

## **Ancient origin of the Parkinson disease gene *LRRK2***

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## Abstract

Dominant mutations in the *LRRK2* gene, a member of the Roco family, cause both familial and sporadic Parkinson disease. *LRRK* genes had been so far detected only in bilaterian animals. In deuterostomes, including humans, two *LRRK* genes (*LRRK1* and *LRRK2*) exist while in protostomes a single *LRRK* gene has been found. In this study, I combine structural and phylogenetic analyses to show that the cnidarian *Nematostella vectensis* has four *LRRK* genes. One of them is a *bona fide* ortholog of the human *LRRK2* gene, demonstrating that this gene has an ancient origin. Two others are, respectively, orthologs of the deuterostome *LRRK1* and the protostome *LRRK* genes. The fourth gene is probably cnidarian-specific. This precise characterization of the early evolution of *LRRK* genes in animals has important implications, because it indicates that the *Drosophila* and *Caenorhabditis* *LRRK* genes, which are studied to gain an understanding of *LRRK2* function, are not true orthologs of the human Parkinson disease gene. Novel functional insights are also gained by the comparison of the structures of *LRRK2* genes in distantly related species.

**Keywords:** Parkinson disease; kinase; GTPase; armadillo repeats; comparative genomics; leucine-rich repeats; paralogy.

## Introduction

The discovery of genes involved in the genesis of familial forms of Parkinson disease (PD) has opened new ways to understand the molecular basis of this pathology. *LRRK2* is the first gene whose mutations have been consistently associated to both the familial and the much more frequent sporadic cases of PD. Dominant mutations in *LRRK2* may explain 13% of the familial and up to 5% of the sporadic PD cases (Berg *et al.* 2005; Mata *et al.* 2006; Taylor *et al.* 2006). This general involvement of the *LRRK2* gene in both types of PD suggests a central role in dopaminergic cell death and the onset of the disease (Mata *et al.* 2006; Taylor *et al.* 2006). In addition, mutations in *LRRK2* may be involved in other neurodegenerative diseases (Chen-Plotkin *et al.* 2007). All these results have led to a great interest in understanding the biochemical and cellular functions of this gene.

LRRK2 protein has a complex structure, which includes several types of repeats and three large domains, called Roc, COR and Kinase domain (Bosgraaf and Van Hastert 2003; Marín 2006; Mata *et al.* 2006). Proteins with a Roc domain, which is a Ras-like GTPase domain, plus a COR domain, a characteristic domain of unknown function, belong to the Roco family (Bosgraaf and Van Hastert 2003). Roco family genes are present in both prokaryotes and eukaryotes. Vertebrates typically contain four Roco genes: *LRRK1*, *LRRK2*, *MFHAS1* and *DAPK1* (Bosgraaf and Van Hastert 2003; Marín 2006). Phylogenetic analyses showed that *LRRK1* is the closest relative of *LRRK2*. This is confirmed by structural similarity, because they both encode a kinase domain of the TKL group that is missing in the other two vertebrate genes. However, LRRK2 proteins have a 650 amino acid-long N-terminal region, formed by repeats, which is absent in LRRK1 proteins (Marín 2006). *LRRK1* and *LRRK2* genes are present in all deuterostomes for which we have complete genomic data, including not only

vertebrates, but also echinoderms. However, protostomes like insects and nematodes have a single *LRRK* gene that is structurally very similar to *LRRK1* (Bosgraaf and Van Hastert 2003; Marín 2006). This led to the hypothesis that *LRRK2* is a deuterostome-specific gene, emerged by duplication after the protostome-deuterostome split (Marín 2006).

The recent release of the first draft of the genome sequence of a cnidarian, the sea anemone *Nematostella vectensis*, has challenged our views of how complex the genomes of archaic animals were. *N. vectensis* has a very elaborate genome, more similar to the human one than to the genomes of protostome model species (Putnam *et al.* 2007). The presence in the anemone of many genes found in vertebrates but absent in *Drosophila* or *Caenorhabditis* suggests that these two model organisms have lost many genes that existed in the ancestor of all animals. This unexpected finding has important implications. It means that *N. vectensis* is a key organism to trace the origin of any human gene. Particularly, we can now test whether a gene present in humans but absent in protostome models (which would be thus in principle considered deuterostome-specific) is, on the contrary, ancient and it has been secondarily lost in protostomes. Moreover, the finding of cnidarian orthologs of human genes may also contribute to the understanding of the functions of our genes by showing what is essential and what may change in them.

