Multiple *Dicer* Genes in the Early-Diverging Metazoa

Danielle de Jong, Michael Eitel,* Wolfgang Jakob,* Hans-Juergen Osigus,* Heike Hadrys,*†
Rob DeSalle,‡ and Bernd Schierwater*†‡*

*Tieraerztliche Hochschule, Division of Ecology and Evolution, Hannover, Germany; †Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT; and ‡American Museum of Natural History, Central Park West, New York, NY

Dicer proteins are highly conserved, are present in organisms ranging from plants to metazoans, and are essential components of the RNA interference pathway. Although the complement of Dicer proteins has been investigated in many "higher" metazoans, there has been no corresponding characterization of Dicer proteins in any early-branching metazoan. We cloned partial cDNAs of genes belonging to the Dicer family from the anthozoan cnidarian *Nematostella vectensis* and two distantly related haplotypes (species lineages) of the Placozoa (*Trichoplax adhaerens* 16S haplotype 1 [H1] and *Placozoa* sp. [H2]). We also identified *Dicer* genes in the hydrozoan *Hydra magnipapillata* and the demosponge *Amphimedon queenslandica* with the use of publicly available sequence databases. Two *Dicer* genes are present in each cnidarian species, whereas five *Dicer* genes each are found in the Porifera and Placozoa. Phylogenetic analyses comparing these and other metazoan Dicers suggest an ancient duplication event of a "*Proto-Dicer*" gene. We show that the Placozoa is the only known metazoan phylum which contains both representatives of this duplication event and that the multiple *Dicer* genes of the "basal" metazoan phyla represent lineage-specific duplications. There is a striking diversity of *Dicer* genes in basal metazoans, in stark contrast to the single *Dicer* gene found in most higher metazoans. This new data has allowed us to formulate new hypotheses regarding the evolution of metazoan Dicer proteins and their possible functions in the early diverging metazoan phyla. We theorize that the multiple placozoan *Dicer* genes fulfill a specific biological requirement, such as an immune defense strategy against viruses.

Introduction

The RNA interference (RNAi) pathway is an ancient and highly conserved mechanism present in most eukaryotes. The pathway plays roles in both gene regulation and defense against viruses via translational repression, mRNA degradation, or genome modification (by the creation of heterochromatin). The process can be triggered by various sources of RNA, including endogenous small noncoding microRNAs (miRNAs), both endogenous and exogenous small interfering RNAs, RNA viruses, transposons, and exogenously introduced double-stranded RNAs (dsRNAs). The RNAi pathway is triggered when larger dsRNA templates are cleaved into smaller RNAs, which pair with accessory proteins to form RNA-induced silencing complexes (RISC) and attach to complementary RNA or DNA sequences. Members of a class 3 RNaseIII-type enzyme family called Dicer generate the small RNAs. Dicer protein members are able to recognize and cleave dsRNAs, help to form the RISC and are thus crucial elements in the initiation of the RNAi pathway (for review see McManus 2004).

Dicer proteins are a widely conserved family, present in many organisms including plants, fungi, and the Metazoa. Typically, Dicer proteins contain a number of different domains: an N-terminal DEAD box, an RNA helicase domain, a Piwi–Argonaute–Zwille (PAZ) domain, a divergent dsRNAs-binding domain (dsRNA bind; previously known as DUF283), two ribonuclease (RNase III) domains, and an additional dsRNAs-binding domain (dsrm) (fig. 1*A*) (Bernstein et al. 2001; Cerutti and Casas-Mollano 2006; Margis et al. 2006). The function of each of these domains are being elucidated; however, catalysis of dsRNA into smaller fragments relies upon the activity of the RNase III domains, which function as a homodimer (Zhang et al. 2004) and are ubiquitous among all Dicer proteins. The

PAZ domain is theorized to be a protein–protein interaction domain and has been shown to bind the end of the target dsRNA and determine the size of RNA fragments produced (typically 21–25 nt) (Macrae et al. 2006). Likewise, the two dsRNAs-binding domains (dsRNA bind and dsrm) most likely bind dsRNA targets (Dlakic 2006).

