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Identification of polymorphic species within groups of morphologically conservative taxa: combining morphological and molecular techniques

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Abstract — Identification of small species with high levels of intra-specific polymorphism within groups of inter-specific morphologically-conservative taxa, presents numerous obstacles for biodiversity and ecological studies. This is particularly true for deep-sea studies that often reveal a great number of species but only few numbers of specimens. It is here proposed to deal with such cases by extrapolating information obtained from highly detailed baseline studies. Such baseline studies should include information about sexual and ontogenetic variation and should include a combination of both morphological and molecular techniques.

Index Terms — baseline studies, polymorphism, sibling species, species identification.

1 Introduction

The identification of species can be problematic enough when dealing with taxa which include a large number of morphologically similar species. The obstacles can increase manifolds with smaller taxa that display few stable characters and show tendencies towards reductions. Adding the complications of substantial sexual and ontogenetic variations, the results are often misleading to the point of being meaningless. This is particularly true for deep-sea studies that often reveal numerous species but only few specimens for each species.

One example of such a problematic group is the Tanaidacea (Crustacea: Peracarida), but there are many other, similar difficult taxa, among the smaller invertebrates. In the Tanaidacea species, differentiation is notoriously difficult,

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and males/juveniles often share no species-specific characters with females; even family level identification of males can be hazardous [1]. In many families, multiple polymorphic males exist- the consequence of a peculiar reproductive strategy involving protogynous hermaphrodites- and this causes additional problems [2]. As males locate females by roaming the substrate, they are exposed to high predation pressure that - combined with their non-feeding life-style - makes the life span of males short. In situations where depletion of males from the population occurs, some females may molt into males at several different instars, each resulting in a morphologically different male (up to four different male morphs have been recorded in one species) [2]. Ontogenetic variations among adult females are also known to cause problems [1].

At the same time, the tanaidaceans are infamous for creating species complexes containing many, often sympatric, species that display a very conservative inter-specific morphology. This again makes species identification exceedingly difficult [1].

Tanaidacea are particularly common in deep-sea substrates, where they constitute a major proportion, up to 22 % by some estimates, of the total fauna (in terms of biodiversity) [3], [4]. It is clearly undesirable for any scientific study (particularly in biodiversity and ecology) that such a large proportion of the fauna cannot be identified, but the solution to this problem is not apparent due to time constraints and lack of available expertise. Many large scale biodiversity programs rely heavily on cheap (and poorly trained/supervised) student help to process the often enormous material of small benthic invertebrates. Clearly, given the problems inherent with taxa displaying such troublesome attributes as described above, such personnel have little chance of successful identification (we have personally observed as much as 50% misidentifications in collections from biodiversity studies).

2 Methods

The methods we suggest here for species identifications are not new but make use of an expanded procedure. Firstly the samples should be screened and identified to order. Thereafter, samples with large number of specimens should be given priority. Those high-value samples should then be sent to taxonomical experts for 'baseline' processing. Do NOT use untrained student assistants for this part. Once the experts have reported the baseline study, make the identifying personnel use these for comparisons with each single species. Singletons should not be dissected, but not assigned species rank either (like for example '*Sp. A*'), until comparisons have been made with other singletons of different instars that may belong to the same species.

2.1 Morphological Methods

The baseline study should be conducted in the utmost details, including the dissection, description, and illustration of ANY appendages (not just those normally regarded as taxonomically informative) for ALL developmental stages encountered. Both the lateral and dorsal view of the body should be

drawn. Dissection should include appendages from BOTH sides of the body. Appendages should be mounted in glycerin dyed with clorasol black, sealed with nail polish, and stored for further studies. All character transformations seen from manca-juvenile-adults should be noted and illustrated as a transformation diagram. The baseline study should result in the manufacturing of a guide to identification of genders and developmental stage, and supplied to the personal conducting the identifications of the entire material.

2.2 Molecular methods

2.2.1 DNA Extraction

The main problem with DNA extraction from such specimens is the very low yield of starting tissue available (for the smaller taxa, the entire animal has to be used, since a leg or other appendages do not yield enough DNA). Therefore, the extraction is crucial for further analyses and usually requires some modifications to frequently used protocols. There are several DNA isolation techniques. Here we describe our modifications to one frequently used protocol: silica columns. The most crucial points are as follows: VERY thorough grinding of samples, prolonged periods in the several steps stated in the DNA extraction Kit (we use JETQUICK Tissue DNA kit) and also prolonged periods for the final elution step.

Insufficient disruption of starting material leads to low yield and purity, therefore this step is crucial; we use hand-made hard-plastic cylinders which are efficient in disruption and homogenization of the hard crustaceans exoskeleton and also-because of their small size- can be used in micro-centrifuges tubes avoiding the risk of contamination (they can also be autoclaved) and avoiding loosing tissue (the same micro-centrifuges tubes can be used for proteinase-k digestion).

Extraction can be performed according the JETQUICK protocol but should be modified by increasing the time length of each step, from incubation with proteinase K to each centrifuge step (we used double time). Due to the low final DNA concentration; the same elution solution should then be used for the DNA elution and the same for the second elution step (pre-warmed at 70ºC for five minutes). Densitometric measurements are not useful for detection of small amounts of DNA [5] so the “Qubit” flurometer is ideal (requires only 1µl DNA elution).