With all this in mind, I decided to search for *LRRK* genes in *N. vectensis*. Surprisingly, this organism has the most complex set of *LRRK* genes described so far for any animal, with four paralogs. Phylogenetic and structural analyses show that a *bona fide* *LRRK2* gene is present in cnidarians. Moreover, new evidence indicates that the protostome *LRRK* genes are paralogs of the human *LRRK2* genes. Finally, I show that the comparison of the cnidarian and vertebrate *LRRK2* genes refines our understanding of the structure and function of these genes.

I discuss the important implications that these findings have for research, relevant to Parkinson disease, on *LRRK2*.

## Methods

### *Sequence retrieval and reconstruction of Nematostella LRRK genes*

The protein sequence of the COR domain, which is exclusive of the Roco family, may be used to find all members of this family present in the databases. The protein sequences of the human LRRK1 and LRRK2 COR domains were used to perform TblastN searches against all databases available at the National Center for Biotechnology Information (NCBI). These searches were finished in September 2007. Many novel sequences, not available at the time of my previous study (Marín 2006) were found. Once excluded some primate proteins which were almost identical to the human ones, I generated a database containing 55 COR domain LRRK sequences. Only 26 of them were included in my previous analyses. Among them, four distinct *N. vectensis* sequences with strong similarity to *LRRK1* and *LRRK2* were detected. Similar searches were performed to detect Roco family-specific kinase and Roc GTPase domains. Again, after excluding some very similar primate sequences, I detected 42 complete kinase sequences, instead of the 17 found in my previous work. Three of them derived from the *N. vectensis* genome. For Roc GTPases, I found 52 instead of the 23 detected in my previous work. Again, three of the new sequences came from *N. vectensis*.

I then performed searches with the full-length protein sequences derived from human, sea urchin (*Strongylocentrotus purpuratus*) and *Drosophila melanogaster* LRRK genes to detect all LRRK-related regions in the *N. vectensis* genome. Combining the results of those

searches with automatic gene structure predictions using GenScan (Burge and Karlin 1997), I built models of the four *N. vectensis* *LRRK* genes. Although none of these models can be considered definitive, and two are obviously incomplete (see details below), the structures defined were sufficiently precise as to test for congruence with the results of the phylogenetic analyses described next.

### ***Phylogenetic analyses***

Analyses of their COR or kinase domains generate consistent phylogenetic reconstructions of the relationships among *LRRK* genes, while the shorter Roc domain is less informative (Marín 2006). I first built phylogenetic trees with the 55 animal *LRRK* COR domains detected plus the domains of the proteins encoded by three *Dictyostelium discoideum* Roco genes (*GbpC*, *Pats*, *Roco10*; see Bosgraaf and Van Hastert 2003; Marín 2006), which were used as outgroups. These 58 protein sequences were aligned using ClustalX 1.83 (Thompson *et al.* 1997) and manually corrected using GeneDoc (Nicholas and Nicholas 1997). A few regions which contained gaps in most sequences were eliminated to obtain a final alignment of 257 amino acidic sites. Trees were then built using that alignment, with three different procedures, namely Neighbor joining (NJ), Maximum parsimony (MP) and Maximum likelihood (ML). The NJ tree was obtained using the routine in MEGA 4 (Tamura *et al.* 2007), MP was performed using PAUP\* beta 10 version (Swofford 2003) and ML reconstructions were established using PhyML (Guindon and Gascuel 2003). For NJ, sites with gaps were included and Kimura's correction implemented. Parameters for MP were as follows: 1) all sites included; 2) randomly generated trees used as seeds; 3) maximum number of trees saved equal to 100; and, 4) heuristic search using the Tree Bisection and Reconnection (TBR) algorithm. This method is more exhaustive, and therefore better, than the one that I used in my previous work. It was possible to use it here because the number of sequences was smaller. Finally, for

ML analyses, the BioNJ tree was used to start the iterative searches and the Blosum62 matrix was chosen to model amino acidic transitions. Reliability of the topologies was tested in all cases by bootstrap analyses. 1000 bootstrap replicates were performed for the NJ and MP analyses and 500 for the ML analyses.

Identical analyses were performed with the kinase and Roc domains. The LRRK kinase and Roc domain sequences detected in the searches detailed above plus three *D. discoideum* outgroups were aligned and dendrograms built following the same methods just described for the COR domains. In this case, the final length of the sequences aligned was 198 amino acidic sites for the kinases and 111 for the Roc GTPases. Dendrograms showing results for COR, kinase and Roc domains were drawn using the tree editor of MEGA 4.