Although the plants *Arabidopsis thaliana* and *Oryza sativa* contain four and five Dicer proteins, respectively (Margis et al. 2006), thus far metazoans were thought to contain only one (e.g., *Caenorhabditis elegans* and vertebrates) (Matsuda et al. 2000; Ketting et al. 2001) or two (insects only) (Lee et al. 2004) *Dicer* genes. It has been suggested that the higher number of Dicers in plants is related to their requirement in immune defense (Blevins et al. 2006; Margis et al. 2006).

Recently, assessing the presence of miRNAs has become a topic of hot research in the early diverging or "basal" metazoans—the cnidarian *Nematostella vectensis* contains at least four miRNAs from three families, whereas the number in the demosponge *Amphimedon queenslandica* (formerly known as *Reniera* sp.) differs from none (Sempere et al. 2006; Prochnik et al. 2007) to eight (Grimson et al. 2008). In the placozoan *Trichoplax adhaerens*, no miRNAs have yet been identified (Grimson et al. 2008). However, despite the large effort currently employed into identifying this aspect of the RNAi pathway, there has been no corresponding characterization of Dicer proteins from any of the early branching metazoan phyla aside from a brief mention of the number of predicted *Dicer* genes from some genome sequencing projects (Grimson et al. 2008; Srivastava et al. 2008). In order to more comprehensively assess the Dicer gene complement in cnidarians, poriferans, and placozoans, we identified *Dicer* genes in the hydrozoan cnidarian *Hydra magnipapillata* and the demosponge *A. queenslandica* with the use of publicly available sequence data sets and cloned partial cDNAs corresponding to genes belonging to the Dicer family from the anthozoan cnidarian *N. vectensis* and two different haplotypes of the Placozoa. The single yet described species of the Placozoa, *T. adhaerens*, is the most simple animal known in terms of morphology
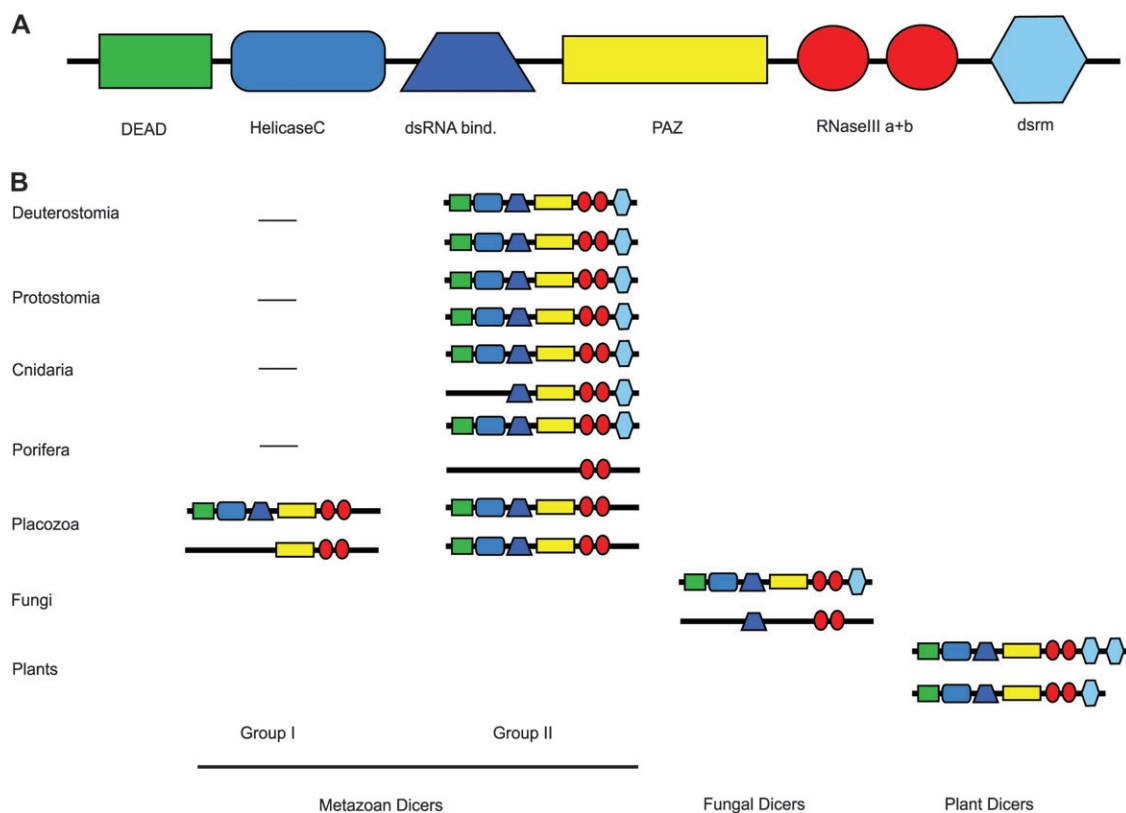
FIG. 1.—Overview of the structure of Dicer proteins found in various groups of organisms. Schematic diagram of the general domain structure of Dicer proteins (*A*). The minimal (least complex) and maximal (most complex) domain structure of Dicer proteins present in different groups of organisms grouped according to our phylogenetic analysis (*B*).

