Conserved miRNA detection in the ESTs of *Ganoderma lucidum*

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Abstract
Ganoderma lucidum is a potentially deadly fungal pathogen belonging to the family Ganodermataceae that affects palms all over the world. Spread through spores, its mode of infection generally starts from the roots and is characterized by the presence of fungal basiocarps on the infected tree. Due to the huge financial losses inflicted upon the farming community, there is a need to find innovative measures to inhibit the spread of this destructive biotroph. miRNA induced silencing is a relatively new technology that is gaining traction in the field of plant pathogen inhibition.

In this study, we aim to identify conserved miRNAs through bioinformatics tools present in the ESTs of G. lucidum. A total of 17 pre-miRNA hairpin loop structures were identified in the genome formed by a total of 9 unique miRNAs. Target annotation revealed that almost all of these miRNAs could have a part to play in propagating the spread of this pathogen. Thus, these miRNAs could be potentially upregulated or downregulated in a pathogen to restrict its parasitic functions.

Keywords: *Ganoderma spp*., miRNA, Detection, ESTs

Introduction

Coconut (*Cocos nucifera L.*) and Arecanut (*Areca catechu L.*) are perennial crops that sustain the livelihood of many small scale farmers in South-East Asia. Thus, it is of paramount importance to formulate and promulgate methodologies aimed towards the protection of these crops from phytopathogens[1]. Among the various diseases affecting these crops, *Ganoderma* is a genus of polyphore mushrooms that causes wood rot [2]. The genus, *Ganoderma lucidum (Polyporus lucidus)* was first described by Peter Adolf Karsten[3], who was a Finnish mycologist. More than 300 species of *Ganoderma* have been identified throughout the world. Earlier, depending on the type of cap that the mushroom produces, two sub-genus for *Ganoderma* were specified (*Ganoderma* and *Elfvingia*) [4]. Recently, the genus has been split into six monophyletic groups based on phylogenetic analysis of mitochondrial small-subunit ribosomal DNA sequences *viz. G. applanatum*, Asian *G. lucidum*, *G. meredithiae*, *G. resinaceum*, *G. tsugae* and *G. colossu* [5].

The Indian subcontinent has high biodiversity in part due to the highly varied climate over different parts of the country. Since first being described in India by Sir Edwin John Butler[6] in 1906, a total of 13 *Ganoderma* taxa and 144 hosts[7] have been identified in India with *G. applanatum* and *G. lucidum* found to affect the most number of hosts. *Ganoderma* spp. have currently been identified as affecting a number of species from cash crops like *Areca catechu* (arecanut), *Camellia sinensis* (tea) etc. to plantation trees like *Albizia lebbeck* (lebbeck tree) and trees in the wild like *Mesua ferrea* (celyon ironwood) [7].

Some methods to prevent and hinder the spread of infection suggested in oil palms include adopting field sanitation along with proper cultivation leaving proper spacing between seedlings, preventing infection in young plants through regular checks, application of fungicides like triadimenol[8] as well as growing seedlings in polybags with uninfected soil[8,9]. With lignin (a complex, phenolic biopolymer) being targeted by the fungi, altering the lignin content in wood has been proposed as a viable option to impede the proliferation of the disease[10].

Biocontrol methods of *G. lucidum* include the treatment of infected coconut palms with *Trichoderma harzianum* strains applied with green leaves, neem cake or farmyard manure with Bordeaux mixture found effective over time. Intercropping with disease resistant plant species like banana in between areca palms on plantations, help break the continuity and prevent the spread of the fungus in between trees[2]. Studies have been done to single out progenies of palms that are naturally resistant to infection by *Ganoderma*. Inherent differences in genetic makeup of oil palms from different regions have helped in the development of more resistant oil palms[11,12]; however, no such genetic resistance has been reported in coconut palms to date.

miRNA identification is a relatively new field of research with the first miRNA being discovered in the early 1990s when the small RNA, lin-14 was found to be present in *Caenorhabditis elegans*[13]. The first discovery of miRNAs in unicellular organisms was in the genome of *Chlamydomonas reinhardtii*[14]. miRNAs are small length non-coding RNAs of approximately 22 nt in length that can regulate the expression of genes by the translational repression or cleavage of mRNAs[15]. miRNAs have a key role to play in the development, growth and behaviour in animals while in plants they handle plant development, resistance and stress responses[16].

