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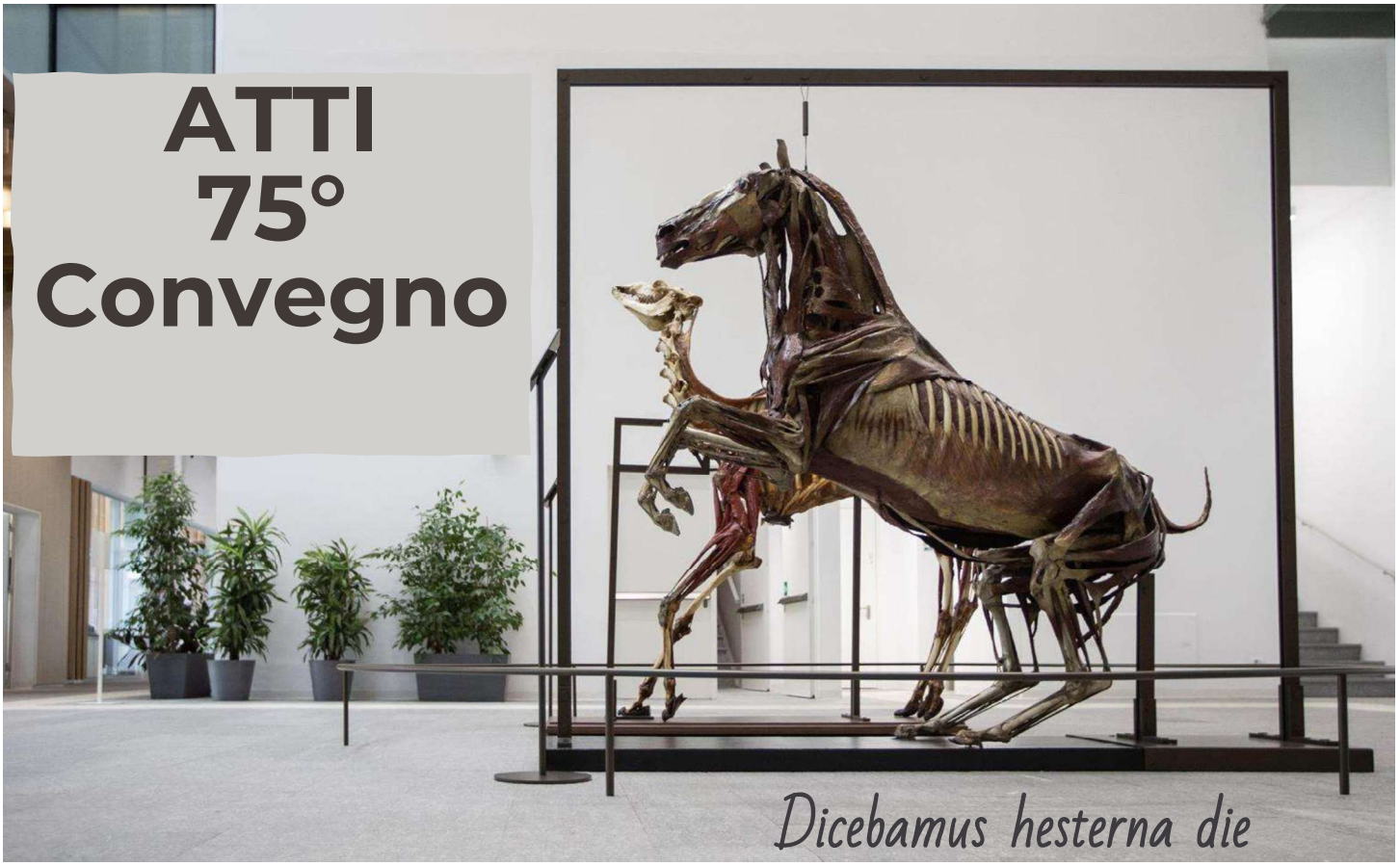

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FINE TUNING OF A SEQUENCING PROTOCOL FOR SPECIES IDENTIFICATION OF MYTILUS SPP. SPECIMENS

