It's a small world—microRNA cuts evolution down to size

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MicroRNAs (miRNAs) are very interesting genetic elements, and are pertinent to the creation/evolution debate. These small non-coding regulatory RNA post-transcriptionally regulate genes by reducing their mRNA levels via base pair complementation, rendering them inactive (gene silencing). They are highly conserved within a large number of taxonomic groups, meaning that their non-mutative characteristics contradict molecular evolution, which depends upon a flux of continuous mutations bringing forth a plethora of new genetic elements. In general, miRNA mutations are harmful, and do not build up new and useful genetic information. Differing miRNA regulatory systems exist in animals, plants, and unicellular organisms, which underscores how fundamentally different these apobaramins (broad taxonomic groups) are from each other. Evolutionary explanations as to how miRNA elements form through harmful inverted duplications are contradictory, and the calculated evolutionary time needed for these elements to form also greatly exceeds the supposed timespan that life supposedly evolved in. MiRNAs are also key parts of irreducibly complex genetic networks wherein both the miRNA element and its target mRNA need to be in place at the same time for the whole system to work.

MiRNAs are about 22 nucleotides (nt) long and represent a growing class of genetic elements that take part in the regulation of many physiological processes and development. They have been recently discovered in unicellular organisms, such as the green alga *Chlamydomonas reinhardtii*,[1,2] and were first discovered in the worm *Caenorhabditis elegans*.[3] They come complete with their own promoter and enhancer elements and their sequences bind via complementary base-pairing to existing genes, typically in the 3-prime untranslated regions.[4] In contrast with siRNAs (small interfering RNAs), miRNAs typically originate from a different gene than the one that they regulate.[5] When they form complementary structures with target mRNA molecules, they ultimately block proteins from being synthesized as a form of post-transcriptional regulation. Largely through the integration of gene expression data, in a form of 'guilt by association' research, miRNAs are implicated in the fine-tuning and regulation of cell trafficking, sensory-signaling, motility, metabolism, gene expression, cell cycle control, circadium rhythm, and virtually every other cell process studied to date.[4,6] However, discovering the many mRNA targets of miRNAs and their exact roles in the cell network has posed great challenges both bioinformatically and experimentally, recently reviewed by Liu *et al.*[7] and Pasquinelli [8]. A sample list of miRNAs taken from the MicroRNA Target Prediction and Functional Study (miRDB) in different species can be seen in table 1.[9]

The reason miRNAs are so interesting to the creation/evolution debate is that they are highly conserved in a large number of organisms and have become targets of study for molecular phylogenies in many taxa.

MicroRNAs are formed out of back-folded hairpin loops after being transcribed from DNA by the enzyme RNA polymerase II (Pol II) to form pri-miRNA. Then an enzyme DCL1 in plants and Drosha in animals processes the pri-miRNA to form pre-miRNA, which are about 70 nt long where it is then exported out of the nucleus by the protein Exportin-5.[4] The pre-miRNA is further processed by a helicase enzyme, thereby losing its loop structure to create the mature form of the miRNA, which is a pseudo double-stranded form of RNA. One strand is the miRNA strand, while the other strand is the miRNA* strand, or 'star' strand. One of the strands then fits into the RISC protein complex (in animals), which then complements imperfectly or perfectly, depending on the target site in the mRNA molecule, degrading it by cleavage. An overview of this process can be seen in figure 1. For a detailed review of this whole model, see Carroll *et al.*[4]

MicroRNA differences in plants, animals, and unicellular organisms

MicroRNAs are different in plants and animals, so much so that miRNA discovery algorithms have had to be modified so as to be able to detect plant miRNAs as compared to those in animals, such as in the algorithm miRDeep-P.[11] MicroRNAs are thought to be so conserved that evolutionists think miRNAs coincided or brought about the evolution of multi-cellular body plans.[12,13]

There are no known experimentally verified orthologous miRNAs between plants and animals.[14] Plant miRNAs mainly target mRNAs of transcription factors. Animal

Table 1. Number of miRNA elements reported in miRDB.