There is great interest in identifying miRNAs exclusive to phytopathogens so as to utilize RNA medicated interference for post translational silencing of genes that would preferably lead to control of the pathogen. The first example of repression (quelling) was noticed in the fungus *Neurospora crassa* by the negation of the expression of *al-1* and *al-3* genes after transforming it with homologous *al-1* and *al-3* sequences from the carotenoid biosynthetic pathway[17].

Two modes of inducing silencing in an organism are introducing a transgene into it which codes for the dsRNA (double stranded RNA) or formation of the dsRNA through convergent transcription on the transgene[18]. Another alternative to induce RNA interference in fungi is through the direct introduction of plasmid constructs with dsRNAs which was demonstrated in a study with the yeast *Schizosaccharomyces pombe*[19]. In this study, we aim to identify conserved miRNA sequences present in the EST sequences of *Ganoderma* spp. through computational methods so as to use this knowledge to potentially eradicate or minimize the damage caused to palms by this pathogen.

Material and Methods

miRBase [20-22] is a database of published miRNA sequences with the current release (Release 21) having a total entry of 28,645 miRNAs. EST sequences for *Ganoderma lucidum* were obtained from the Joint Genome Institute's (JGI MycoCosm) new fungal genomics resource[23]. A local BLASTN[24, 25] search was run using stringent parameters (e-value < 0.001, percent identity of 100 and a word match of 7) with the EST sequences set as the query and and a database formed from the mature miRNA sequences. The results were obtained in the form of an excel file utilizing the tabular format output available in BLAST.

The next step in positively identifying the presence of mature miRNAs is to identify its possible precursor and confirm if it forms a credible hairpin loop structure. A custom PERL program was developed to extract seventy nucleotides both upstream and downstream of the match area to analyze if there were any pre-miRNA like hairpins formed by the matched sequence to confirm the presence of conserved miRNAs. Previous studies have indicated that the length of precursor miRNAs (pre-miRNAs) varies a lot depending on the species e.g. animal miRNA precursors fall in a range of 45-215 nt with a mean of ~87 while the precursors in plants show a range in between 55-930 nt with a mean of ~146 nt[26]. Sequence and structural features of pre-miRNAs can be evaluated with the indicators of GC content, minimum free energy value (mfe), hairpin mfe and the ratio of core mfe to hairpin mfe (ch_ratio)[27].

Those sequences with a GC content between 30% - 60% , any duplicate sequences or those with a length < 45 nt were filtered out using the PRINSEQ tool (v0.20.4)[28] and only the remaining sequences were used for the rest of the analysis. The miPRED tool[29] was utilized to identify if the miRNAs formed real or pseudo hairpin loops and the RNAfold tool present in the Vienna RNA website (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi)[30] was used to attain the hairpin loop structure of the pre-miRNAs (Fig 1). The structures were checked for less than 4 mismatches in the base-pairing between the miRNA and the other arm of the hairpin (miRNA*).

The size and frequencies of asymmetric bulges were noted and the criteria followed was that these bulges were at a minimum[31]. Gene transcripts of *Ganoderma* spp. were downloaded from the JGI MycoCosm database (http://genome.jgi-psf.org/programs/fungi/index.jsf) and the targets of the miRNAs obtained were determined with the psRNA Target tool[32]. Protein sequences corresponding to the genes that were obtained as targets were also retrieved off the database. A BLASTP search was conducted with these sequences against the non-redundant protein database of NCBI to determine the homologs of the proteins and understand the possible function that the protein could play in the fungi.

