Published in final edited form as:

Differentiation. 2010 July ; 80(1): 1–8. doi:10.1016/j.diff.2010.02.003.

Basic Helix-Loop-Helix Transcription Factor Gene Family Phylogenetics and Nomenclature

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Abstract

A phylogenetic analysis of the basic helix-loop-helix (bHLH) gene superfamily was performed using seven different species (human, mouse, rat, worm, fly, yeast, and plant *Arabidopsis*) and involving over 600 bHLH genes [1]. All bHLH genes were identified in the genomes of the various species, including expressed sequence tags, and the entire coding sequence was used in the analysis. Nearly 15% of the gene family has been updated or added since the original publication. A super-tree involving six clades and all structural relationships was established and is now presented for four of the species. The wealth of functional data available for members of the bHLH gene superfamily provides us with the opportunity to use this exhaustive phylogenetic tree to predict potential functions of uncharacterized members of the family. This phylogenetic and genomic analysis of the bHLH gene family has revealed unique elements of the evolution and functional relationships of the different genes in the bHLH gene family.

Keywords

basic Helix-Loop-Helix transcription factor; bHLH; human; mouse; rat; worm; fly; yeast; plant; nomenclature; phylogenetics

INTRODUCTION

A recent phylogenetic analysis of the basic helix-loop-helix (bHLH) gene superfamily including seven different species re-evaluates the classifications of these genes based on an unbiased sequence comparison across the full length of the proteins [1]. Previous classifications of the bHLH genes were based on a combination of functional characteristics, such as DNA binding activity, dimer partners, and expression patterns [2,3]. The organization of bHLH genes into clades [1] does not make assumptions about gene function and resulted in the formation of six clades. Clade A contains primarily mammalian genes and no plant genes, while clade F only contains plant genes. The other four clades had a mixture of different species' genes, and

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specific ancestral genes are identified [1]. The mouse bHLH gene family is shown in Figure 1. Several members of the bHLH superfamily have been subdivided based on the inclusion of additional functional motifs, including leucine zipper, PAS domain, and Orange domain that provide additional functionality to the transcription factors, as well as alter the DNA binding activity of the bHLH domains [4–7]. These various classes of bHLH genes were categorized appropriately in the new phylogenetic analysis [1].

The bHLH motif was first identified in 1989 and initial classification schemes were based on ubiquitous (Class A) and tissue-specific (Class B) expression [8]. The classification system was expanded with the first large scale phylogenetic analysis by Atchley and Fitch (1997). This system used a comparison of the bHLH domains to a Class A set of bHLH genes that included tissue-specific proteins [9], a Class B Group of functionally unrelated proteins [10], a Class C that contained the PAS domain proteins [4], and Class D that are inhibitory (e.g. lack basic domain, Ids). In the most recent phylogenetic analysis, the complete amino acid sequence was used to identify new clades that do not correlate with this previous classification [1], Figure 1. Due to the inclusion of uncharacterized (e.g. EST) bHLH genes and the development of a more accurate relationship of all the genes between species, a new nomenclature is suggested. The previously proposed nomenclature is not unified and provides multiple names for the same gene between species and within the same species. Currently, no functional or structural relationships are considered. The new nomenclature uses the name “bHLH” for the entire gene family and then a letter representing the clade distribution for the gene (e.g. bHLHa for Clade A genes), followed by a number within the clade to help cluster related genes (e.g. bHLHa1-bHLHa46). Homologous genes from multiple species are given the same name and the variants of a single gene have letters [e.g. E12 (bHLHb21a) and E47 (bHLHb21b)]. The old and new nomenclature for all seven species are provided in Supplemental Table S1 and Supplemental Table S2, including recent additions and corrections. The old and new names for the mouse bHLH proteins are shown in the phylogenetic tree in Figure 1.

