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In Vitro and In Silico Annotation of Conserved and Nonconserved MicroRNAs in the Genome of the Marsupial *Monodelphis domestica*

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Abstract

The gray short-tailed opossum, *Monodelphis domestica*, is the world's most widely utilized marsupial model for biomedical research. Recent completion of the initial *M. domestica* genome assembly offers the first opportunity to examine genome-wide phenomena in a marsupial. Using in silico methods, we have mapped 124 conserved microRNAs (miRNAs) to 94 loci in the *M. domestica* genome. In addition, using RNA pooled from 5 tissues, we cloned 85 miRNAs. Seventy-two of these are conserved miRNAs that we had mapped in silico. The additional 13 are nonconserved candidate miRNAs in 11 loci. Nine of these 13 are also found in the wallaby (*Macropus eugenii*) genome. Two of the candidate miRNA clones, located on the X chromosome, are part of a cluster containing a total of 24 potential miRNAs spanning more than 100 kb.

Metatherian (marsupial) mammals have long served as important models in biomedical research and, more recently, have assumed increasingly important roles in evolutionary studies as well (Samollow and Graves 1998; Graves and Westerman 2002; Samollow 2006). Molecular studies, bolstered by recent paleontological discoveries, have dated the divergence of metatherian and eutherian mammals at 170–190 million years ago (Kumar and Hedges 1998; Luo et al. 2003; Woodburne et al. 2003). The metatherian–eutherian divergence is proximate enough to the Cretaceous eutherian radiation, which occurred between 80 and 100 million years ago, that shared ancestral mammalian characteristics that persist in these mammalian sister groups, yet it is sufficiently ancient to enable distinct variations on the basic mammalian themes to have evolved between them. It is for this reason that metatherian mammals are invaluable components of comparative studies in many branches of the biological sciences.

Chief among extant marsupials utilized in such research is a small South American species, the gray short-tailed opossum, *Monodelphis domestica* (for reviews, see VandeBerg 1990, 1999; VandeBerg and Robinson 1997; Samollow 2006). Its small size (70–160 g), favorable breeding characteristics, and rapid maturation make *M. domestica* the standard laboratory-bred marsupial worldwide. Its importance is further evidenced by the recent completion of a full-genome sequence assembly—the first marsupial genome to be sequenced (Mikkelsen et al. 2007).

Completion of the initial *M. domestica* genome assembly opens the door to examining genome-wide phenomena and characteristics in a marsupial for the first time. MicroRNAs (miRNAs) are an abundant and important class of small, regulatory RNAs first identified in the early 1990s (Lee et al. 1993). Since then, thousands of miRNA loci have been found in animals, plants, and viruses (Du and Zamore 2005; Ouellet et al. 2006). Although primarily found to be regulators of gene expression involved in cellular development and differentiation, miRNAs have been found to play a significant regulatory role in a host of other functions including apoptosis and cancer (Zhang et al. 2007).

We have used the *M. domestica* genome assembly and a combination of in silico and in vitro methods to annotate 137 miRNAs in the *M. domestica* genome. Taking likely tandem and polycistronic transcripts into account, these miRNAs map to 105 loci. These include 124 conserved miRNAs in 94 loci of which 73 (59%) were directly cloned from an RNA pool composed of 5 tissues. Direct cloning also identified 13 new, candidate marsupial miRNAs in 11 loci.

Materials and Methods

In Silico Identification

All searches reported here were carried out against the Broad Institute *M. domestica* genome assembly (MonDom4 in Ensembl). Pre-miRNA sequences from chicken, mouse, and human were taken from Release 7.0 of miRBase (Ambros et al. 2003; Griffiths-Jones 2004). Each species-specific pre-miRNA sequence list was screened against MonDom4 via BLAST in Ensembl. *Monodelphis domestica* BLAST hits were then assembled by locus, and the identity of each putative miRNA locus was verified by alignment with the orthologous miRNAs and also by BLAST against miRBase. An archive of each verified *M. domestica* locus was then assembled from MonDom4 scaffolds. Each miRNA sequence was recorded along with at least 300 bp of 5′ and 3′ flanking sequence from the scaffold. In many cases, and especially for tandem and polycistronic miRNA transcripts, additional upstream and downstream sequence was archived.

