

Peer-Review Record:

Disrupted tRNA Genes and tRNA Fragments: A Perspective on tRNA Gene Evolution

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Reviewer 1: Anonymous

Reviewer 2: Anonymous

Editor: Niles Lehman (Guest Editor of Special Issue “The Origins and Early Evolution of RNA”)

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First Round of Evaluation

Round 1: Reviewer 1 Report and Author Response

- (1) This review pushes the facts too hard trying to make a case about ancientness, without properly considering or weighing alternatives. Origin-of-life writing needs to be especially careful about rigor.

Response: Thank you for your critical comments. In this review article, I have described one possible perspective on tRNA evolution based on the literature and a summary of recent progress in the field. Therefore, the title of the review contains the word “perspective”. I have also mentioned that “Akio Kanai formulated the hypothesis presented” in the author contributions section (Page 9, Line 267). I agree with the reviewer that weighing the alternatives is an important part of a review. Therefore, I have added some of the reviewer’s suggestions to the revised manuscript. I have also deleted descriptions relating to tRFs that are too speculative (see Response to (2)).

- (2) An example is taking observations from modern biology to be vestiges of LUCA, that are better interpreted as relatively recent innovations. Thus the tRFs are seen in their best light in regulatory roles, which tend to be more recent adaptations than ancient relicts.

Response: I agree with this comment. I have clearly stated that the recently identified tRFs have their own specific functions (including in RNA silencing) other than as adapter molecules in translation (Page 7, Lines 186–187). I have also deleted one of the original conclusions, “(a) tRNA fragments may reflect the prototype tRNA molecule,” from the revised text (Page 9, Line 254).

- (3) The fact that many processing and intron location events have occurred at the anticodon loop is taken to have special evolutionary significance concerning the two halves. There may instead be convergence of multiple events to the same region for structural/mechanistic reasons; the anticodon loop is the most exposed part of the tRNA, finely structured so as to facilitate its main translation function (viz kissing complexes). The post-anticodon cleavage site found frequently for tRFs may simply be a phosphodiester bond in tRNA that is well-configured for hydrolysis. (see Fouace 2004 Polyamine derivatives as selective RNaseA mimics.)

Response: Yes, the structural/mechanistic reason is an alternative explanation of the introns found in the anticodon loops of tRNAs. I have added this explanation to the revised manuscript (Page 4, Lines 104–106). However, in the case of Fouace (2004), many RNA cleavage sites are mapped to sites other than the anticodon loop of the tRNA. In our analysis, we have shown that tRNA introns are located in almost all positions in certain archaeal species (Sugahara et al. 2008 Mol. Biol. Evol. 25(12): 2709–2716).

- (4) The strongest objection to the proposed ancient anticodon-split half-tRNAs is that none of these mainly single-stranded half-RNAs can have any clear function in isolation. In contrast the acceptor stem half-tRNAs of Di Giulio which would alone be chargeable and able to deliver amino acids, that is they allow a stepwise enhancement of function. The evolution of a large system of many non-functional tRNA fragments that must find their proper base-pairing partners seems quite implausible to me. Anyway the preponderance of evidence clearly says that LUCA tRNAs were unitary.

Response: Thank you for these comments.

- *First, it has been reported that the complete cloverleaf structure of tRNA is not necessary for tRNA function. For example, (i) a mini-helix RNA was efficiently aminoacylated by Escherichia coli leucyl-tRNA synthetase (Larkin et al. 2002 NAR 30: 2103–2113); (ii) the D-arm mini-helix of tRNA^{Tyr} is sufficient for its importation into the mitochondria of the kinetoplastid protozoan, Leishmania tropica (Mahapatra et al. 1998 NAR 26: 2037–2041); (iii) the top-half tRNA mini-helix is a good substrate for the eubacterial CCA-adding enzyme (Shi et al. 1998 RNA 4: 276–284); and (iv) a ribozyme of only five nucleotides effectively generated an aminoacyl-RNA (Turk et al. 2010 PNAS 107: 4585–4589). However, I agree that there are no such short functional tRNAs in the current species. I have mentioned this in the revised manuscript (Page 7, Lines 196–197).*
- *Second, it has been reported that most tRNA sequences have vestiges of double hairpin folding (Tanaka & Kikuchi 2001 Viva Origino 29: 134–142). In that report, each hairpin corresponded to either the 5' or 3' tRNA half, and Di Giulio's model is supported by this finding. I have mentioned this in the revised manuscript (Page 8, Lines 214–217) and have added this citation to the revised manuscript (Page 11, Lines 375–376). A similar review article entitled "Transfer RNA: From Minihelix to Genetic Code" has also been published (Schimmel & Ribas de Pouplana 1995 Cell 81: 983–986).*
- *Third, in previous research, we mimicked a split tRNA by artificially separating the tRNA sequences of seven primitive archaeal species at the anticodon loop, and analyzed the sequence similarity and diversity of the 5' and 3' tRNA halves. A network analysis revealed topological*

differences between the tRNA halves, suggesting different evolutionary backgrounds for the 5' and 3' tRNA halves (Fujishima et al. 2008 PLoS One 3: e1622).