### ***Structural analyses***

Domains in LRRK proteins were characterized using InterProScan (Zbodnov and Apweiler 2001), which includes in a single search comparisons with the patterns of several structure databases, such as SMART, Pfam, etc. Repeats in the N-terminal region of *N. vectensis* LRRK2 protein were determined by combining two methods: 1) aligning that region with the sequences of other nine LRRK2 proteins (from vertebrates and echinoderms), using ClustalX 1.83; and, 2) automatic detection of repeats with HHrep (Söding *et al.* 2006).

## **Results**

As already indicated, four *Nematostella vectensis* COR domain sequences which had very strong similarity to those encoded by *LRRK* genes were detected in the databases (expect

values ranged from  $1 \cdot 10^{-16}$  to  $8 \cdot 10^{-41}$ ). I built models for the four corresponding proteins using comparisons of the *LRRK* genes in vertebrates and invertebrates and the genomic *Nematostella* sequences plus *ab initio* analyses of the gene structures using GeneScan. Two of these models may correspond to full-length proteins. The third, which corresponds to the *LRRK2* gene of *N. vectensis* (see next paragraph) lacks a small part of the Roc domain, unavailable so far in the databases, but seems otherwise complete. The fourth, which I have called *Nv LRRK3* for reasons that will soon become clear, lacks its C-terminal end, including part of the kinase domain characteristic of LRRK proteins, and may also lack its N-terminal end. The structures deduced for these four models can be compared with representative human, *S. purpuratus* and *D. melanogaster* LRRK proteins in Figure 1.

One of the proteins found in *N. vectensis* showed a very high sequence similarity to LRRK2 proteins in TblastN analyses. Structural analyses demonstrated that it also has the characteristic domains that I described for deuterostome LRRK2 proteins (Marín 2006). Particularly, it has a set of 11 N-terminal repeats (schematized in Figure 1) that are very similar to those found in other LRRK2 proteins (Figure 2). Phylogenetic analyses based on COR domain sequences and kinase sequences, shown in Figures 3 and 4, confirmed the close relationship of the LRRK2-like protein in *N. vectensis* with deuterostome LRRK2 proteins. They appear together in a monophyletic group which is supported by the three methods of phylogenetic reconstruction in the independent analyses using both domains. Thus, I consider the gene encoding that protein as the *bona fide* ortholog of *LRRK2* in *Nematostella vectensis*, hence the name *Nv LRRK2* used from now on to name it. The analyses of the shorter Roc domain sequences did not provide any additional information (no significant bootstrap support was found for the critical internal branches of the tree; see Supplementary Figure 1), and were not further considered.



There is some controversy in the literature regarding the repeats found in the N terminus of LRRK2. I indicated in my previous study that they are gene-specific. I used the patterns deduced from the human and *S. purpuratus* LRRK2 proteins to search for similarities in other proteins, but did not detect any significant match. On the contrary, Mata *et al.* (2006) suggested, without any indication of how they obtained the evidence, that the N-terminal region of human LRRK2 contains a set of armadillo repeats. I decided to check for this possibility using the set of 10 sequences shown in Figure 2. InterProScan analyses of those sequences failed to detect any armadillo repeat in 9 of them. The exception was the *S. purpuratus* sequence, in which InterProScan detected, albeit with a low score, two armadillo repeats in positions 99-141 and 506-549. This approximately corresponds to repeats number 3 and 12 in the *S. purpuratus* protein. Thus, only 2 out of the 135 repeats shown in Figure 2 are detected in the structural analyses performed against multiple databases by the InterProScan tool as canonical armadillo repeats. These results refute the hypothesis of the LRRK2-specific repeats being armadillo repeats. The exceptional detection of those repeats in automatic analyses is explained by the fact that both the LRRK2-specific repeats and the armadillo repeats contain multiple hydrophobic residues in somewhat similar positions. However, armadillo repeats are just one among several different types of repeats which are able to generate long alpha helical structures known as “Armadillo-like helical domains” (InterPro domain no. IPR011989; Groves and Barford 1999). Most significantly, InterProScan detected these domains in 8 of the 10 sequences shown in Figure 2. The exceptions were the two fish sequences, which I reconstructed from genomic data and may be incomplete (see gaps in Figure 2). Thus, we may conclude that LRRK2 proteins contain a peculiar Armadillo-like helical domain, absent in the rest of LRRK proteins. This kind of structure is considered to be a protein-protein interaction surface (Groves and Barford 1999).