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The genus *Mytilus* comprises eight species of mussels of commercial relevance, which have different geographic origin and distribution and can hybridize in case of coexistence (1). To date, events of habitat contamination with allochthonous species occur with increasing frequency, due mainly to anthropogenic transfer, posing threats to local ecosystems (2). Moreover, such events favour species mislabeling, which is reported as the most frequent issue affecting seafood products (3). To prevent these problems, EU Regulation 1379/2013 promotes the use of DNA-based methods. PCR-RFLP is to date one of the most efficient techniques used in *Mytilus* spp. species identification (4-5). However, it has some drawbacks linked to limited resolution and subjective interpretation of the results. To improve such aspects, an optimized protocol for the sequencing of short fragments was developed. A total of 50 samples were analyzed. Of them, 12 were DNA samples belonging to various *Mytilus* spp. that were already identified by PCR-RFLP in a previous study (5). The other 38 were tissue samples, of which 10 belonging to specimens directly collected from production sites in Chile, and 28 belonging to fresh and pre-cooked specimens collected from national market. For these samples, the total DNA was extracted using the DNeasy Mericon Food kit (Qiagen). The target PAP region was amplified from all the 50 samples (DNA and tissue) using the primer pair designed by Satto et al. (4). All PCR products were analyzed through capillary electrophoresis to verify the successful amplification, and then sequenced using Sanger technique. Twenty samples (41%), of which 10 from production site and 10 from market samples, were randomly selected to perform the RFLP analysis to be compared to the sequencing results. Ten out of the 12 species (83.3%) identified by PCR-RFLP in the previous study were confirmed by sequencing. The remaining 2 samples, identified as *M. chilensis* x *M. edulis* by PCR-RFLP were instead identified as *M. chilensis* x *M. trossulus* by capillary electrophoresis and confirmed by sequencing. For the remaining 38 samples, sequencing identified 20 tissue samples (52.6%) as *M. galloprovincialis,* 7 as *M. chilensis* (18.4%), 6 as hybrids *M. chilensis* x *M. galloprovincialis* (15.8%), 2 as *M. edulis* (5.3%), 2 as hybrids *M. chilensis* x *M. trossulus* (5.3%) and 1 as hybrid *M. galloprovincialis* x *M. edulis* (2.6%). The PCR-RFLP confirmed the sequencing results for 19 of the tested samples and failed the unambiguous identification of one sample which was identified as hybrid by sequencing. Overall, the species identification by PCR-RFLP failed in 3 out 32 (9.4%) tested samples (12 DNA and 20 tissue). These findings suggest that the optimized protocol relying on Sanger sequencing has some practical advantages. First, the use of capillary electrophoresis to visualize the PCR products allowed the clear distinction of *M. edulis* and *M. trossulus* which, having a difference in amplicon length of only 12 bp (177 bp *vs* 165 bp), are very hard to discriminate with a standard agarose gel electrophoresis. Moreover, the use of sequencing allowed the unambiguous identification of pure species and hybrids, especially in the case of *M. galloprovincialis* x *M. edulis,* in which a double peak at the SNP site is clearly visible. Considering the ever-dropping costs linked to sequence-based technology, we propose this sequencing protocol as a valid, consistent, and reliable alternative to the currently used methods.

[1] Gaitàn-Espitia *et al*., *Scientific Reports*, 6, 26853, 2016.
[2] Wonham, *Journal of Shellfish Research*, 23/2:535–543, 2004.
[3] Luque e Donlan, *Biological Conservation*, 236:556–570, 2019.
[4] Fernández-Tajes *et al.*, *European Food Research and Technology*, 233/5:791–796, 2011.
[5] Satto et al., Analisi biomolecolare per l'identificazione di *Mytilus galloprovincialis*, *Mytilus edulis* e *Mytilus chilensis*. VI *SIRAM*, 51–52, 2017.
[6] Giusti *et al.*, *Food Control*, vol. 134, pag. 108692, 2022.