Results and Discussion

The BLASTN analysis yielded a total of 1,710 matches of miRNA mature sequences using *Ganoderma* ESTs as input which included duplicate matches. Filtration of the matches obtained for GC content and removal of duplicates further reduced the number of viable matches to 511. miPred analysis of these sequences yielded a total of 17 probable miRNA precursors that formed true hairpin loops and a total set of 9 unique miRNAs. The prediction confidence values of all the non-pseudo miRNA hairpin loops fell between 50% to 55% except for two loops which had a value above 60% and one loop with a value above 70%. Mfold analysis for structure prediction was done on the 17 miRNAs obtained after MiPred using default values (Table 1). Among all the matches, miR-7058 gave three, miR-5336 gave two, miR-7084 gave four and miR-2673 gave three unique matches to different EST sequences respectively.

The sequences with lower minimum free energy values (MFE) were preferred when considering multiple precursor sequences for the same miRNA. miR-7058 and miR-7084 are listed on miRBase as miRNAs that have been identified in *Mus musculus* previously[33]. miR-5336 and miR-2673 have been previously detected in cattle tick (*Rhipicephalus microplus*)[34] and *Medicago truncatula*[35] respectively. miRNAs are involved in diverse functions across all species like signaling pathways, cell proliferation etc. Conservation of miRNAs among species has been identified from plants [a study found upto 682 miRNAs in 155 plant species[36]] to primates [a work detailed the presence of 122 miRNAs in 10 primate species[37]]. In some cases, as much as 133 human miRNA sequences have related miRNA sequences in *C. elegans* and *Drosophila melanogaster*.[38]

A search with the precursors obtained after the analysis, on miRBase, matched with the precursors of the predicted mature sequences. Each mature miRNA can have multiple precursors which is why miRBase concentrates on identifying mature sequences if the precursor search function is utilized. As long as the chemical composition and the length of the miRNA stay sufficient to interact with the multi-protein complex (RNA induced silencing complex or RISC), there are multiple ways to generate them depending on a variety of factors like if the miRNAs are in plants or animals[39]. It is also possible for the same precursor to give rise to multiple miRNAs.

A case study that identified 19 miRNA precursors in *Arabidopsis thaliana* has documented that these precursors gave rise to a multitude of miRNAs[40]. The miRNA precursor on cleavage by RNAases first gives rise to a miRNA-miRNA* duplex. Any one of the arms of the miRNA precursor hairpin is capable of acting the role of a miRNA but is decided through a process called arm sorting while the other arm is degraded or takes part in forming a functional product if it has the ability to associate with the RISC proteins[41, 42].

Default parameters set in the psRNATarget tool used to search for targets on the gene transcripts resulted in six of the nine miRNAs previously predicted finding and giving targets (Table 2). Multiple targets were predicted by all six miRNAs with miR-2673b giving a total of 142 targets with varying expect values (E-value) and maximum energies required to un-pair the target sites (UPE) which calculates the accessibility of the targets to the small RNAs. It has been found that different ways of interactions exist between miRNAs and their targets which could be a one to one, many to one or one to many relationship[43]. In different species, there is also a varied variety in the targets that the same miRNA could regulate. An example would be *let-7* which has been identified in a wide variety of genomes with a wide variety of functions.

In *C. elegans*, it controls heterochronic genes that control the timing of molecular development[44] and in *D. melanogaster*, *let-7* plays a role in the maturation of neuromuscular junctions and the wing's cell cycle[45]. Only the best target predicted for each miRNA were carried on for further analysis. The second best targets were taken only in those cases where the homologous proteins could not be found for a particular protein encoded by a gene. Default parameters were used for the BLASTP searches and the proteins that could serve as potential miRNA targets were recorded.