Species	Number of hairpins
Animals	
Homo sapiens	1,600
Pan troglodytes	655
Mus musculus	855
Monodelphis domestica	156
Gallus gallus	684
Xenopus laevis	22
Danio rerio	344
Caenorhabditis elegans	223
Drosophila melanogaster	238
Plants	
» Dicots	
Arabidopsis thaliana	299
Glycine max	506
Brassica oleracea	6
Populus trichocarpa	13
Medicago truncatula	675
» Monocots	
Triticum aestivum	42
Oryza sativa	591
Hordeum vulgare	67
Brachypodium distachyon	135
Zea mays	172
» Conifers	
Picea abies	40
» Mosses	
Physcomitrella patens	88
» Chlorophytes	
Chlamydomonas reinhardtii	50
Mycetozoa	
Dictyostelium discoideum	2

miRNAs target their corresponding mRNAs at multiple sites due to slight wobbling, while plant miRNAs match almost perfectly at one site, after which they both cleave the mRNA. From this it follows that the minimum free energy (MFE) distribution of binding in plants is broader on average with a lower mean binding free energy than in animals, binding much tighter with its target, due to more hydrogen bonds being involved. The extent of the base pairing between the mature and star strands of the duplex miRNA is also more variable in plants than in animals.[15] This makes it easier, for example, for bioinformatics programs to discover plant miRNAs than those of animals. Plant miRNAs are also longer on average, and their conserved core sequence is also different.[16,17] Plant miRNAs contain introns and are more heterogenous in their polyadenylation sites.[1] See table 2 for a list of differing characteristics between miRNA of animals and plants.

MiRNAs are different not only between animals and plants, but also between plants and unicellular green algae, such as *Chlamydomonas reinhardtii*,[1] from which they supposedly evolved. There are several characteristics which make algal miRNAs different from those in plants. A large fraction of *Chlamydomonas* miRNAs are intronic in origin, while in the DNA of plant miRNAs, they reside in intergenic regions. Algal miRNAs also take part in metabolic and physiological processes compared to those in plants which mainly complement with transcription factors. No miRNA homologs have been found between *Chlamydomonas* and land plants. Furthermore, there are also no detectable homologs between *Chlamydomonas* and 3 other alga species (*Volvox carteri*, *Ostreococcus tauri*, and *Ostreococcus lucimarinus*). Tarver *et al.*[14] also reports a small number of experimentally verified miRNAs from 6 other protist species.

Interestingly enough, structures in bacteria called non-coding RNAs or ncRNAs have also been discovered which somewhat resemble microRNA structures in higher organisms. Different kinds are 50–500 bp long, and are present in 200–300 copies per bacterial genome, and regulate many bacterial genes. They are also capable of inhibiting mRNAs by degrading them similar to plants and animals, or by masking ribosomal binding sites.[18] They also form hairpin and loop structures and regulate metabolism in a number of ways. The Hfq enzyme in *E. coli* is responsible for the formation of duplexes between ncRNAs and the mRNAs of protein-coding genes, which thereby destabilize the mRNA or modify translational efficiency.[19] This is evidence of a highly complex regulatory system already present in bacteria, at the root of the supposed phylogenetic tree.

Due to this, evolutionists lightly assume that miRNAs must have evolved separately three different times in animals, plants, and unicellular organisms. According to evolutionary theory, it is highly unlikely that an evolutionary trajectory would repeat itself, let alone at least three separate times, furthermore one right after the other. The question begs itself: if miRNAs had already evolved in green algae, then why did such a basic and fundamental system of gene regulation evolve a second time, unnecessarily? If plants evolved from green algae-like organisms, then why are their genomic distributions of miRNA so different? It would only