(see Schierwater 2005). Although their exact phylogenetic position remains highly controversial, they are clearly one of the earliest branching metazoan phyla and may even have originated at the very root of the Metazoa (Dellaporta et al. 2006; Schierwater et al. 2009). These animals have proven to be amenable to experimental molecular studies (Martinelli and Spring 2003; Jakob et al. 2004; Hadrys et al. 2005), and there are indications that the RNAi pathway functions as it does in other organisms; putative members of the pathway are present in the *T. adhaerens* genome (Drosha and Argonaute—data not shown and Grimson et al. 2008) and addition of dsRNA can induce gene-specific silencing in *T. adhaerens* (Jakob et al. 2004). The fact that these genes are expressed in *T. adhaerens* (and also *N. vectensis*) strongly suggests they are also functional, unless they are (very new) pseudogenes.

The results of phylogenetic analyses incorporating our new sequence data suggest the duplication of a single hypothetical metazoan "*Proto-Dicer*" gene early in evolution giving rise to the major metazoan Dicer family which we have termed Dicer "Group II" and an (as of yet) Placozoa-restricted Dicer protein family (Dicer "Group I"). We show that the *Dicer2* genes present in insects represent a lineage-specific duplication. We also show that in each basal metazoan phyla sampled, multiple *Dicers* are present (clearly in contrast to "higher" phyla) and are the result of lineage-specific duplications. A hypothetical function of these duplications is discussed.

Materials and Methods
Data Sets

Genomic and expressed sequence tag (EST) sequence data were accessed from the available databases at National Center for Biotechnology Information, Compagen (www.compagen.org), the Department of Energy Joint Genome Institute (http://genome.jgi-psf.org), and the Computational Biology and Functional Genomics Laboratory (http://compbio.dfci.harvard.edu/tgi/). The raw data sets from the Cnidaria included 10,272,644 genomic reads and 163,221 ESTs, from *H. magnipapillata*, 2,817,779 genomic reads (comprising 356 Mbp) and 166,595 ESTs for *N. vectensis* (release v1.0), from the Placozoa (*T. adhaerens*), 940,892 genomic reads (comprising 105.6 Mbp) and 14,572 ESTs (release v1.0), and from the Porifera, 2,823,539 shotgun sequences and 83,040 ESTs (*A. queenslandica*). Coverage of the *N. vectensis* genome is currently 7.8×, whereas for the *H. magnipapillata*, *T. adhaerens*, and *A. queenslandica* genome projects, the coverage at present is estimated to be approximately 6-fold, 8-fold, and 12-fold, respectively.

Database Searches and Phylogenetic Analysis

For database searches, a local Blast platform, the public Blast platform at NCBI, or the Blast platform provided on the appropriate database were used (see previous