A BLASTP analysis of target protein of miR-7058-3p found it homologous to ribosomal proteins in poryporales fungi. Specifically, the NHP2/L7ae family proteins bind to the kink-turn (K-turn) motifs in ribosomal RNA[46]. The K-turn plays a very important structural role in the RNA structure by introducing a tight kink in the axis of helical RNA. Down-regulation of L7Ae proteins can lead to the disorganization of functional small nucleolar RNAs (snoRNAs) that guide the modifications of other RNA[47].

In cattle tick (*Rhipicephalus microplus),* miR-5336 was found to be expressed specifically in the male sex. On BLASTP analysis, the target gene of the miRNA was found to express GATA-type sexual development transcription factors. Sexual development in fungal pathogens is well characterized and their virulence is usually associated with sexual development[48]. If the sexual development of *Ganoderma* spp. could be influenced, then the targeting of this miRNA could be a useful tool to the control of the disease.

miR-2673b targeted the gene expressing the Utp-14 domain containing protein. UTp-14 is one of atleast 40 proteins along with U3 snoRNA that makes up a large ribonuclear protein complex involved in the processing of small subunits (SSU) in the ribosomal RNA (rRNA)[49]. Alteration or inhibition of the protein could lead to errors in rRNA modification which could cause havoc in the fungal molecular machinery.

Dbl homologous domains (or RhoGEF) that activate Rho proteins through the binding of GTP (Guanosine-5'-triphosphate) and deactivate them through binding with GDP[50], were found to be targeted by miR-6873-3p. These class of proteins are also called guanosine nucleotide exchange factors (GEFs). The gene targeted by miR-9098 was found to express an acetyl-CoA synthetase-like protein which is a molecule that plays a vital role in metabolism [51].

Caspase/metacaspase domains were inferred to be targeted by miR-4968-3p. Metacaspases are present in fungi and are arginine/lysine-specific[52]. Their main function is to induce programmed cell death in fungal populations like in the case of yeast where it induces apoptosis due to adverse environmental factors which degrade subpopulations into compounds that could be used by other healthy cells in the environment thus ensuring the endurance of yeast colonies[53]. Upregulation of caspase activity could promote apoptosis in fungal pathogens which is also a mechanism used by certain antifungal agents [54].

Computational prediction of miRNAs is a necessary aspect because the decrease in sensitivity and specificity make methods like Northern Blot, SAGE and DNA microarrays unreliable[55]. Base pairing between small RNAs and its targets make miRNA silencing an efficient method of gene regulation. In fungi, RNAi can affect the basic functioning of a cell like in the case of *Neurospora crassa* where knocking down FRH (an RNA helicase) affects the circadian clock of the organism[56], but in some cases, fungi lack the RNA silencing machinery like *Ustilago maydis* where untimely termination of transcription happens due to the expression of heterologous genes[57]. This suggests that fungi could do without the RNA silencing pathway for

development and if a silencing pathway is deemed too cumbersome, it could evolve to find alternatives for gene expression regulation.

Recent studies have demonstrated the use of RNAi technology in different biotechnological functions like bioconversion, biomaterial production etc. An example would be the case of Manganese peroxide (MnP) which is an enzyme used as a catalyst in operations like the pulping of lignocelluloses. Silencing of the *mnp3* gene that transcribes MnP reduced the capacity of *Pleurotus ostreatus* fungus to decolorize azo dye whose decolorization is positively dependent on the concentration on Mn^{2+} in a medium[18,58].

Hence, even with the limitations of RNA silencing pathways like incomplete regression of gene expression or the possibility of the same miRNA targeting unintentional genes[59], the development of rapid gene expression analysis platforms make this technology a very attractive prospect when compared to contemporary gene disruption studies. The results obtained in the course of this work could be further confirmed *in vitro* through lab work. Successful usage of miRNAs in fungal pathogen control has been previously demonstrated and the development of a suitable methodology for the utilization of miRNAs to control *Ganoderma* spp. could help in boosting production of crops.

Table 1
Pre-miRNA sequences having real hairpin loops as predicted by miPRED with the MFE and p-values.