Based on these considerations, I propose a modified model for tRNA evolution. Here, I also propose that the LUCA may have had cloverleaf tRNAs, like the current tRNAs. However, even so, it is possible that combinations with different 5' and 3' tRNA halves generated a variety of tRNA species a little later in evolution. I speculate that the 3' tRNA halves appeared first in the early evolution of the tRNA molecule (perhaps during chemical evolution). In the revised manuscript, I have clearly discussed these matters (Page 8, Lines 230–234).

- (5) While ancient relationships between archaeal and eukaryotic systems of tRNA introns and their splicing endonucleases are well-supported, it is sloppy to place the bacterial group I introns in this same context. Instead the available evidence suggests that the various bacterial tRNA group I introns are not pre-LUCA ancient. Each uses a different subsite within the anticodon loop. No evidence is presented for sequence/mechanistic relationships between group I introns and the arch/euk systems. Quite the opposite of evidence for ancientness, it seems more likely that, as group I introns generate new tRNA site specificities, they favor the anticodon loop for mechanistic reasons. For group I introns it may be harder to set up splice site base-pairings at other tRNA sites, and again, the anticodon loop is the most exposed part of the tRNA. An interesting counterpoint is that in tmRNA (who has no anticodon loop), a group I intron is found in the T-loop (the second best region for group I intron function?).

Response: In this section, I have not insisted that sequence/mechanistic relationships existed between the group I introns and the archaeal/eukaryotic systems. My point is that introns in the anticodon loop region are conserved in the three domains of life: Bacteria, Archaea, and Eukaryota. In previous research, we have also shown that archaeal intron-containing tRNAs contain a wide diversity of small tRNA introns that differ in their numbers and locations. The comparative genomic data suggest that many tRNA introns located at noncanonical positions are ‘translocatable introns’ and may have appeared at a later stage of tRNA evolution (Fujishima et al. 2010 Mol. Biol. Evol. 27: 2233–2243). Therefore, the introns located in the anticodon loop region are relatively ancient in an evolutionary sense. However, I respect the alternative explanation that many processing events and intron insertion events have occurred in the anticodon loop for structural/mechanistic reasons. I have added these explanations to the revised manuscript (Page 3, Lines 96–100; Page 4, Lines 104–106).

- (6) The split/permuted tmRNA gene would make a good counterpoint to the split/permuted tRNA genes, as a point of comparison.

Response: Thank you for this suggestion. I think it would be confusing to readers if I mentioned the evolution of tmRNAs in this short review. Therefore, I have omitted it from the discussion.

- (7) Line 122. Weak point. This doesn’t show evolutionary relationship between the genes. The alternative hypothesis is that a few archaeal tRNA genes split after the establishment of Archaea, adapting to the existing tRNA-processing mechanism, of which there is only one known (BHB-based). Which is more likely, that a novel split gene adapts to the existing mechanism, or “invents” a new machinery?

Response: We have also demonstrated that the intervening nucleotide sequences of split tRNAs show high identity to tRNA intron sequences located at the same positions in intron-containing tRNAs in related archaeal species (Fujishima et al. 2009 PNAS 106: 2683–2687). I have added this discussion to the revised manuscript (Page 4, Lines 128–131).

Round 1: Reviewer 2 Report and Author Response

- (1) This is an excellent review on a topic very important to understand the origin of the tRNA molecule. I think that the manuscript might be published in the presentsent form. Therefore, I suggest to accept the manuscript in the present form.

Response: I greatly appreciate the reviewer's positive and encouraging comments. Thank you very much.

Second Round of Evaluation

Round 2: Reviewer 1 Report and Author Response

- (1) Some improvements have been made to the text. However some of the author's responses seem not to support the author's proposal.

Response to (3) in Round 1: The non-anticodon loop archaeal introns are considered by the author recently evolved, and so do not pertain to the ancient use of the anticodon loop site.

Response: Thank you again for your comments.

In previous research, we have shown that archaeal intron-containing tRNAs contain a wide diversity of small tRNA introns that differ in their numbers and locations. The comparative genomic data suggest that many tRNA introns located at noncanonical positions are "translocatable introns" and may have appeared at a later stage of tRNA evolution (Fujishima et al. 2010 Mol. Biol. Evol. 27: 2233–2243). Therefore, we suggested that the introns located in the anticodon loop region are 'relatively ancient' in an evolutionary sense.