section). Genomic contigs were assembled manually as required and coding sequence predicted using the Genescan (Burge and Karlin 1997), Genomescan (Yeh et al. 2001), or GeneMark.hmm (Lomsadze et al. 2005) programs. The various protein domains were identified with the use of PFAM protein family database (Finn et al. 2006) and resulted in an initial matrix with 645 proteins (available upon request). Protein sequence alignments of the RNase III (a) and (b) domains (without the intervening linker) were created using MAFFT (Thompson et al. 1994; see supplementary data set 1, Supplementary Material online). Missing data were denoted with question marks in the alignment. The phylogeny of helicase superfamily proteins was generated using Neighbor-Joining analyses (PAUP*; Swofford 2003) with the archaeal helicases used as outgroups. A 50% jackknife tree was generated with 100 repetitions of character removal to determine the level in the tree where robustness fades (supplementary fig. 1, Supplementary Material online). A second trimmed matrix was used to examine the relationships of proteins within the Dicer family (supplementary data set 2, Supplementary Material online) using Bayesian inference with MrBayes v3.2 (Ronquist 2004) and the plant Dicers as outgroup. The parsmodel option was used as a model and 4 million Markov chain Monte Carlo generations were used and the first 10% (400,000) of the trees removed as burn-in. The Bayesian posteriors were calculated from the saved trees from MrBayes runs using the majrule option in PAUP*. Only nodes with posterior probabilities greater than 0.75 were retained in the final tree. For more detail of Bayesian posteriors at all nodes in the tree, see supplementary data set 3 (Supplementary Material online). It should be noted that the nomenclature of the newly identified Dicer genes from these organisms is based solely on the order in which they were identified, and the use of the same alphabetical letter or number for genes of different species does not necessarily denote orthology. Accession numbers of all sequences used in the analyses is shown as supplemental table 1 (Supplementary Material online).

Isolation of Partial Dicer cDNAs from N. vectensis and Placozoa

RNA was extracted from a single *N. vectensis* polyp (Hannover culture; Nv0204) starved for 3 days prior to the procedure, using the QIAGEN RNeasy Mini Kit. Similarly, RNA was extracted from a culture of starved placozoans using approximately 350 adult animals each from two different haplotypes (*T. adhaerens*, 16S haplotype 1 [H1] and *Placozoa* sp., 16S haplotype 2 [H2]). Note that these two haplotypes reflect two different species lineages and possibly even two different families (Eitel M, Guidi L, Balsamo M, Schierwater B, in preparation) and as such are termed *Trichoplax adhaerens* (*T. adhaerens*) or *Placozoa* sp. H2 in the text and figures. cDNA was generated from reverse transcription of total RNA using the GeneRacer RACE Ready cDNA synthesis kit (Invitrogen) following the manufacturer's recommendations. Initially, we amplified small fragments of a *Dicer* gene (*NvDcr2*) from *N. vectensis* cDNA with primers based on genomic DNA sequence, to create a cDNA contig of approximately 5,000 bp (which included the RNase III (a) and (b) domains). Following this, we focused on the characteristic RNase III domains for subsequent cloning attempts. Subsequently, cDNA corresponding to the RNase III (a) and (b) domains of a second *N. vectensis Dicer* gene (*NvDcr1*) and each of the five placozoan *Dicer-like* genes from two haplotypes (*TaDclA–E* and *PlacoDclA-E*; including the intervening linker) were isolated using primers based on *T. adhaerens* genomic DNA sequence. A complete list of primer sequences and polymerase chain reaction (PCR) Protocols are available on request. Following PCR, products were cloned using the pGEM-T cloning system (Promega) and two to five clones from each fragment were sequenced on both strands using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI PRISM 310 Genetic Analyzer or were sequenced using the services provided by Macrogen. The sequences were manually checked and assembled with the use of SeqMan (DNAstar package).

Results and Discussion
Multiple *Dicer* Genes in the Early-Branching Metazoa

We isolated partial cDNAs of five *Dicer* genes in each of the two placozoan haplotypes and partial cDNAs of two *Dicer* genes in the anthozoan, *N. vectensis*. The sequences of these cloned cDNAs have been deposited into the NCBI GenBank database (EU394521–EU394532). These data, taken together with the results of our genomic database searches, reveal that the cnidarians *N. vectensis* and *H. magnipapillata* possess two *Dicer* genes each, whereas the poriferan *A. queenslandica* and the two placozoan haplotypes investigated possess five *Dicer* genes each. We would like to note that this differs from other predictions of the same data sets; the number of *Dicer* genes in *T. adhaerens* is denoted as three in the supplementary data for the recent whole-genome sequencing project (Srivastava et al. 2008) and four in *A. queenslandica* (Grimson et al. 2008). The reasons for this are most likely differences in prediction programs (although strangely, the *T. adhaerens* and *A. queenslandica* Dicer genes are not significantly different to others so as to appear unrecognizable upon a simple Blast similarity search). In any case, it serves as a reminder that automated annotation of whole-genome sequence may not always provide accurate answers regarding gene number or sequence; careful manual annotation might be indispensable in certain cases.