S. N.	miRNA	EST sequence id	Pre-miRNA sequence extracted	MFE	p-values
1	miR-7058-3p	GL3IV2H02HF5LZ	CTGGAAGAAAGGAAGGAACTGCTGGAAGTTGCCGGAGCCGTCGCGGAACTGGGTGAAATTGGTGGGGTTCTGGACAGTTGCAACAATAC	-22.00	0.001
2	miR-5336	GL3IV2H02HUYJF	ACCTTTACCATATCATACCGCATACCGACCACGCATACCACATTACCCGCACCTCATTCCATATGGGCTTTTGATCTCATTCGGCGCGGTGTCTGAATCCGGCATGTACACAATCAA	-15.90	0.002
3	miR-7084-5p	GL3IV2H02GI1FP	GTTTGTCGTCCGATCTTCACGCGCTGGCTGTTGACGAGTGATATATGCTTGGGGACGAAAGAGTCGTTAAGAGGATAGAGGTAGAGAGCAGGAAGGGGCACAGCGGCGTG	-28.60	0.002
4	miR-7084-5p	GL3IV2H02JPYIT	TGCCTGCCCCCGTTCTCCACCCCGCACGCCGCTGTGCCCCTTCCTGCTCTCTACCTCTATCCTCTTAACGACTCTTTCGTCCCCAAGCATATATCACTCGTCAACAGCCAGCGCGTGAAGATCGGACGACAAACC	-20.10	0.001
5	miR-2673b	GL3IV2H02GM9T1	CGTAATCCAAAACCGTTGGTGCAAGGCATGTGATGCTTCGTACCGTTTGTTACGAGGTTAGGGGGGTGGGGAAGAGGAAGAGGAAGAGAA	-19.10	0.001
6	miR-5336	GL3IV2H02JGPF1	GTGAGATTGATTGTGTACATGCCGGATTCAGACACCGCGCCGAATGAGATCAAAAGCCCATATGGAATGAGGTGCGGGTAATGTGGTATGCGTGGTCGGTATGCGGTATGATATGGTAAAGGTA	-33.40	0.001

7	miR-6873-3p	GL3IV2H02GZ4D5	GAGGTCAGCCGCTGTACTTACCTCATGAAGGCCTTCCTCACTCGTTTGAACCGCGGCCTCGCGGGCGGCAGAGAGAAAGACAGAGAGAAGGACCCTCAAGCTCAGAAGGAAAAGATACCCCAGCTCCCTCCCCT	-37.90	0.001
8	miR-7084-5p	GL3IV2H02F6N33	GTTTGTCGTCCGATCTTCACGCGCTGGCTGTTGACGAGTGATATATGCTTGGGGACGAAAGAGTCGTTAAGAGGATAGAGGTAGAGAGC	-19.50	0.002
9	miR-4968-3p	GL3IV2H02IEU2B	GAACGGCTTCCAACAGCATGCGGACCCGCAACATCGACAGAATCTCCTAGGATCGCTAGCTGGAGGGCACAGCAACAGCAGCAGCAGCCGGGTTNCTGAATCTGGGCAGCAGGCGAGGAATGTG	-32.00	0.002
10	miR-7058-3p	GL3IV2H02HENEQ	CCGTCCTTGACATTGCTGATCCCGGAGCGCCGAGGTCGATATTAACGCAGAACGCACCCTGCCGGTGGTCTGGAAGAAAGGAAGGAACT	-24.60	0.001
11	miR-7058-3p	GL3IV2H01DA7RP	GTCCTTGACATTGCTGATCCCGGACGCGCCGAGGTCGATATTAACGCAGAACGCACCCTCGCCGGTGGTCTGGAAGAAAGGAAGGAACTGCTGGAA	-22.20	0.002
12	miR-7084-5p	GL3IV2H01DR7BZ	GTTTGTCGTCCGATCTTCACGCGCTGGCTGTTGACGAGTGATATATGCTTGGGGACGAAAGAGTCGTTAAGAGGATAGAGGTAGAGAGCAGGAAGGGGCACAGCGGCGTGCGGGGT	-32.00	0.001
13	miR-9098	GL3IV2H01C3ZP3	TCATAGTTCTTATCAGCCGCCTTGTCGAAGACTGTCCTGCAACGCCGGTTGTACACCTTTGGTAGTCCTCCTTGGTCGGGACGTAGTTGGCCGCGTGGATGC	-25.40	0.002
14	miR2673b	GL3IV2H01D3C4A	CGTAATCCAAAACCGTTGGTGCAAGGCATGTGATGCTTCGTACCGTTTGTTACGAGTTAGGGGGGGTGGGGAAGAGGAAGAGGAAGAGAATGGAATA	-18.50	0.001
15	miR2673b	GL3IV2H01A0QOC	TCGTAATCCAAAACCGTTGGTGCAAGGCATGTGATGCTTCGTACCGTTTGTTACGAGTTAGGGGGGTGGGGAAGAGGAAGAGGAAGAGAATGGAA	-19.80	0.001
16	miR-7886-5p	GL3IV2H01DDN49	AAACCTCCCTTCAACCCTGGACCTCGACGCCGACTCGGACGGCCGCCGAGCGCACATGCACGAGGCAGCAATGAGTCAAAATGATGCTGGTGAAGAGAGGGGTGAGGGGACGTACTGGTGAGC	-46.40	0.001
17	miR-2139	GL3IV2H01EGFY0	TCCCGTTCGAAGATGGCCATGCACTCCTGTGCCCATGTTCAGGTGGAGCGAGAACTTGTCGCGCTGCTCCTGGTAACTGTGGTAGATTGGGCGAGCATGTCCG	-33.60	0.002