Several large and small scale phylogenetic analyses of the bHLH transcription factor family have been performed that only use the bHLH domain sequence [2,11,12]. A recent analysis in plants used the entire coding sequence [13]. Due to the number of additional domains being associated with the bHLH proteins, utilizing the entire coding region in a large-scale phylogenetic analysis for classification is needed [1]. The unified bHLH gene family nomenclature suggested allows for structural and functional gene relationships within and across species to be considered, Supplemental Table S1. Recently the Human Genome Organization (HuGO) has adopted this nomenclature and integrated it into the GeneBank information. The Arabidopsis

(<http://www.arabidopsis.org/portals/nomenclature/guidelines.jsp>) (Table S5), mouse (<http://www.informatics.jax.org/mgihome/nomen/>), *C. elegans* (<http://www.wormbase.org/>) (Table S4), *Drosophila*

(http://flybase.bio.indiana.edu/static_pages/docs/nomenclature/nomenclature3.html) (Table S3) and human (<http://www.bioscience.org/services/genenome.htm>) nomenclature committees have adopted the proposed nomenclature. In the event an EST or un-named gene was identified the nomenclature was adopted as the official name. In the event a previous name existed the new nomenclature was adopted as an alternative name. If the new nomenclature is adopted by the research community in the future the official name assignment will be considered by the appropriate nomenclature committees. The updated nomenclature is proposed to assist in future analysis of cellular differentiation and developmental processes influenced by bHLH proteins.

SPECIES PHYLOGENETIC TREES

The various bHLH proteins for each of the species is shown in Supplemental Table S1 and provides a comparison between the species using the new nomenclature, Supplemental Table 2. Species-specific phylogenetic trees for mouse (Figure 1), human (Figure 2), drosophila (Figure 3) and *C. elegans* (Figure 4) were constructed to show the clade distribution of the bHLH genes in the specific species. As expected, the *C. elegans* have the fewest number of bHLH genes, while a larger diversity of gene types are found in drosophila, and the most found in humans (~130), although the number found in mouse is nearly identical (~117). This pattern is suggestive of gene family duplication and diversification as the metazoans diversified. Interestingly, the similar level of diversity among all mammals studied (human, mouse, rat), suggest that most of the functional types of bHLH transcription factors likely originated early in or prior to mammalian diversification. In contrast, *Arabidopsis*, a flowering plant, has even more (~141) copies of bHLH genes than the mammals (~130 in humans), but many of these plant genes are found in clades completely separate from the metazoan genes [1]. This suggests that while a few copies were present in the shared ancestor of plants and animals, most of the gene diversity was derived later in eukaryotic diversification. This diversification was likely associated with the origins of multicellularity and different lineages of multicellular organisms. The duplication of different copies of bHLH leads to only marginal overlap in gene family membership between these disparate lineages.

Gene diversity within clades also varies among species. While the patterns are very similar between human and mouse (Fig. 1–Fig. 2), the diversity in *C. elegans* is very different (Fig. 4). The largest obvious difference, other than fewer total genes, is the paucity of bHLH-B clade genes. The functional significance of this lack of Clade B genes is unclear. In drosophila, the bHLH gene family is reasonably diverse, but it should be noted that while drosophila has more diversity than *C. elegans*, and less than mammals, the distribution of gene copies in the phylogenies are quite different between the Ecdysozoa (the clade that includes both arthropods and nematodes) and mammals. For instance, the drosophila bHLHa28 is sister to the clade of mammalian bHLHa26/a27 (HAND) genes, suggesting that the drosophila copy diverged prior to this duplication. However, there does not appear to be an ortholog in *C. elegans*. Since *C. elegans* and drosophila share a more recent common ancestor than either does with the mammals, this is suggestive that this copy might have been lost in the *C. elegans* lineage or has not been recognized as a bHLH gene family member yet. Alternatively, there are some clades with multiple copies in drosophila or *C. elegans*, or both, but without any known mammalian copies. This suggests duplications and possible functional diversification in the Ecdysozoa or within more recently diverged lineages. In order to address the timing of duplications and deletions, and their possible functional significance, more detailed sampling of the bHLH gene family will be necessary outside of the commonly used model systems. Further phylogenetic analysis will be required to better predict functional diversification and the differences between duplications and deletions in the bHLH gene family.