In Vitro miRNA Cloning

miRNA cloning was carried out against an RNA pool composed of brain, heart, lung, liver, and kidney from an adult female animal using the miRCat small RNA Cloning Kit protocol (Integrated DNA Technologies, Coralville, IA).

One hundred micrograms of total pooled RNA (20 μg from each tissue) was run on a 12% denaturing (7 M urea) polyacrylamide gel. RNAs in the 20–24 nt size range were then recovered from the gel. The small RNAs were 3′ ligated with the preactivated linker 5′-rAppCTGTAGGCACCATCAATddC-3′, where rApp is the pre-activating adenylation pioneered by the laboratory of David Bartel (Lau et al. 2001) and ddC is a 3′ block to prevent circularization of the linkered RNAs. The reaction was carried out at 22 °C for 2 h with T4 RNA Ligase (Epicentre Biotechnologies) in the absence of adenosine triphosphate (ATP). Linkered RNAs (~40 nt in length) were recovered from a second 12% denaturing (7 M urea) polyacrylamide gel. These RNAs were then 5′ ligated with the RNA/DNA chimera 5′-TGGAATucucgggcaccaaggu-3′, where uppercase letters are DNA nucleotides and the lowercase letters indicate RNA nucleotides. This reaction was also carried out at 22 °C for 2 h with T4 RNA Ligase (Epicentre Biotechnologies) in the presence of 10 mM ATP.

A reverse transcription reaction was performed on the double-ligated RNAs using the primer sequence 5′-GATTGATGGTGCCTACAG-3′ and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The RT reaction was then polymerase chain reaction (PCR) amplified with the RT primer as the reverse PCR primer and the sequence 5′-TGGAATTCTCGGGCACC-3′ as the FOR PCR primer. The PCR conditions are 95.0 °C$^{5:00}$[95.0 °C$^{0:30}$; 52.0 °C$^{0:30}$; 72 °C$^{0:30}$]$_{35}$72.0 °C$^{7:00}$.

Amplicons from the PCR contain flanking *Ban*I restriction endonuclease recognition sites (G|GYRCC). A *Ban*I (New England Biolabs, Ipswich, MA) digestion was performed, and the reaction was extracted in one volume of phenol:chloroform:isoamyl alcohol (25:24:1). The extracted restriction fragments were concatamerized overnight at room temperature in the presence of 2000 U of T4 DNA Ligase (Epicentre Biotechnologies). The concatamers were "polished" by incubating the entire ligation reaction with *Taq* DNA polymerase for 10 min at 72 °C. This reaction filled in the restriction ends and added nontemplated adenines. This permitted direct concatamers cloning in pGEM T-EASY (Promega, Madison, WI). Concatamer-containing clones were then sequenced on an Applied Biosystems Model 3130xl Genetic Analyzer.

Results

In Silico Annotation of Conserved miRNAs

Screening the MonDom4 genome assembly with conserved vertebrate miRNA precursor sequences (pre-miRNAs) returned a total of 94 miRNA loci containing a total of 124 miRNAs. Of these, 74 correspond to solitary, 15 to tandem, and 5 to polycistronic (3 or more miRNAs in the same transcript) transcripts in the mouse and human genomes. Among the tandem and polycistronic transcript loci identified in the *M. domestica* genome are 2 in which one of the miRNAs present in the mouse and human genomes was absent from *M. domestica*. These are the tandem *let7f-2/miR-98* of which *miR-98* is not present and the polycistronic locus *miR-25/miR-93/miR-106b* of which *miR-106b* is not present in *M. domestica*. Both of these loci are found in introns in mouse and human genomes, with the former located within HUWE1 and the latter within *MCM7*. In each case, the inter-miR spacing in the mouse and human genomes was noted and sufficient additional sequence from the appropriate *M. domestica* scaffold was searched to confirm the absence of an *M. domestica* ortholog. Apart from the 2 instances noted, all other tandem or polycistronic miRNA loci were found to contain the same miRNAs in the *M. domestica* genome that they do in other mammalian genomes. In addition, with few exceptions, there is reasonably good conservation of spacing between the pre-miRNAs in the putative primary transcripts (pri-miRNAs). Exceptions include the tandem *miR-200b/miR-200a* transcript in which the 2 pre-miRNAs are located 696 and 665 bp apart in human and mouse, respectively, but 2239 and 2573 bp apart in chicken and *M. domestica*, respectively; the tandem *miR-212/132* transcript in which the spacing is 748 bp in *M. domestica* but only 263 and 204 bp in human and mouse, respectively; the tandem *miR-191/miR-425* transcript in which the spacing is 2181 bp in *M. domestica* but 394 and 385 bp in human and mouse, respectively; and the polycistronic cluster *let7a-1/let7f-1/let7d* wherein *M. domestica* displays inter-miR spacing of 662 and 4715 bp compared with 311 and 2401 bp in human and 262 and 1715 bp in mouse.