Phylogenetic Analysis of Dicer Proteins

Previous phylogenetic analyses supported by comparable domain organization have suggested a monophyletic origin of plant and animal Dicer proteins (Cerutti and Casas-Mollano 2006). We conducted similar phylogenetic analysis, with the inclusion of sequences from the basal Metazoa. Initially, we conducted a Neighbor-Joining phylogenetic analysis with 645 protein sequences from the DEAD/DEAH Box, MDA5 RIGI IGP2, Archaeal and invertebrate helicase, and Dicer families, which all

belong to the helicase protein superfamily. This analysis (supplementary fig. 1, Supplementary Material online) clearly shows that the newly identified putative Dicer proteins in Placozoa, Porifera, and Cnidaria belong to the same Dicer family already identified in the plant and opisthokont lineages and not to any other members of the helicase superfamily. We then trimmed this larger helicase matrix down to 112 proteins from the Dicer family only and conducted phylogenetic analyses to examine the relationships between the Dicer proteins of plants, fungi, and Metazoa. Our results show that metazoan Dicers form two distinct clades—one containing *Dicer* genes solely from the Placozoa (Dicer Group I) and the other comprising *Dicer* genes from the Placozoa and all other metazoan phyla (Dicer Group II). An independent duplication event in the lineage leading to the fungi has also resulted in two distinct fungal Dicer families (which we have termed "Alpha" and "Beta"; figs. 2 and 3).

Dicer Genes in the Basal Metazoa and Their Relationship to Other Metazoan Dicers

Our results suggest a single duplication event of a hypothetical *Proto-Dicer* gene early in metazoan evolution to give rise to two types of metazoan *Dicer* genes, Group I and Group II, and show that the Placozoa are the only known extant metazoan phyla which possesses both Group I and Group II genes. The most parsimonious interpretation of this data is that the Placozoa are basal to the Porifera and there was a loss of a Group I *Dicer* gene early in the evolution of the Metazoa. Although data from this study and from Schierwater et al. (2009) clearly supports this hypothesis, it is important to consider that this may simply reflect undersampling, especially in the basal metazoan lineages.

Although discrete from the situation, we see in the Metazoa, our analyses also show a duplication event in the ancestor of the fungi, giving rise to two separate fungal Dicer families and further diversification within these families (figs. 3 and 4). Interestingly, however, our survey of available choanoflagellate data failed to identify any sequences with homology to any fungal or metazoan *Dicer* genes, suggesting lineage loss (see also Grimson et al. 2008).

Lineage-Specific Duplications within the Basal Metazoa

Within the Bilateria, *Dicer* genes are only present in single copies, with the exception of the insect *Dicer2* genes, which arose via a lineage-specific duplication event. Within the early diverging Metazoa, other lineage-specific duplications of *Dicer* genes are clearly apparent; *N. vectensis* and *H. magnipapillata* contain two independently duplicated *Dicer* genes each, and the five sponge *Dicers* also appear to have arisen via lineage-specific duplications (all belonging to Dicer Group II). Within the Placozoa, the situation is slightly more complex; four independently duplicated placozoan *Dicer* genes (*Dcl1A*, *B*, *C*, and *E*) belong to the hypothetical Dicer Group I, whereas a single gene belongs to Dicer Group II (*Dcl1D*) based on our classification. Recent

studies conducted on EST and genomic sequence data sets of several of the early diverging phyla have shown a more complex set of genes and gene families than historically assumed. For example, cnidarians, poriferans, and placozoans have been shown to possess homologs of components of a diverse range of metazoan signaling pathways (Samuel et al. 2001; Kortschak et al. 2003; Kusserow et al. 2005; Technau et al. 2005; Nichols et al. 2006; Adamska et al. 2007; Matus et al. 2008; Srivastava et al. 2008), and many of the genes likely to play key roles in development have been independently duplicated (Samuel et al. 2001; Ball et al. 2004; Hislop et al. 2005). The *Dicer* gene family therefore represent another example of genetic complexity in morphologically "simple" animals.