bHLH FAMILY FUNCTIONAL RELATIONSHIPS

The bHLH transcription factors represent an ancient family found in fungi, plants, and animals that are essential for organisms to respond to a broad set of environmental factors. Individual members of the family are able to direct differential gene expression that allows cells to respond to select nutrients in the environment (glucose, sterols, inositol, choline, iron, and calcium), promote cellular and systemic protection from hypoxic conditions and xenobiotics, and coordinate organismal growth in response to light. Other members are required for diversification of the cell types during embryogenesis through cell type-specific specification, differentiation, proliferation, and apoptosis. A comprehensive analysis of the bHLH gene family in metazoans revealed multiple rounds of expansion [3,14]. The first round occurred

with the split of the metazoans from the choanoflagellates and the second round was associated with the increase in specialized cell types in higher metazoans. The most recent phylogenetic analysis of bHLH genes by [1] using 541 known bHLH genes across both plants and animals divides the genes into new clades based solely on the sequence comparison of the entire protein, instead of the highly conserved bHLH domain. The organization of these clades provides new insight into the functional relationships and potential ancestral origins of the bHLH genes.

The best-described role for bHLH genes during embryogenesis is the development of the neurogenic circuitry [15–17]. The differentiation and specification of individual neurons as well as their patterning in the central and peripheral nervous system are dependent on the Atonal-related [Atonal (bHLHa12, bHLHa13, and bHLHa21), NeuroD (bHLHa1, bHLHa2, bHLHa3, and bHLHa4), Neurogenin (bHLHa6, and bHLHa7), Mist (bHLHa15)], Acheate-scute complex (AS-C) (bHLHa42, bHLHa44, bHLHa45, bHLH46, bHLH47), Hairy/Enhancer of Split (HES) [Hairy and E(spl) (bHLHb37, bHLHb38, bHLHb39, bHLHb40, bHLHb41, bHLHb42, and bHLHb43), Hey (bHLHb31, bHLHb32, bHLHb33)], N-Twist (bHLHa31) and NHLH (bHLHa34, and bHLHa35) genes. Orthologs for these subfamilies have been identified in all cnidarians and bilaterians underscoring the fundamental role of these bHLH proteins in neurogenesis [14]. The Atonal-related AS-C, and NHLH genes are grouped in Clade A, consistent with their linked functions. Interestingly, the HES genes are present in Clade B. The HES genes are downstream targets of the Notch signaling pathway, where they function to inhibit the transcription of proneural genes [18]. This predicts that HES genes are a later addition that increased the complexity of the proneural regulatory pathway.

In addition to the HES genes, Clade B also contains the Id/emc family (bHLHb24, bHLHb25, bHLHb26, bHLHb27) of transcriptional repressors. A previous classification of bHLH genes placed the Id/emc family into a separate class (Class D) based on the absence of the basic residues within the bHLH domain required for DNA binding [2]. Repression by these factors occurs by forming heterodimers with bHLH transcriptional activators and disrupting DNA binding. Inclusion of these factors in the same clade as the HES subfamily predicts a previously unappreciated ancestral link between these two subfamilies of transcriptional repressors. Hes and E(spl) genes direct repression by binding the transcriptional co-repressor, *Groucho*, through a carboxy-terminal tetrapeptide WRPW motif [19]. However, several members of the HES subfamily (HES1, 3, 5, and 6 and Hey1 and 2) have also been reported to repress transcription by a mechanism analogous to Id/emc proteins [20–22] supporting the functional link between these gene subfamilies.

Notably absent from Clade B are BHLHB2/DEC1 (bHLHe40) and BHLHB3/DEC2 (bHLHe41) (1). These transcriptional repressors play a pivotal role in regulating circadian rhythm control, metabolite-sensing, hypoxia response and the development of the vascular, neural, muscle and cartilage tissue [23–25]. Based on the sequence comparison of the bHLH domain alone, BHLHB2 and BHLHB3 have been grouped with the Hes and E(spl) genes [26]. However, when using the entire protein for a phylogenetic analysis, BHLHB2 and BHLHB3 fall in Clade E that includes the bHLH-Pas genes that are essential for circadian oscillation, hypoxia response, and vascular and neuronal development (Figure 1) [27]. This reveals a closer phylogenetic link between bHLH and bHLH-Pas genes participating in environmental adaptation and differentiation than previously appreciated.