Genome Context and Synteny

Among the currently annotated miRNAs in species listed in miRBase, about two-thirds are located in intergenic space,

whereas the remaining one-third are located in introns. Of the 94 conserved miRNA loci identified in the *M. domestica* genome, 23 (24.5%) are known to map to introns of genes in the mouse genome, human genome, or both. We used mouse and/or human mRNA sequences for these 23 genes to determine whether the corresponding *M. domestica* ortholog miRNAs are also intronic. Confirmation consisted of determining the map position and strand orientation of the appropriate mouse and/or human mRNA via BLAST of MonDom4 and matching this with the map position and strand orientation of the *M. domestica* pre-miRNA. Using this method, we confirmed 20 of the 23 putative intronic miRNAs in *M. domestica* to introns of presumed orthologous genes in MonDom4 (Table 1).

Monodelphis domestica chromosome coordinates were obtained for 117 of the 124 miRNAs identified in the BLAST searches of MonDom4. The remaining 7 are currently unassigned. The 117 chromosome coordinates were compared with the chromosome coordinates of each human ortholog listed in miRBase. Both sets of coordinates were then located on a 50 K *M. domestica*/human synteny map produced at the Broad Institute via BLAST (Kamal M, personal communication). Of the 117 miRNAs for which chromosome coordinates were obtained in both the human and the *M. domestica* genomes, a syntenic match, that is, a match in the appropriate coordinate space, was found for all 117. A relative placement map of these 117 miRNAs on *M. domestica* chromosomes is shown in Figure 1. Also shown are the relative genome placements of the genes encoding the 5 primary miRNA processing proteins DROSHA, DGCR8, Exportin 5, Dicer 1, and AGO2. Note that the coordinates of the 20 intronic miRNAs for which gene context synteny could be confirmed in Table 1 also provide the relative genome placements of their host genes in the *M. domestica* genome.

Table 1. *Monodelphis domestica* miRNAs localized to conserved positions within introns

miR ID	Chromosome	Confirmed[a]	Gene (HGNC approved gene symbol)[b]
let 7f-2	Un	Yes	HECT, UBA, and WWE domain containing 1 (HUWE1)
7-1	6	Yes	Heterogeneous nuclear ribonuclearprotein K (HNRPK)
10b	4	Yes	Homeobox D4 (HoxD4)
25	2	Yes	Minichromosome maintenance deficient 7 (MCM7)
32	6	Yes	C9orf5
101-2	Un	Yes	RNA terminal phosphate cylcase-like 1 (RCL1)
103-1	1	Yes	Pantothenate kinase 3 (PANK3)
103-2	1	Yes	Pantothenate kinase 2 (PANK2)
107	1	Yes	Pantothenate kinase 1 (PANK1)
128b	6	Yes	Cyclic AMP-regulated phosphoprotein 21 (ARPP21)
148b	8	No	Coatomer protein complex, subunit zeta 1 (COPZ1)
152	2	Yes	Coatomer protein complex, subunit zeta 2 (COPZ2)
186	2	Yes	Zinc finger protein 265 (ZNF265)
196b	8	Yes	Homeobox A10 (HoxA10)
203	1	Yes	LOC374569
211	1	No	Transient receptor potential cation channel, subfamily M, member 3 (TRPM3)
208	1	Yes	Myosin heavy polypeptide 6 (MYH6)
214	2	Yes	Dynamin 3, opposite strand (DNM3os)
218-1	5	Yes	Slit homolog 2 (SLIT2)
449	3	Yes	FLJ37927
191/425	Un	Yes	DALR anticodon binding domain containing 3 (DALR3)
23b, 27b, 24-1 6	6	Yes	C9orf3
17 cluster	7	No	C13orf25

[a] Confirmation based on chromosome coordinates of the miRNA and the result of BLAST with either mouse and/or human mRNA sequence of the gene. Excluding open reading frames, FLJ37927, and LOC374569, these results were also confirmed through text searches in Ensembl.