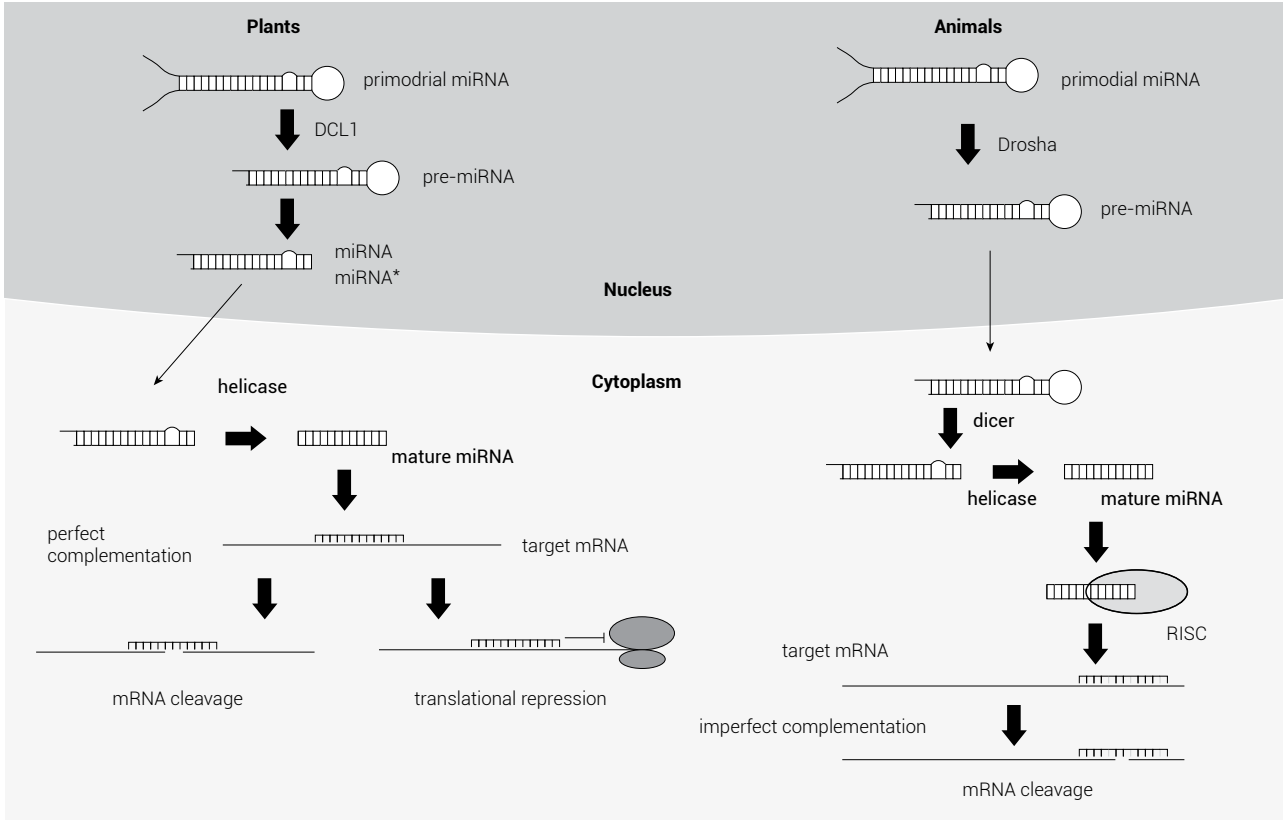

Figure 1. Process of formation of miRNA elements and their mode of regulation in animals and plants (adapted from Mallory[10] and Chen[22]).

be logical that we would find the footprints of algal miRNAs in much the same place in plant genomes.

The fact that there are such great differences between the miRNAs of plants, animals, and unicellular organisms show that these organisms constitute separate, disjunctive domains of life. It would take such a great number of fundamental mutations to transform one system of miRNA into another as to make it impossible. In other words, miRNAs show that animals, plants, and green algae are separate apobaramins.

Catch-22—miRNAs regulate the proteins for their biogenesis

It is interesting to note that the DCL1 and AGO1 mRNAs which take part in miRNA biogenesis are themselves regulated by miRNAs. The DCL1 mRNA is regulated by miR162, while AGO1 is regulated by miR168. In *dcl-7* mutants DCL1 mRNA accumulates to high levels, suggesting that these molecules act in a negative feedback loop.[21] This then provokes the following question: which evolved first, the DCL1/AGO1 proteins or their respective miRNAs? If the DCL1/AGO1 proteins did, then this means that they would increase miRNA levels to such a degree so as to suppress all mRNA targets almost completely. However, if the miR162/miR168 evolved first, then their existence would be useless, and they would serve no function. This goes to show that the auto-regulation of miRNA proteins is an irreducibly complex system, needing both components of the system to be present in the beginning to be functional.

