Bae did an excellent work between manuscript versions. The current version of the manuscript is written in a much clearer way, provides a more meaningful background, and carries out additional analyses to add statistical support to their findings. They have carefully addressed all my comments, and provided a rational argument when rejecting any of them, on which I agree.

Please, find below a few minor comments. As in the previous round of revisions, I recommend some (OPTIONAL) analyses to add statistical support, since your results are already quite solid. Moreover, I suggest some grammatical changes which I consider would help to improve the readability of the manuscript.

I consider that the present paper is close to have a perfect shape for its publication, and I really look forward to see it available to the world.

Cheers and thank you

INTRODUCTION

Line 19: A little bit redundant. Change to: improved efficiency of the redesigned AscCOI2 pair, with ascidians….

Line 41: The sentence looks weird. I think there is a comma that should be placed in another place. Can you have a look at it, please?

Line 45: There is a main topic change. I would recommend to create a new paragraph devoted to metabarcoding. Remove “Hence”.

Line 49: Many of which

Line 52. Or particularly difficult or very difficult. I would keep particularly.

Line 53: change “quickly” to “fast” to avoid three adverbs finished with “ly” in the same sentence.

Lines 50-53: Reorganize and modify as “Additionally, biodiversity assessment is particularly difficult in marine ecosystems, in which DNA degrades relatively fast (Collins et al., 2018; Wood et al., 2020). For this reason, the effectiveness of the DNA metabarcoding tools cannot be successfully used unless they are designed and selected appropriately.”

Line 67: Capital letter in “However”

MATERIAL AND METHODS

Line 115: Careful with the citation name. Possibly a manager mistake. (Pagès et al., 2024)

RESULTS

Line 177: by sequence length you mean primer length??? If so, make it clear by splitting the sentence in two, as the first half of the sentence looks like it refers to the primers and the second to the amplicons. Something like: “The primers AscCOI2 had the same length as AscCOI. Nevertheless, the target region, Tm (ºC), and resulting amplicon size differed between primer sets. “

Line 184: <0.001. Remove the comma after AscCOI

Lines 203-209: Remove the word significantly as there is no statistical support, unless you run an (OPTIONAL) GLMM:

- Bonding ~ primer set F/R + (1|species)

(OPTIONAL) Lines 212-224. I think you can run a couple of GLMM here to statistically support your findings:

- Ascidians -> Penalty_score ~ primer pair + (1|species)
- Target vs non target -> Penalty_score ~ strand(F/R) + taxon + (1|species)

Line 225-227: This sentence sounds weird. Rephrase for clarity please. “For Ascidiacea, 3,934 (99.64%) sequences failed and 14 (0.35%) provided insufficient evaluation data when using the primer pair AscCOI.”

Line 231. Remove a space in “99. 78%”)

Line 235: “0%”

Lines 250-261 and throughout the manuscript: Remove decimal “0” in 1.0%, 60.0%, etc…

Line 256: Remove “corresponding to non-related taxa”. For this analysis you used only ascidian species, and although they might be distantly related, they are still related taxa.

DISCUSSION

(OPTIONAL) Maybe it is a matter of style and personal taste, but I personally do not like Figure and table citations on the discussion section. To me, these figures and tables have been already presented in the results section, and the discussion section should be written to argue about them. Feel free to keep the citations or

remove them as I think they will not make the difference, but is a common practice in most of papers :)

Line 290: throughout ascidians

Line 296-298: I do not understand what do you mean by relatively PCR conditions. Rephrase for clarity please

Line 300 and throughout the manuscript: use ascidians (in plural) or Ascidiacea (taxonomic) when applicable instead of the singular ascidian. Use the singular when followed by a species (e.g: ascidian species are interesting / ascidians are interesting)

Line 305: differentiating

Line 331: “This primer pair specificity instead” of “This primers pair specificity”

Line 343-345: (OPTIONAL) You can add a sentence justifying that the difference between your thresholds (0-6.5%) and the Hebert’s one (1-3%) might be because you use a mini-barcode instead of the whole Folmer primer pair sequence, and that it is important to acknowledge the new barcode gap instead of using standard barcode gaps even if working with the cox1 for proper evaluation.

Line 372: Add “%” after the value 47.99