[b] Identification of the gene is from miRBase and subsequently confirmed with MapViewer in GenBank. Gene names and symbols are taken from the HUGO Gene Nomenclature Committee (www.genenames.org).

miRNA Cloning

Direct cloning from the *M. domestica* RNA pool yielded mature miRNA sequences for 73 of the 124 conserved miRNAs (58.9%). A number of these miRNAs were found in multiple clones. For example, *miR-122a* was found in 48 clones, *miR-29b-1* was found in 14 clones, and *miR-148a* was found in 10 clones. Another miRNA, *miR-126*, was found in 18 clones, but only 4 were the mature sequences, whereas 14 clones were the star sequences, *miR-126**, on the opposite strand of the hairpin. Of the remaining conserved miRNAs, 51 were observed in 3 or fewer clones.

In addition to the 73 conserved miRNAs cloned, there were 17 clones that contained RNA sequences we have designated as new candidate miRNAs in *M. domestica*. Two of these, designated Mdo-27 and Mdo-10, were each cloned 3 times. None of the other candidate miRNAs was observed more than once. To be considered a candidate miRNA, an otherwise unidentified cloned sequence had to satisfy 2 criteria. First, the cloned sequence could not match any known miRNA in miRBase or other noncoding RNA in RNAdb (Pang et al. 2007). Second, a sequence at least 60 bp