The structures predicted for two other *N. vectensis* proteins were very similar to those characteristic of LRRK1 proteins in deuterostomes or LRRK proteins in protostomes. Going from the N terminus to the C-terminal region, they have ankyrin repeats, leucine-rich repeats and the typical Roc, COR and kinase domains (Figure 1; *Nv LRRK1*, *Nv LRRK4*). The fourth gene was, as already indicated, only partially characterized (Figure 1; *Nv LRRK3*). The reconstructed region is uninformative with respect to its possible relationships to other *LRRK* genes. Phylogenetic analyses place two of these genes in positions that strongly suggest that they are either the ortholog of deuterostome *LRRK1* genes (hence the name *Nv LRRK1*) or the ortholog of protostome *LRRK* genes (*Nv LRRK3*). The fourth gene (*Nv LRRK4*) appears in an intermediate position between *LRRK1* and *LRRK2* (Figures 3, 4). Apart from these cnidarian genes, the rest of new LRRK sequences found in my searches and detailed in Figures 3 and 4 fit very well with the picture of *LRRK* gene evolution described in Marín (2006). All new genes in vertebrates, from mammals, reptiles and fishes, were obvious orthologs of either *LRRK1* or *LRRK2* and additional genes in insect and nematode species were also very similar to the protostome *LRRK* genes that I described before.

The trees in Figures 3 and 4 can be interpreted as a whole considering the phylogenetic relationships among species. The simplest hypothesis to explain the topology of those trees is that there were at least three genes prior to the cnidarian-bilaterian split. These three genes gave rise to the branches annotated as LRRK1, LRRK2 and LRRK3 in Figures 3 and 4. Thus, two of these genes correspond to the *LRRK1* and *LRRK2* genes in vertebrates, which are found also in echinoderms and cnidarians. The third gene would correspond to the one that was hitherto defined only for protostomes and called *LRRK* (Marín 2006). The results shown in Figure 3, however, strongly suggest that a gene, which I think is appropriate to call *LRRK3*, existed in the

ancestor of all eumetazoans. It has been conserved in cnidarians, echinoderms and protostomes, but lost in vertebrates. Figure 4 confirms that a monophyletic branch, including protostome and *S. purpuratus* *LRRK* genes, exists. In this case, however, the current lack of an alignable *N. vectensis* *LRRK3* kinase domain precludes further confirmation that this gene is older than the protostome/deuterostome split. Notice that the only small incongruence in the *LRRK3* branch is the atypical position of the *Caenorhabditis* proteins. However, it is well known that *Caenorhabditis* genes evolve rapidly (Mushegian *et al.* 1998; Stein *et al.* 2003), so this result can be simply explained by acceleration in the rate of evolution of *LRRK3* genes in nematodes. Finally, the *Nv LRRK4* gene, which is the only one found outside the three main branches which define the *LRRK1-LRRK3* genes (Figures 3, 4), may be a *Nematostella*-specific paralog.

These results mean that protostomes and vertebrates do not contain orthologous *LRRK* genes. Protostomes have kept *LRRK3*, at the same time losing the *LRRK1* and *LRRK2* genes, while vertebrates have kept *LRRK1* and *LRRK2* but lost *LRRK3*. Among the groups for which we have data, the three genes are present only in cnidarians and echinoderms. Therefore, these results demonstrate that *LRRK2* is an ancient gene, emerging in ancestral animals prior to the cnidarian-bilaterian split. They also indicate that no true ortholog of *LRRK2* exists in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*.

## Discussion

Tracing the origin of a human gene may greatly contribute to our understanding of its functions. In a series of works, my group has described the origin and evolution of several Parkinson disease genes such as *parkin*, *DJ-1* and *LRRK2* (Marín and Ferrús, 2002; Marín *et al.*

2004; Lucas *et al.* 2006; Marín 2006; Lucas and Marín 2007). These studies have contributed to delineate the best simple model organisms in which to study the functions of the human genes. In the case of *LRRK2*, I warned against the potential problems of using *Drosophila melanogaster* or *Caenorhabditis elegans* as models, given that it was unclear that those species had true *LRRK2* orthologs (Marín 2006). The only *LRRK* gene present in protostomes encodes for a protein that not only is very dissimilar in sequence but also is structurally different from *LRRK2*. Thus, I suggested concentrating on deuterostome species as models (Marín 2006). Recent papers, however, described apparently promising results using protostome models. Sakaguchi-Nakashima *et al.* (2007) showed that mutations in the *C. elegans LRK-1* gene, the only *LRRK* gene present in this species, generate anomalies in the localization of synaptic vesicle proteins. This result, which involves *LRRK* gene products in the regulation of vesicular transport, agrees with functional data for human *LRRK2*, which implicates it in synaptic vesicle recycling and neurite outgrowth (MacLeod *et al.* 2006; Hatano *et al.* 2007). Even more striking were the results obtained by Lee *et al.* (2007), showing that loss of function of the *LRRK* gene of *D. melanogaster* leads to the death of dopaminergic neurons in the fly brain and anomalies in locomotor activity.

