and P4 photographed.
- Same individuals then sequenced for COI and genetically identified.
- This work still in progress
Results of the combined morphological and genetic analysis (COI Sanger sequencing)

Algeria (Annaba):
All specimens (5) identified as indicus → quasimodo

Aegean Sea (Saronikos):
Specimens identified either as indicus (12) or parvus (6) → quasimodo

Napoli:
Specimens identified either as indicus or parvus → quasimodo

N. Adriatic:
Most of the specimens identified as indicus → quasimodo but two were indeed indicus
All specimens identified as parvus → parvus

Black Sea
All specimens identified as parvus → parvus

- The genetic results in agreement with our genetic findings from SESAME and MetaCopepod.
Indicative photos of specimens identified morphologically as *P. indicus*

<table>
<thead>
<tr>
<th>Locality</th>
<th>Morpho-species</th>
<th>Genetic species</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aegean Sea</td>
<td><em>P. indicus</em></td>
<td><em>P. quasimodo</em></td>
<td></td>
</tr>
<tr>
<td>Napoli</td>
<td><em>P. indicus</em></td>
<td><em>P. quasimodo</em></td>
<td></td>
</tr>
<tr>
<td>Algeria</td>
<td><em>P. indicus</em></td>
<td><em>P. quasimodo</em></td>
<td></td>
</tr>
<tr>
<td>N. Adriatic</td>
<td><em>P. indicus</em></td>
<td><em>P. indicus</em></td>
<td></td>
</tr>
</tbody>
</table>
Indicative photos of specimens identified morphologically as *P. parvus*

<table>
<thead>
<tr>
<th></th>
<th>P3</th>
<th>P4</th>
<th>Morpho-species</th>
<th>Genetic species</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0177</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><em>P. parvus</em></td>
<td><em>P. quasimodo</em></td>
<td>Aegean Sea</td>
</tr>
<tr>
<td>P0189</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td><em>P. parvus</em></td>
<td><em>P. quasimodo</em></td>
<td>Napoli</td>
</tr>
<tr>
<td>P0211</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><em>P. parvus</em></td>
<td><em>P. parvus</em></td>
<td>N. Adriatic</td>
</tr>
<tr>
<td>SB3A-1</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><em>P. parvus</em></td>
<td><em>P. parvus</em></td>
<td>Black Sea</td>
</tr>
</tbody>
</table>
Conclusions

• Geographic distribution of the *P. parvus* species complex in the Mediterranean revised based on genetic data (barcoding and metabarcoding)

• Morphological characters used to discriminate the 3 species are not reliable.

• Further study is needed - use of nuclear markers to test for introgression and hybridization, detailed microscopy (SEM)

• More extensive sampling in the Mediterranean and the world oceans combined with DNA metabarcoding analysis could further elucidate the distribution of the *P. parvus* species complex.
• Acknowledgements

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Thank you for your attention!