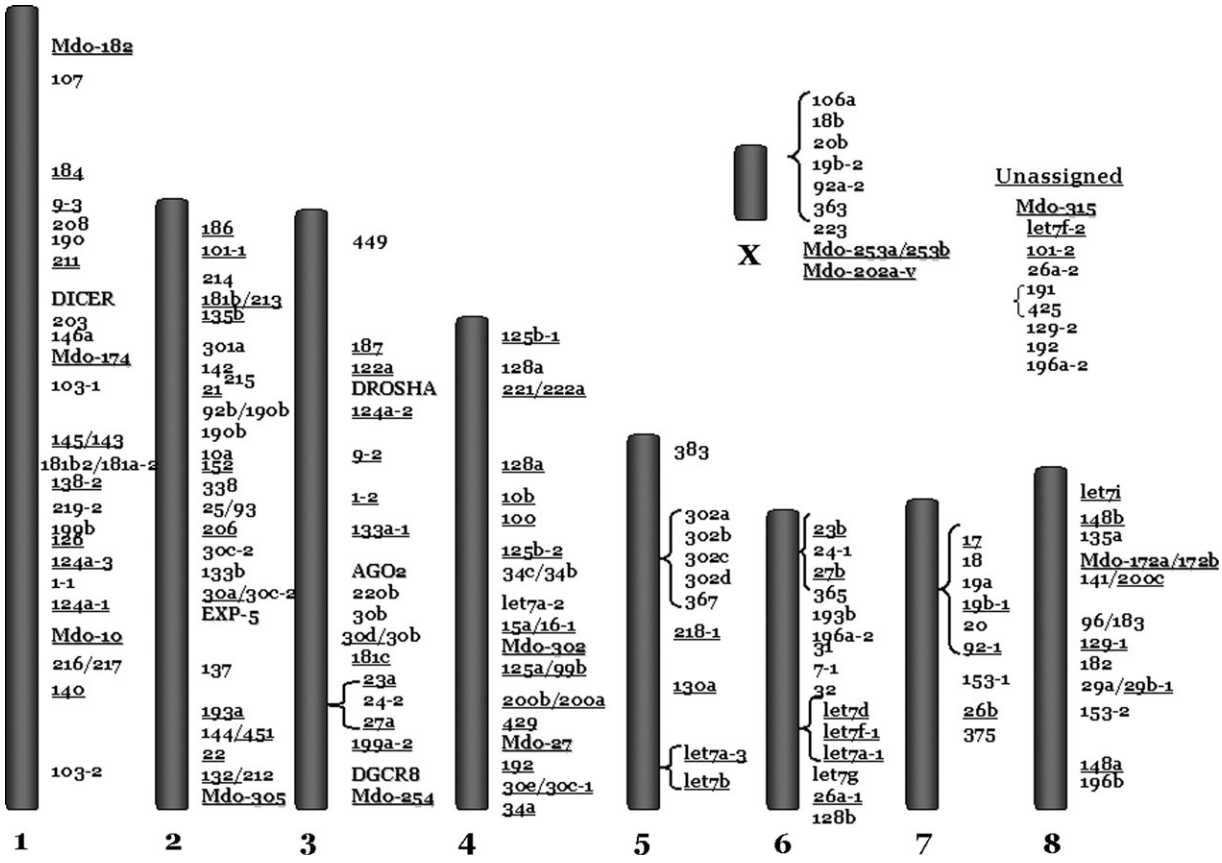

Figure 1. Chromosome locations of 136 miRNAs in *Monodelphis domestica*. Underlined miRNAs have been annotated *in silico* and validated by direct cloning of the mature miRNA sequence. Loci designated Mdo and underlined are the 13 new candidate miRNAs. Locations of the 5 major miRNA processing proteins are indicated as well.

in length that includes the cloned putative mature sequence had to form a thermodynamically stable hairpin structure as determined by the RNA folding program mFOLD (Zuker 2003). This latter criterion is crucial as it is the formation of a stable hairpin within the primary miRNA transcript in the nucleus that guides the first stage of miRNA processing by DROSHA and DGCR8 (see Du and Zamore 2005). Thirteen sequences that met both of the above stated criteria are shown in Figure 2. The candidates designated Mdo-253a and Mdo-253b have the same precursor sequence and are located 5 kb apart on the X chromosome. The candidates designated Mdo-172a and Mdo-172b are a likely tandem transcript as they lie less than 100 bases apart on chromosome 8 and share an identical mature sequence. A number of otherwise unidentified RNA sequences from our clones were not accepted as candidates because they failed the second criterion.

Discussion

Using standard bioinformatics tools and the recently completed draft genome assembly of the marsupial *M. domestica* (MonDom 4.0), we have identified and annotated 105 miRNA loci containing 137 miRNAs. Of these loci, we were able to map 98 (116 miRNAs) to the *M. domestica* genome. The remaining 6 loci (7 miRNAs) remain unassigned.

Using an RNA pool composed of 5 major organs, we cloned mature sequences for 73 of the 124 conserved miRNAs (58.9%). Among these sequences were several that occurred in many of the clones. One of these, *miR-122a*, which was seen 48 times, is a liver-specific miRNA associated with hepatitis C viral replication (Jopling et al. 2005). Another sequence, *miR-29b*, which was observed 14 times, has been shown to control expression of a specific branched amino acid complex (Mersey et al. 2005). Other sequences observed 8 or more times are *miR-148a* (10 times), *miR-23a* (9 times), *miR-26a* (9 times), and *miR-124a* (8 times). One locus, *miR-126*, was found a total of 18 times, but in only 4 of these instances were they the mature 126 sequence. The other 14 instances were the imperfectly complementary sequence from the opposite strand of the hairpin referred to as 126*. One other observation of both strands of a miRNA being expressed in our clones was a single occurrence of *miR-30a-3p* and a single occurrence of *miR-30a-5p*, which are also imperfect complements.