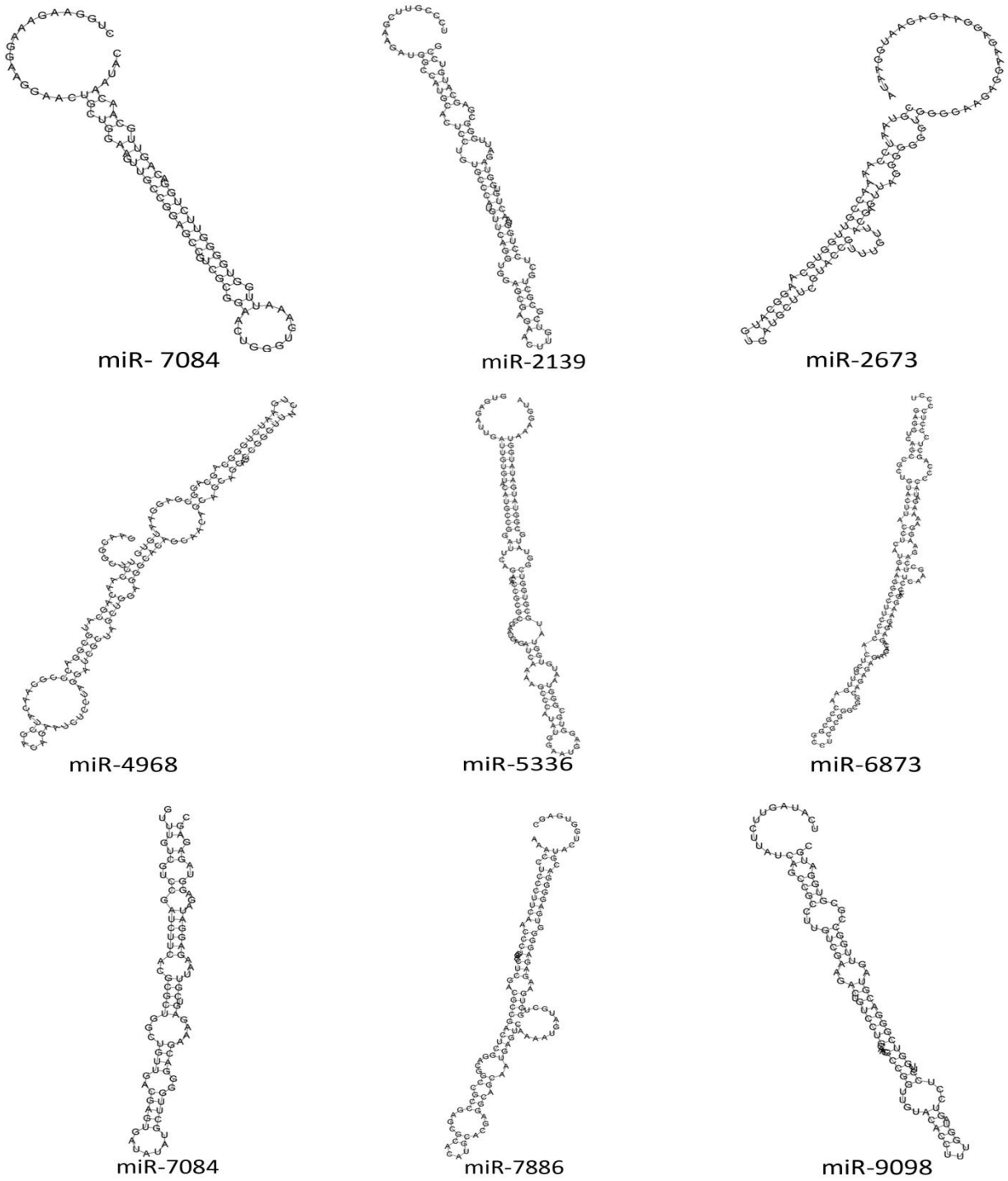

Figure 1: Precursor structures of the conserved miRNAs detected in *Ganoderma* EST

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Table 2
Targets predicted and their potential targets

miRNA_Acc	**miR-7058-3p**	**miR-5336**	**miR2673b**	**miR-6873-3p**	**miR-4968-3p**	**miR-9098**
Target_Acc.	jgi|Gansp1|127319|fgenesh1_pm.3_#_374	jgi|Gansp1|123428|fgenesh1_kg.13_#_171_#_isotig04216	jgi|Gansp1|128986|fgenesh1_pm.6_#_7	jgi|Gansp1|154084|gm1.6546_g	jgi|Gansp1|166792|estExt_Genemark1.C_12_t10481	jgi|Gansp1|145949|estExt_fgenesh1_pg.C_100308
Expectation	2.5	2	0	0	2.5	2.5
UPE	18.674	17.804	6.762	24.833	14.066	17.984
miRNA_start	1	1	1	1	1	1
miRNA_end	20	27	21	20	22	20
Target_start	1464	1332	1037	46	1719	1059
Target_end	1483	1358	1057	65	1740	1078
miRNA_aligned_fragment	CUCGUUCCUUCCUUUCUUCC	UUUGAUCUCAUUCGGCGCGGUUUCCUC	CCUCUUCCUCUUCCUCUUCCA	UUCUCUCUGUCUUUCUCUCU	CAGCAACAGCAGCAGCAGCAGA	UGACCUUGGUCGGGACGUAG
Target_aligned_fragment	GGAGGAGAGGGAAGAACGAG	GCGCAACCCGCGCCGCAUGAGAUCGAG	UGGAAGAGGAAGAGGAAGAGG	AGAGAGAAAGACAGAGAGAA	UCUGUUGUUGUUGUUGUUGUUG	CUACGGCUCGACCGAGGUCG
Inhibition	Cleavage	Cleavage	Cleavage	Cleavage	Cleavage	Cleavage
Target	NHP2/L7ae family proteins	GATA-type sexual development transcription factors	Utp-14 domain containing protein	Dbl homologous domains	Caspase/metacaspase domains	acetyl-CoA synthetase-like protein

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