Mutations in microRNAs are destructive

In general, mutations in the miRNA biogenesis and regulation machinery tend to be harmful in nature, which is not surprising since miRNAs regulate such fundamental processes as development of the organism. In plants, such mutations include death in early embryogenesis,[22] changes in leaf and flower morphology,[10,20] and infertility.[23] This is proof that random mutations do not build up genetic information, but tend to erode already existing genetic information.

Conservation of microRNAs

MiRNAs are highly conserved, more so in plants than in animals. It is quite remarkable that such short genetic elements show such a high level of conservation all throughout the plant and animal kingdoms. The question begs itself: if such short genetic elements are so impervious

Table 2. Differences in miRNA sequences between animals and plants.

	Animals	**Plants**
Length of precursors	45–215 nt	55–930 nt
MFE distribution	Narrow, higher mean value	Broader, lower mean value
Base positions in nucleus of miRNA	Positions 2–8	Positions 2–13, 16–19
Nuclear localization signal	Missing	Present in dicer
Enzyme localization	Drosha, cytoplasmic	Dicer, nuclear
Mode of regulation of mRNA	Multiple sites	One specific site, cleavage
Introns	Missing	Present

to change, then what about longer elements? If virtually the same genetic element is conserved throughout the course of evolution in different taxonomic groups, retaining its function, then this means that evolution, in effect, did not occur. As noted in the creationist literature, genetic conservation flies squarely in the face of evolution, which demands continuous genetic change through mutations.[24]

For example, Bonnet *et al.*[25] found a set of 91 miRNA elements supposedly conserved between *Arabidopsis,* a dicot, and rice, a monocot species. Dicots and monocots supposedly diverged from each other some 140–150 million years (Ma) ago. This was based on the study of a set of 61 chloroplast genes.[26] The miR165/miR166 miRNAs are complementary to the mRNA of class-III HD-ZIP transcription factors, which are found in flowering plants, gymnosperms, ferns, lycopods, and bryophytes. These miRNAs are supposed to have been in existence for 400 Ma.[27] In figure 2 we can see a multiple alignment of the binding site in the HD-ZIP transcription factors showing almost perfect conservation. Arif *et al.*[28] report 13 miRNAs conserved between the moss *Physcomitrella patens* (which is supposed to have arisen 450 Ma ago) and *Arabidopsis* and rice.

Durrett *et al.*[29] calculated that given neutral point substitutions, it would take an miRNA 375,000 years to form if there was already a core pre-miRNA sequence present to evolve from, but a striking 650 Ma without it. For comparison, Berezikov *et al.*[30] supposes that 1 miRNA arises per million years during drosophilid evolution. According to Meunier *et al.*,[31] the number of new miRNA families in therians, monotremes, and birds is 0.83, 0.23, and 0.3 new families (respectively) per million years. It has been estimated that 1,336 miRNAs may be human/primate-specific.[32]

Through simple multiplication, we can calculate the time needed for the evolution of these 1,336 human miRNAs. If all of these elements evolved from a core miRNA-like element, they would need at least 1,336 x 0.375 Ma = 501 Ma to evolve. Otherwise, if they all evolved from scratch (without a core miRNA-like element to start out from), it would take them 1,336 x 650 Ma = 868.4 Ga, which is about 58 times the supposed age of the universe (15 Ga)! Even if only 1% of these miRNAs evolved from no previous core miRNA-like elements (1% of 1,336 ≈ 13, with 1,336 – 13 = 1,323 left), it would still take (13 x 650 Ma) + (1,323 x 0.375 Ma) = 8.9 Ga for all of them to form, which is supposedly older than earth itself!

Duplication and divergence

Several evolutionary theories purport that miRNAs evolved by a process of inverted duplication and divergence, whereby the miRNA target sequences would tend to form clusters.[16] According to the duplication and divergence model of evolution, miRNAs would originate from a duplication event of a protein coding gene (its target gene), and would lay close by. Since they are complementary to their target gene, they would then be detectable.

However, there are a number of problems with the duplication and divergence model of miRNA evolution. According to data from Chen and Rajewsky,[22] the distribution of these sequences is close to random. Furthermore, very many times, miRNAs are isolated, and derive from independent transcription units. This proves that miRNA sequences are not the result of a duplication event, but came into existence separately from their target genes.