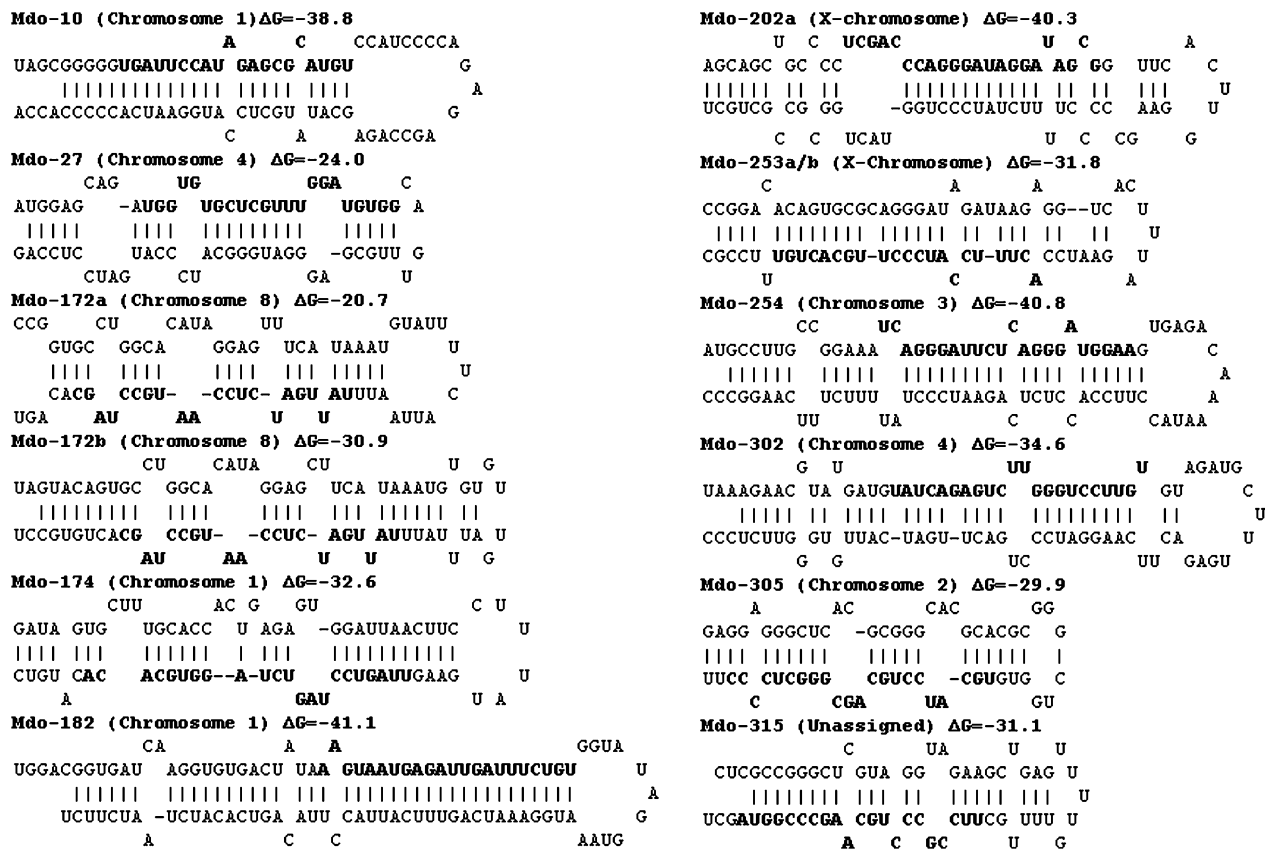

Figure 2. Stem-loop precursor sequences of 13 new candidate miRNAs in *Monodelphis domestica*. The chromosome assignment is given along with the thermodynamic stability of the hairpin (ΔG) in kilocalories per mole. RNAs were folded in mFOLD as linear sequences at 37 °C.

In addition to their characteristic stem-loop structures, another characteristic feature of both plant and animal miRNAs is a tendency toward extreme sequence conservation, especially on the mature sequences (Pasquinelli et al. 2000; Floyd and Bowman 2004; Berezikov and Plasterk 2005). This conservation is an aid to in silico identification of conserved miRNAs as has been seen here. It also is an aid in identifying potential new miRNAs as has also been shown here. None of the 13 new candidate miRNAs reported here produced matches in any database regardless of whether the cloned mature sequence or the entire putative precursor sequence was used as the query. Thus, we suggest that the 11 loci containing these miRNAs evolved in the marsupial genome after the divergence of metatherian and eutherian mammals. We tested this idea by searching the only other marsupial genome currently available that of the Tammar wallaby, *Macropus eugenii*, which is found in an unassembled state in the GenBank Trace Archive. The 12 different precursor sequences were used as queries. This search returned 9 matches (75%) in the wallaby genome. Matches were not found for Mdo-27, Mdo-202a, and Mdo-253a,b. We carried out a similar query in the wallaby genome using the precursors for the 124 conserved *M. domestica* miRNAs reported here in order to get a sense of the coverage currently represented in the Trace Archive in GenBank. This query returned 109 matches (88%). As coverage of the wallaby genome further improves, we hope to be able to evaluate the remaining novel candidate miRNAs. On the basis of the available sequences, however, the conclusion that these candidates are marsupial-specific miRNAs is reasonable.