Selective Loss of the PAZ Domain in Some Sponge Dicer Proteins

Although the complete coding sequences have not yet been ascertained, structural features can be deduced from the predicted proteins. Each of the basal metazoan Dicer proteins show a typical domain structure (although all lack a C-terminal dsrm motif), indicating that the proteins most likely function as other known Dicer proteins and that the hypothetical metazoan *Proto-Dicer* almost certainly harbored a full (or near full) domain complement (fig. 1). Interestingly, the *A. queenslandica* AqDcr2B and AqDcr2C proteins appear to lack a PAZ domain. Although Dicer proteins which lack a PAZ domain are found in ciliates (e.g., *Tetrahymena thermophila*; Malone et al. 2005), algae (e.g., *Chlamydomonas reinhardtii*; Schroda 2006), and fungi (e.g., *Neurospora crassa* and *Schizosaccharomyces pombe*; Catalanotto et al. 2004), to our knowledge all metazoan Dicer proteins so far investigated contain PAZ domains (fig. 1). Therefore, *A. queenslandica* AqDcr2B and AqDcr2C are the first reported metazoan Dicer-like proteins to lack a PAZ domain, although postulating theories as to the significance of this would be purely speculative and is therefore not discussed here. In addition, it should be noted that this observation is based solely on genomic predictions, and as of yet, we have no further data in support of these predictions.

Why So Many Dicers?