One of the outcomes of the analysis by [1] is the distribution of a family of bHLH genes that contain another functionally important motif known as the Leucine Zipper (Zip), which participates in both hetero- and homodimerization. Though these genes share two functional domains they are distributed among four clades. The best described of the bHLH-Zip genes belong to the Myc/Mad/Max network. Max (bHLHb6) serves as an obligate heterodimer partner for members of both the Myc and Mad subfamilies in the regulation of cell proliferation,

differentiation, and death [28]. Myc genes are transcriptional activators, while Mad genes function as repressors through a Sin3a-mediated mechanism. An interconnected network uses a Max-like factor (Mlx/ bHLHd13) as an obligate partner for Mad factors and a separate family of transcriptional activators that regulate lipid and glucose metabolism [SREBF1 (bHLHd1), SREBF2 (bHLHd2), MondoA (bHLHe36), and MondoB (bHLHd14) [29,30]. Both Max and Mlx fell into clade D, while the Mad and Myc subfamilies were in clades C and E, respectively (Figure 5). Interestingly, the Mad-like factor, Mnt was present in clade D instead of clade C with the Mad factors. This predicts Mnt shares functional similarity with other Mad transcriptional repressors, but a phylogenetic relationship closer to Max. Similarly, SREBF1 and 2, as well as MondoA are found in clade D, predicting that these transcriptional activators have a closer relationship to Max than the Myc genes. A second bHLH- Zip family called Mitf-Tcf [Mitf (bHLHe32), Tcf3 (bHLHe33), Tcf3b (bHLHe35), and Tcf3c (bHLHe34)] forms heterodimers similar to the Myc/Mad/Max network. Natural and induced mutations predict that this family regulates neural crest and osteoclast differentiation [31–33]. Though these genes do not interact with Myc factors, the phylogenetic analysis places them in the same clade. Twist (bHLHa38) and members of the Twist subfamily are known to alter their function based on binding partner [34–36]. Consistent with other analyses, the Twist subfamily fell into the same clade [1]

CONCLUSION

The ability of bHLH factors to alter their DNA binding specificity based on their dimer partners has long been recognized as a mechanism to maximize the diversity in target gene transcription that can be controlled by a relatively small number of genes. The new organization of the bHLH genes suggests novel linkages between subfamilies that were not previously appreciated, but now uncovered by their clade association. This represents a potentially fertile area of study for further investigation into the interactions and functions of bHLH genes. The unified nomenclature proposed now assists comparative studies between species and provides a more accurate functional relationship of the bHLH genes. Clearly as research in the bHLH area advances the gene associations and nomenclature will need to be corrected and evolve. Consideration of this phylogenetic analysis provides insights into the evolutionary biology of this gene family and potential functional relationships of specific bHLH genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ms. Heather Johnson for assistance in preparation of the manuscript. None of the authors have any financial interests or disclosures. This work was supported by NIH grants to Michael K. Skinner.

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Figure 2. Human bHLH super-tree for the 5 different clades and all 118 genes and relatedness presented. Multiple listings with the same new nomenclature are the same gene, and some new genes listed in Table S1 are not listed on the tree.

Drosophila bHLH Phylogenetic Tree

Figure 3. Drosophila bHLH super-tree for the 5 different clades and all 56 genes and relatedness presented.

C. Elegans bHLH Phylogenetic Tree

Figure 4.
C. elegans bHLH super-tree for the 5 different clades and all 30 genes and relatedness presented.

Figure 5. Clade distribution of bHLH-Zip genes that heterodimerize with Max or Mlx. Max and Mlx are obligate dimer partners for both transcriptional activators and transcriptional repressors.