X Chromosome miRNA Cluster

The candidate miRNAs Mdo-172a and Mdo-172b were identified as a tandem transcript when a BLAST of the Mdo-172a hairpin forming pre-miRNA (shown in Figure 2) against MonDom4 returned 2-chromosome 8 locations within 100 bp of each other. A BLAST of the pre-miRNA sequence for Mdo-253 against MonDom4 also returned 2 identical hits on the X chromosome, but these are about 5 kb apart. We have designated these as Mdo-253a and Mdo-253b. These 2 loci flank the location obtained for the other X chromosome miRNA candidate, Mdo-202. When the Mdo-202 pre-miRNA was BLASTed against MonDom4, the result was not 2 but 22 hits spanning more than 100 kb of the X chromosome. The original candidate clone is designated Mdo-202a, and the remaining candidates are designated Mdo-202b to Mdo-202v (Figure 3A).

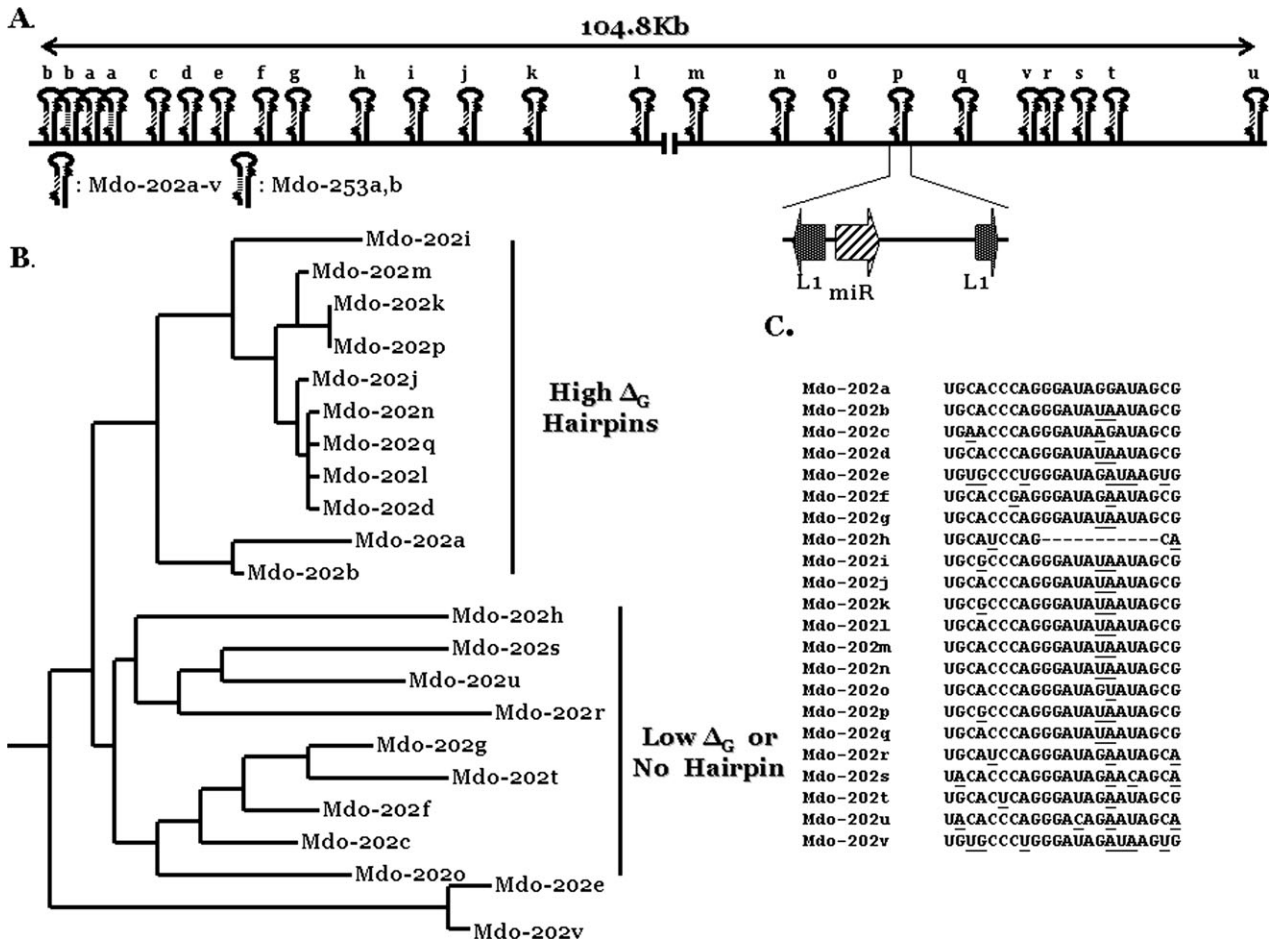

Figure 3. The large potential miRNA cluster on the *Monodelphis domestica* X chromosome. (**A**) A map of the relative spacing and order of the 22 members of the Mdo-202 cluster and the 2 Mdo-253 loci. Also shown is the consensus organization of the Mdo-202 cluster repeat unit annotated in CENSOR using Mdo-202p as the example. (**B**) A neighbor-joining tree resulting from a CLUSTAL W alignment of the 22 Mdo-202 cluster pre-miRNAs. (**C**) An alignment of the 22 orthologous mature miRNA sequences corresponding to the original Mdo-202a sequence cloned from the *M. domestica* RNA pool.

An alignment of the 22 Mdo-202a-v pre-miRNA sequences using CLUSTAL W (Thompson et al. 1994) indicates 2 distinct clades. One of these, Clade 1, contains the original Mdo-202a as well as 10 other precursors. Clade 2 contains 9 of the 11 remaining precursors with Mdo-202e and Mdo-202v belonging to neither clade (Figure 3B). Submitting all 22 Mdo-202 pre-miRNAs to mFOLD showed that all the members of Clade 1 have thermodynamically stable hairpins (ΔG range: −34.6 to −40.3 kcal), whereas 5 members of Clade 2 (202c, 202f, 202h, 202o, and 202r) fail to form miRNA hairpins at all, and the 3 that do (202g, 202s, and 202t) are not as stable as Clade 1 hairpins (ΔG range: −28.8 to −32.5 kcal). Mdo-202e and Mdo-2092v both form hairpins with ΔG values of −29.3 kcal. These 2 have the most divergent mature sequences with 7 base differences compared with Mdo-202a (Figure 3C). It should be noted that the only