One important and significant finding of this study is the fact that, unlike all other metazoan phyla with the exception of the insects, the basal metazoans possess multiple *Dicer* genes. Notably, although *N. vectensis* and *H. magnipapillata* possess only two *Dicer* genes each, five *Dicer* genes are present in both *A. queenslandica* and the Placozoa. One function of Dicer proteins is to generate miRNAs which modulate gene expression. In animals, this initially requires the actions of the proteins Drosha and Pasha to create primary miRNA, a template for Dicer, whereas long dsRNA, such as that obtained exogenously, requires Dicer only (see Tomari and Zamore 2005). Both processes require the action of the RISC central component Argonaute. However, although the genome of
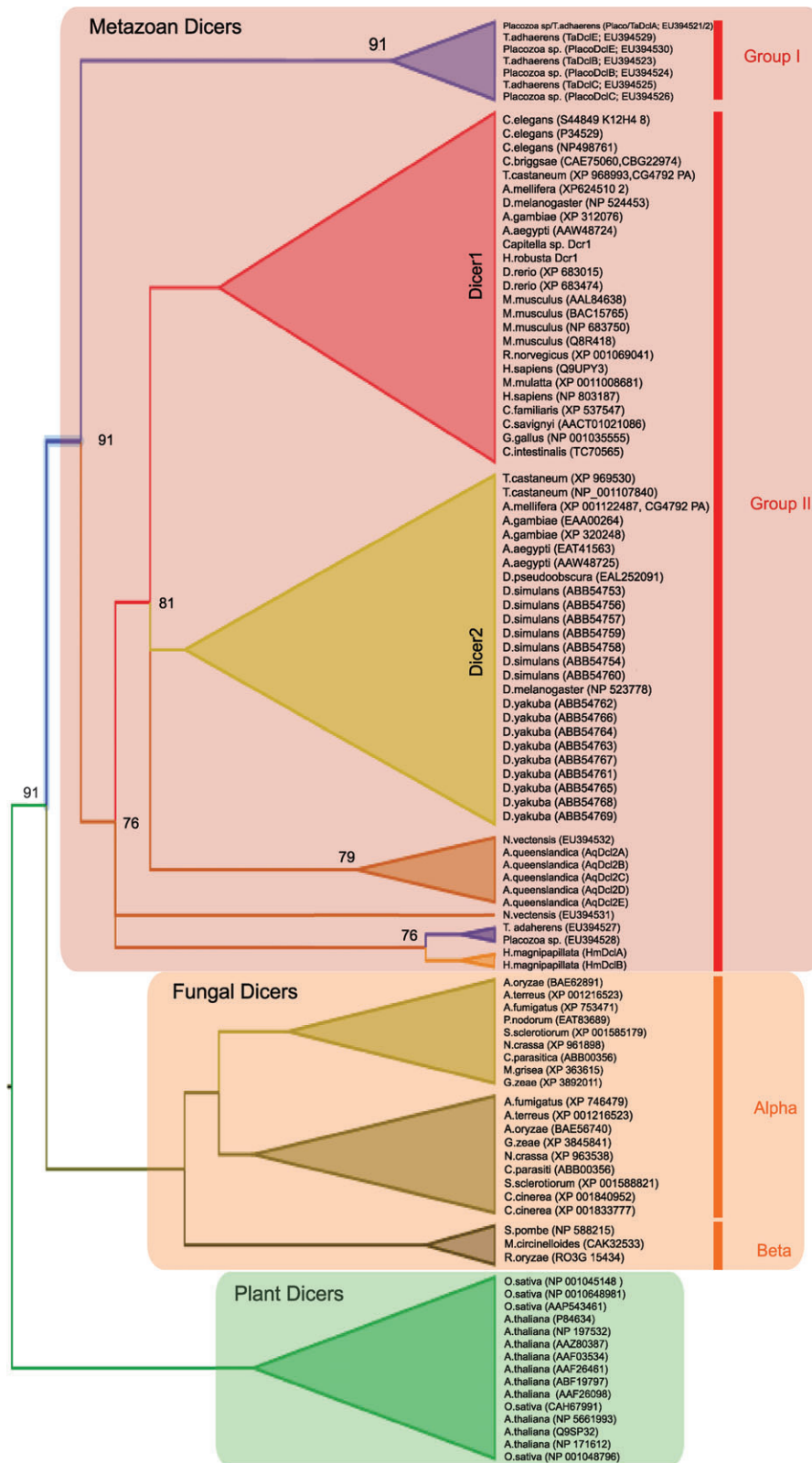
FIG. 2.—Bayesian phylogenetic analysis of Dicer proteins of various organisms. Metazoan, fungal, and plant sequences are boxed in red, orange, and green, respectively. The purple shaded triangles show placozoan proteins, orange triangles show cnidarian and sponge proteins. Numbers on the nodes represent the posterior probability using parsmodel after 4 million generations. The first 400,000 trees were removed from computing the Bayesian posteriors as burn-in. Only nodes with Bayesian posteriors greater than 75% were retained in this tree. Any node shown in the tree that does not have a number has Bayesian posteriors of 1.0. For complete list of proteins in the analysis and raw Bayesian posterior values for individual nodes within the large clades represented by shaded triangles, see supplemental data sets 2 and 3 (Supplementary Material online).
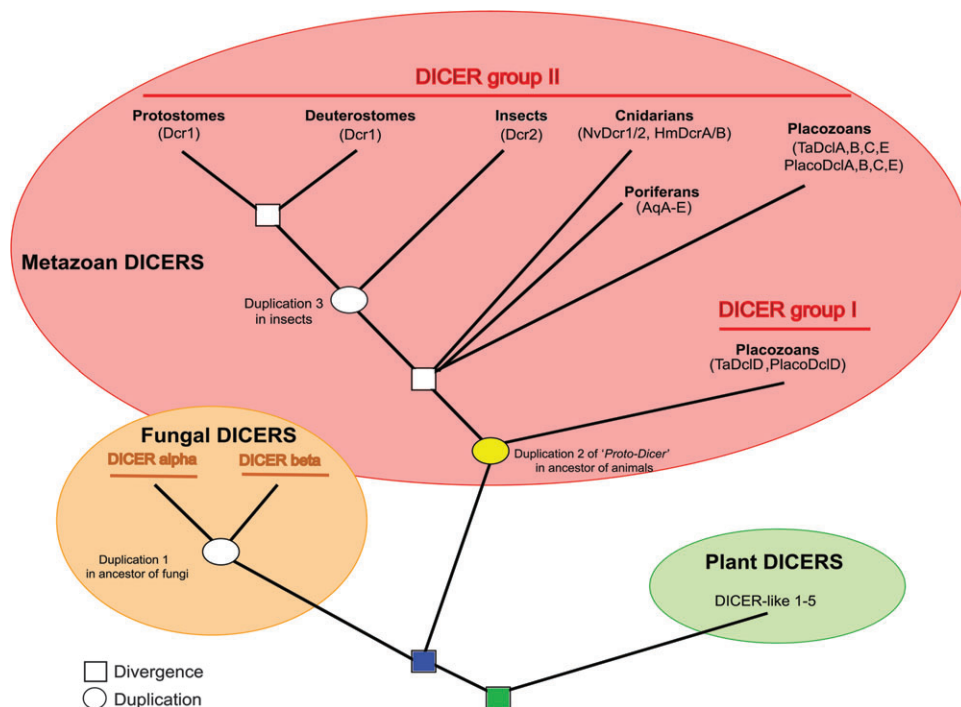
Fig. 3.—Tree-based scenario for the evolution of Dicer proteins. Boxes indicate a divergence event (i.e., divergence by cladogenesis). Circles represent putative duplication events. Change in colors represent major cladogenetic events or ancestors in the tree of life; green represents the plant–opisthokont divergence; dark blue represents the fungi–animal divergence; yellow represents the hypothetical "*Proto-Dicer*" duplication.