Secondly, inverted duplication events are generally harmful to genes; therefore they result in the silencing of the gene. This is a paradox, since evolutionists suppose this is how miRNA sequences arose. There are no convincing examples of miRNA arising by inverted duplication in animals.[22] Therefore evolutionists suppose that the RNA-mediated silencing evolved before gene duplication events happened in plants.[32] This again contradicts how evolutionists believe that new miRNA elements have formed quite recently in the past. For example, Fahlgren *at al.*[34] argued that in the genomes of *Arabidopsis thaliana* and

Figure 2. Multiple alignment of the miR165 element which targets the class-III HD-Zip transcription factor mRNA. Sequences taken from supplemental data in Floyd[27]. Multiple alignment made by the T-COFFEE software (Taly[36]).

lyrata there recently (~10 Ma in evolutionary time) arose 32 families of miRNA elements.

Another evolutionary idea is that miRNAs arose randomly from already existing hairpins through intermediary forms, which are described vaguely and have been assigned no specific function. If we take a mature miRNA to be 21 nt (its duplex form being 42 nt since in its primordial form it exists as a hairpin structure), this means that at random we would expect to find 1 mature miRNA every $2^{42} \sim 10^{12}$ nucleotides. This is much, much larger than the vast majority of all genomes. The problem with miRNAs forming from random hairpin structures is that if a mutation (e.g. an nt substitution) happens within one strand of the hairpin, a complementary mutation of the corresponding type would also have to happen on the other strand of the miRNA in order to keep the complementarity of the miRNA duplex (A:T, C:G). Furthermore, if a substitution occurs within either the miRNA or its target genes, this would weaken the complementarity between the miRNA and miRNA*, or the miRNA/miRNA* and its target. It is obvious from this that the miRNAs can only undergo devolution, and not evolution.

Conclusion

MicroRNAs play an interesting role in the regulation of genes. Since they regulate such fundamental processes as development, the cell cycle, and tissue differentiation, their mutations can be very detrimental. Their sequence and thus their function are highly conserved, as can be seen in the case of miR165. Their high degree of conservation and therefore presence in a number of different taxonomical units speaks against evolution, which demands a constant flux of change through mutations in the genome. Their sequences, genomic distribution, and physio-chemical characteristics between animals, plants, and unicellular organisms are so divergent that they can be used to differentiate between these three apobaramins. Therefore miRNA regulatory systems are mosaic in this sense, which speaks against these systems having three separate evolutionary trajectories leading up to their independent evolutionary formation. Indeed, Peterson *et al.*[13] postulate that miRNAs form part of the ancestral regulatory apparatus in eukaryotes. Therefore, distinct miRNA elements in plant and animal species would be due to loss of genetic elements, which heavily bespeaks of devolution and not upwards evolution. Furthermore, according to Peterson *et al.*,[13] miRNAs also increase genetic robustness and decrease variation, which is fundamental to evolutionary development.

Evolutionary explanations as to how miRNAs formed through random mutations are contradictory or highly improbable and very tentative at best. Ultimately miRNAs do not explain how genes evolved, since these genetic elements only regulate the expression of these genes. Furthermore, since miRNA sequences are very highly similar to their target mRNA sequences, they essentially add no new information to the genome. According to Peterson *et al.*,[13] the role of miRNAs is to fine-tune the expression of protein-coding genes already in place in the genomes of organisms. This canalization process poses an obvious problem to evolutionary theory: since it must eventually end, this means that evolution itself must also end. The calculated time needed for miRNAs to form is excruciatingly much too long for evolution to have occurred on this planet.

On the other hand, the miRNA regulatory system can be viewed as an irreducibly complex system. Both the miRNA and its target mRNA sequences are needed to be in place

at the same time for them to work properly. Indeed, many miRNAs (such as miR-15 and miR-16 in human) serve as oncogenes and tumor suppressors, as they are downstream elements in pathways that regulate development, apoptosis, and differentiation.[35] The deletion of these elements leads in many cases to cancer. Without the miRNA apparatus in place, target mRNA levels would be miss-regulated, resulting in the loss of fine tuning and dynamic response capabilities in the cell's regulatory network.

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