member of this entire cluster known to be expressed at this time is the original clone, Mdo-202a. Further study of this cluster including expression profiling in other tissues and, potentially, in other marsupial species is warranted as this is one of the largest miRNA clusters known in either animal or plant genomes. The largest known miRNA cluster, with 54 members, was found on human chromosome 19, is only in primate genomes, and is expressed only in placenta (Bentwich et al. 2005). This cluster was created through multiple replication events mediated by flanking *Alu* retrotransposons, and recent studies indicate that retrotransposons such as *Alu*s, *L1*s, and *L2*s are intimately involved in both the birth of miRNA loci via duplications and the miRNA target sites (Smalheiser and Torvik 2005, 2006; Borchert et al. 2006). The complete 105 kb sequence containing the *M. domestica* X chromosome miRNA cluster from Mdo-202b through Mdo-202u was submitted for repeat element annotation in Censor web server at the Genetics Information Research Institute (Jurka et al. 2005). A consensus repeat unit about 1.1 kb in length emerged in the annotation. This consensus unit consists of an Mdo-202 pre-miRNA flanked by *L1* retrotransposon

fragments oriented tail to tail (Figure 3A). Thus, like the 54 member primate miRNA cluster, the Mdo-202 cluster appears to have evolved via retrotransposon-mediated duplications.

The metatheria represent 1 of 3 mammalian reproductive strategies and occupy an intermediate position with respect to evolutionary divergence times between the egg-laying monotremes and the Eutheria (van Rheede et al. 2006). The present catalog of conserved miRNAs in *M. domestica* is likely not complete, but it represents the beginning of a miRNA profile of the base of the Therian clade. Moreover, given the crucial developmental roles being assigned to miRNAs in both plants and animals (Bartel 2004; Ouellet et al. 2006; Zhang et al. 2007), the discovery of marsupial-specific miRNAs will also shed light on miRNA evolution as well as on the possible marsupial-specific processes they are regulating.

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miRNA sequences reported on here have been deposited in miRBase (http://microrna.sanger.ac.uk/sequences/index.shtml).

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