The results shown here further support the idea that *LRRK* genes in *Drosophila* and *Caenorhabditis* and *LRRK2* genes in humans are paralogous. Therefore, these recent results should be reevaluated, taken this fact into account. In my opinion, all the results in invertebrates and the results obtained for *LRRK1* (Korr *et al.* 2006; Greggio *et al.* 2007; Taylor *et al.* 2007) and *LRRK2* (summarized in Marín 2006; Thomas and Beal 2007) in vertebrates are compatible with all the *LRRK* gene products in animals being involved in neuronal functions. This is consistent with their presence only in animals with a developed nervous system, from cnidarians to vertebrates. However, that these neuronal-specific functions are the same for all

*LRRK* genes in different animal species is not supported by the available data. For example, the *Drosophila* results indicated above -- which are so far the only indication that *LRRK* genes in protostomes might be useful models to understand why *LRRK2* mutations are involved in Parkinson disease -- are in obvious contradiction with the evidence obtained in our species. In the fly, overexpression of the *LRRK* gene does not lead to any obvious phenotype, whereas loss of function causes dopaminergic cell death, among other problems (Lee *et al.* 2007). In humans however, it is unknown if loss-of-function mutations in *LRRK2* lead to dopaminergic cell death. On the contrary, it seems that it is increased/constitutive activity of the *LRRK2* protein (probably through increased kinase function) what leads to the loss of dopaminergic cells in patients with PD (West *et al.* 2005; Gloeckner *et al.* 2006; Smith *et al.* 2006; Guo *et al.* 2007; Li *et al.* 2007). In summary, researchers in this field should be very careful before extrapolating results obtained for protostome *LRRK* genes to humans.

I suggested in my previous study that *LRRK2* originated recently, by a duplication which occurred after the protostome-deuterostome split. This hypothesis has been refuted by the new findings in *Nematostella*. I now favor a more complex explanation: first, two duplications led to the presence of three genes before the protostome-deuterostome split; second, after that split two of those genes were lost in protostomes and the third one was lost in deuterostomes. This was obviously impossible to foresee, not knowing the cnidarian data. It is a good example of the radical change in our interpretations that genomic analyses of basal organisms may provide. As I already commented in the introduction, so far the scientific community has been working under the impression that we should expect a progressive increase in genome complexity when we get closer to humans, perhaps with some anomalies here and there caused by particular features in the ways of life of some species, by species-specific genome duplications, etc. Thus, a typical reasoning was that, whenever single genes

are found in invertebrates and multiple genes are detected in vertebrates, the most parsimonious explanation was the occurrence of vertebrate-specific duplications. However, the unexpected complexity of the genome of this simple anemone may lead to a paradigmatic change, in which *Drosophila* or *Caenorhabditis* are viewed as possessing genomes in which many of the genes of their animal ancestors were lost. This may lead to a reevaluation of the evolution of many gene families in the light of cnidarian data, as I have done in this study.

The characterization of orthologous genes in very distant animals may also significantly contribute to our understanding of the biochemical potential of their products. Here, by comparing the N-terminal region of the *LRRK2* orthologs, I have shown that they contain an Arm-like surface. Mutations in this surface have been involved in familial PD (Nichols *et al.* 2007). Given that this is considered to be a protein-protein interaction surface, as well as its absence in *LRRK1* proteins, it is a good candidate to perform experiments aimed at identifying cellular partners specific to *LRRK2*.