- (2) Response to (4) in Round 1: Example (ii) is irrelevant, a modern transport function (evolved after capture of mitochondria), not a charging/translation function. Examples i and iii make the point that the Di Giulio (and Maizels/Weiner and Schimmel/Ribas de Pouplana) top-half tRNA already is a mini acceptor helix that is plausibly aminoacylable, while in contrast the 3' tRNA fragment proposed here has no acceptor helix. Example iv is a provocative result but not resembling a tRNA acceptor end free CCA tail.

Response: The sentence relates to example (ii), and its reference in the original manuscript is deleted in the newly revised manuscript (Page 7 Line 194; Page 11). Other examples have shown that the complete clover-leaf structure of tRNA is not necessary for some tRNA functions. These examples show that a mini-helix RNA played important roles in these functions. It has also been reported that most tRNA sequences have vestiges of double hairpin folding (Tanaka & Kikuchi 2001 Viva Origino 29: 134–142). Tanaka & Kikuchi reported that each hairpin corresponded to either the 5' or 3' tRNA half. In other words, either the 5' or 3' half of the tRNA molecule forms an alternative secondary structure with a mini-helix

RNA. However, it is also true that there are no such short functional tRNAs in the current species. I have added this information to the revised manuscript (page 8, lines 216–218; page 7, line 197).

- (3) The Schimmel/Ribas de Pouplana model is different from the current proposal, containing an acceptor helix.

Response: The Schimmel/Ribas de Pouplana model is not mentioned in the revised manuscript.

- (4) Complex half-tRNA systems are proposed to operate both early and again late in evolution (pre- and post-LUCA)?

Response: I cannot postulate the exact time point at which the tRNA halves combined (Page 8, Lines 236–237), but I speculate that the system generated certain kinds of diversity in the tRNA molecules both early and late during evolution.

- (5) Response to (5) in Round 1: The use of the same region (the anticodon loop) by two unrelated systems (group I introns and archaeal/eukaryotic introns), and in multiple independent instances by group I introns, does not argue that there is a single site used only for historical reasons, but favors the idea that there are structure-based preferences for continued re-utilization of this target region.

Response: In Section 2, I summarize the reports relating to tRNA introns in the three domains of life. The fact that group I introns in Bacteria are also located in the anticodon loop region is interesting and informative for readers. Again, I respect the alternative explanation that many processing events and intron insertion events have occurred in the anticodon loop for structural/mechanistic reasons. I have added these explanations to the revised manuscript (Page 4, Lines 105–108).

- (6) Two major problems with the main proposal: (I) the proposed half-tRNAs in isolation should be non-functional, lacking an acceptor stem, and could function only in a complex world that supplies partner tRNA halves that themselves suffer the same problem. Evolutionary pathways with stepwise function are more convincing than scenarios requiring high complexity dependencies before any function is possible. (II) Allowing multiple available partners would make it difficult to control the linkage between amino acid identity and the anticodon sequence, as is necessary for reliable translation. Earlier proposals by Di Giulio and others, with functional half-tRNAs and unitary tRNAs that link charging to a single anticodon, are far more plausible than the current one.

Response:

- *For comment (I), please see my response to (2).*
- *For comment (II), I understand the reviewers concern. Actually, so far, all alternative split tRNA genes encode tRNAs with certain synonymous anticodons. However, in previous research we mimicked a split tRNA by artificially separating the tRNA sequences of seven primitive archaeal species at the anticodon loop, and analyzed the sequence similarity and diversity in the 5' and 3' tRNA halves. A network analysis revealed topological differences between the 5' and 3' tRNA halves, suggesting their different evolutionary backgrounds. Furthermore, the combinations of “specific” 5' and 3' halves corresponded to the variation in amino acids in the codon table. We not only found universally conserved combinations of 5' and 3' tRNA halves in tRNA^{iMet}, tRNA^{Thr}, tRNA^{Ile}, tRNA^{Gly}, tRNA^{Gln}, tRNA^{Glu}, tRNA^{Asp}, tRNA^{Lys}, tRNA^{Arg}, and tRNA^{Leu}, but also*

phylum-specific combinations in tRNA^{Pro}, tRNA^{Ala}, and tRNA^{Trp}. Our results support the idea that tRNA emerged through the combination of separate genes, and this explains the sequence diversity that arose during archaeal tRNA evolution (Fujishima et al. 2008 PLoS One 3: e1622). Therefore, I suggest that the combination of 5' and 3' tRNA halves was not random during evolution. I have added a brief discussion of this to the newly revised manuscript (Page 8, Lines 227–229).

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