2.2.2 PCR

The basic “PCR rules” HAVE to be employed when dealing with these kind of samples, e.g., cleaning the bench top with alcohol before setting up reactions, using plugged tips for all PCR reagents (to avoid contamination), always including a sample without template as a negative control to check for contamination of the reagents. The most crucial points are as follows: short length of PCR products (optimum of 300-350 bp) and higher number of PCR cycles.

The amount of DNA used will depend on the concentration of the sample. It is best to use a “hot start” *Taq* that will provide increased sensitivity, specificity

and yield. Due to the high numbers of PCR cycles needed the quality of the *Taq* is also important (we used Platinum *Taq* DNA Polymerase). Finally in order to avoid adding enzyme inhibitors that may be present, we recommend the use of a high PCR final volume (20 µl).

2.2.3 DNA Sequencing

At this stage, the products must be checked for both quantity and quality. Agarose gel electrophoresis can be used to visualize the amount and size of DNA fragments present in the sample, and since usually the amount of final PCR concentration is low when using these type of samples, we recommend to dry up the total PCR product (use a vacuum centrifuge) into a loading agarose gel volume and excised the PCR gel band. We used several different commercial Gel extraction kits, with no significant results among them. The only modification is the final elution step, which should be no higher than 10µl (we used 5 µl). The DNA sequencing can proceed as usual hereafter.

3 Discussion

Given the large material of 'difficult taxa' often encountered during biodiversity/ ecological studies (particularly from deep-sea environments), the limited expertise available on many such taxa, and the financial restraints, it is not possible to have specialists processing all the material. Therefore we propose to deal with these problems by extrapolating information obtained from the highly detailed baseline-studies described above. We are not so much suggesting new 'methods' for species identifications, but rather a different overall procedure of dealing with large amounts of small troublesome taxa. Instead of dealing with samples from one end to the other, we suggest discriminating between samples of 'low' and 'high value', the latter to be dealt with in great details by specialist, and with priority over 'low' value samples. High value samples are those which contain lots of specimens. Particularly deep-sea collections often reveal many species but few specimens and thus offer only few such targets for detailed studies of inter-specific variation. However, due to the patchy distribution often encountered in the deep sea, a few samples (maybe 1 in 100) will contain lots of specimens and most often these will belong to one or two species. These are the samples worth their weight in gold, and those species of which much material exist should be examined (and the species described/redescribed) in great detail, including dissections, illustrations and descriptions of several individuals, of several developmental stages, and of both sexes. At the same time specimens (males, females, juveniles, and mancae) should be processed for molecular studies to verify con-specificity with absolute certainty. Since most families have not been studied in such detail, these baseline studies are needed to provide the detailed information required for processing other species of the same phylogenetic groups but encountered in fewer numbers during the specific survey. Once such a baseline study has been made, other members of the same family can be processed 'normally' by comparing the characters of whatever instar or gender with the information provided by the baseline study.

If an adult female singleton is encounter, then it can be compared with an adult female from the baseline study; if a manca is encounter, it can be compared with a mancae from the baseline study and so forth. We will thus have the information at hand which is needed for firstly correctly identifying the actual instar/gender, and, secondly, for species identification knowing now which characters are stable or not. These may well vary between higher taxonomical groups but are likely to be similar (or more similar at least) within phylogenetically close groups.

We would like to end this paper with a note on descriptions of new taxa. The senior author recently participated in a workshop regarding the description of peracaridean crustacean. The participants received the following request by the one of the organizers:

"We recently collected several thousand deep-sea species of which we estimate half of them to be new to science. We would like to describe these new species but it is a monumental task that we just don't have the time for. We would therefore like the participants of this workshop to come up with some guidelines to how to describe 'bulk' new species in a short abbreviated and timely fashion".

After a short debate the participants unanimously came up with the only possible answer:

"Please don't do that!".

While the person in charge of this overwhelming material only had good intentions and was indeed faced with an impossible task, abbreviated descriptions can only lead to chaos. If descriptions of such small and difficult creatures are to have any value what so ever- now and in the future- it is absolutely paramount that new species are described thoroughly and in minute details.

References

[1] K. Larsen, "Morphological and Molecular Investigation of Polymorphism and Cryptic Species in Tanaid Crustaceans: Implications for Tanaid Systematics and Biodiversity Estimates". *Zoological Journal of the Linnean Society,* vol. 131(3), pp. 353–379, 2001.

[2] J. Sieg, "Evolution of Tanaidacea". In: F. R. Schram (ed.) *Crustacean Issues* 1, *Crustacean Phylogeny,* Rotterdam, 1983.

[3] T. Wolff, "Diversity and composition of deep-sea benthos". *Nature*, London, vol. 267, pp. 780–785, 1977.

[4] K. Larsen, *Deep-Sea Tanaidacea (Peracarida) from the Gulf of Mexico*. Crustaceana Monographs. (Brill, Leiden), vol. 5, p. 387, 2005.

[5] U. M. Csaikl, M. Bastian, R. Brettscheider, S. Gauch, A. Meir, M. Schauerte, F. Scholz, C. Sperisen, B. Vornam and B. Ziegenhagen, "Comparative analysis of different DNA extraction protocols: A fast universal maxi preparation of high quality plant DNA for genetic evaluation and phylogenetic studies". *Plant Molecular Biology Reporter*, vol. 16(1), pp. 69–86, 1998.