T. adhaerens possesses recognizable homologs of Argonaute and Drosha, a homolog of Pasha is not identifiable. The most simple explanation for not finding a homolog of Pasha might be that it escaped whole-genome sequencing; although the coverage is approximately 8-fold, it is certainly incomplete. It may also be possible that a different mechanism is used for miRNA production in this organism. A third explanation is that placozoans are not able to produce miRNAs and, therefore, lack any form of miRNA-mediated gene regulation. This is indeed suggested in a recent article which failed to identify any miRNAs in *T. adhaerens* despite a widespread screen which was able to identify candidates in both *N. vectensis* and *A. queenslandica* (Grimson et al. 2008), a claim supported by a second study (Hertel et al. 2009). If this is the case, it suggests that the Dicer duplication we see in the Placozoa is not likely to be a reflection of an increased level of gene regulation mediated by miRNAs. A logical theory is that placozoans use RNAi as a large part of their defense against viruses. In plants, the presence of multiple Dicer-like proteins reflects, in part, complex antiviral strategies (Bernstein et al. 2001; Xie et al. 2004; Gasciolli et al. 2005; Margis et al. 2006). For example, in *A. thaliana*, the Dicer-like 2 (Dcl2) protein responds to the turnip crinkle virus but not the cucumber or turnip mosaic viride, which are specifically targeted by Dicer-like 4 (Dcl4) (Xie et al. 2004). The use of RNAi as a viral defense mechanism has also been shown in fungi, for example, *Cryphonectria parasitica* (Segers et al. 2007) and metazoans, for example *Drosophila melanogaster* (Li et al. 2002; Wang et al. 2006), *C. elegans* (Lu et al. 2005; Wilkins et al. 2005), and mouse (Muller and Imler 2007).

The reason for the *Dicer* duplication in the Porifera and Cnidaria is not so clear, with the full subset of machinery required for the synthesis of miRNAs from stem–loop precursors encoded in their genomes and putative miRNAs identified in each of these phyla (Sempere et al. 2006; Prochnik et al. 2007; Grimson et al. 2008). Although it clearly requires further research, we believe it is possible that because the semisessile and phagocytic Placozoa are exposed to a high viral load, the duplication of *Dicer* genes may constitute part of a specific immune defense strategy against viruses. This would suggest that the Placozoa and Porifera have relatively simple innate immune systems, although to date, there has been no research in support of this. Recent investigation into the innate immune system of cnidarians has shown that in general they possess a relatively complex innate immune system (Hemmrich et al. 2007; Miller et al. 2007; Sullivan et al. 2007), a situation mirrored in the marine deuterostome *Strongylocentrotus purpuratus* (Rast and Messier-Solek 2008). In these animals at least, although they must be exposed to a similarly high viral load, perhaps the need for a viral defense system mediated by Dicer is negligible.

Conclusion

In this study, we identified several new sequences that have previously been overlooked in several genome projects and cloned partial cDNAs from two placozoan species lineages and an anthozoan cnidarian. Phylogenetic analyses incorporating this new data have allowed us to formulate new hypotheses on the ancestral repertoire of

Dicer proteins in animals. We show that the complexity of the *Dicer* gene complement of the early branching metazoans is striking and changes our view on the presence and evolution of metazoan Dicer proteins. Ultimately, further research in this area will lead to a greater understanding of RNAi and the evolution of its roles in gene regulation and immune defense.

Supplementary Material

Supplementary figure 1, data sets 1–3, and table 1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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