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## Figure legends

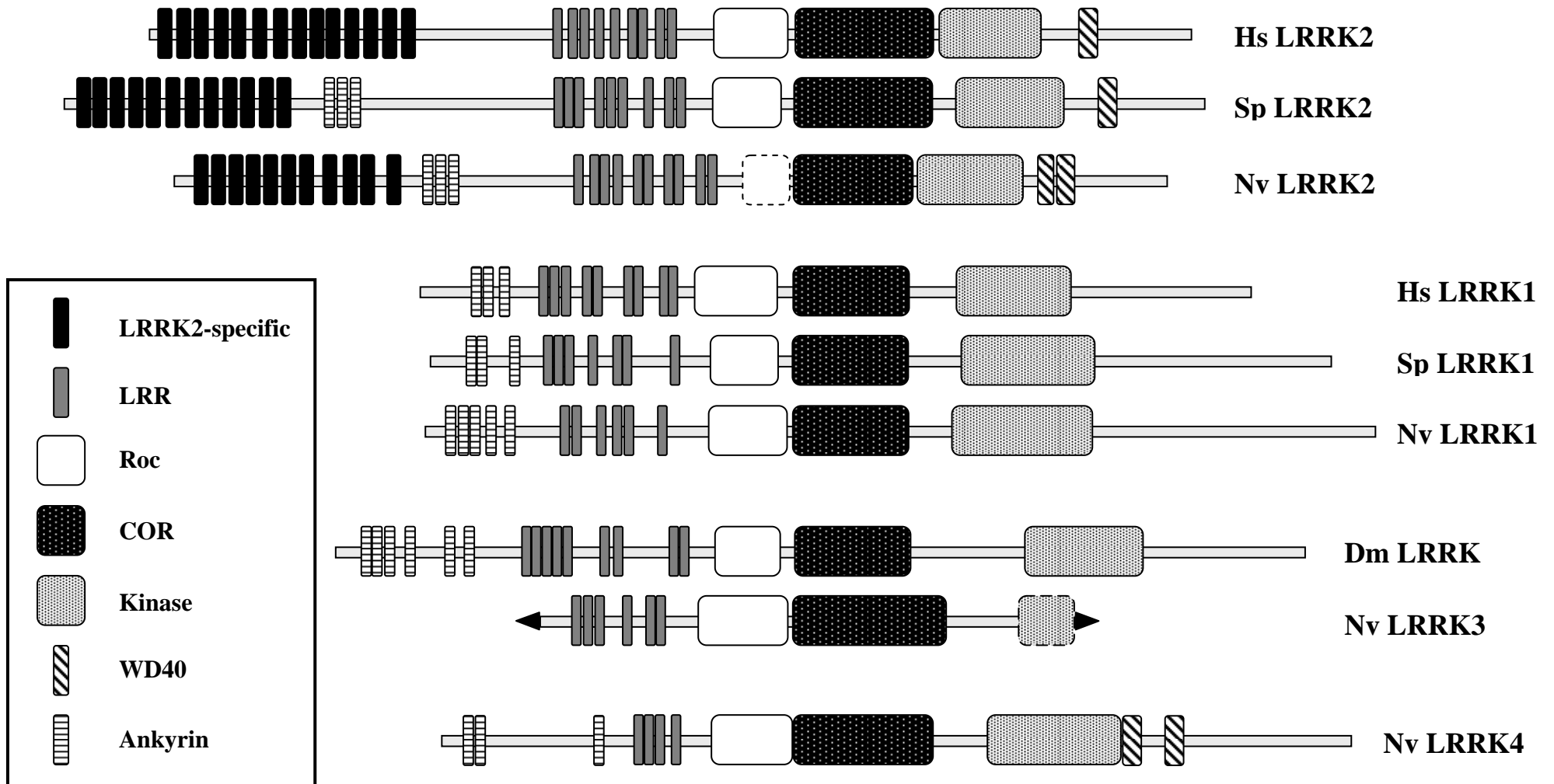
**Figure 1.** Structures of selected LRRK proteins. The three main domains (Roc, COR, kinase) plus the repeats characteristic of these proteins (LRRK2-specific, Leucine-rich repeats [LRR], WD40, Ankyrin) are indicated. Species abbreviations are as follows: Hs: *Homo sapiens*; Sp: *Strongylocentrotus purpuratus*; Nv: *Nematostella vectensis*; Dm: *Drosophila melanogaster*. Broken lines refer to domains not fully characterized due to lack of data. The arrows in Nv LRRK3 indicate that the full-length protein cannot be reconstructed with the available information and both ends of the protein are missing.

**Figure 2.** N-terminal repeats specific to LRRK2 proteins in five mammals (*Homo sapiens*, *Mus musculus*, *Canis familiaris*, *Bos taurus*, *Monodelphis domestica*), a bird (*Gallus gallus*), two fishes (*Danio rerio*, *Tetraodon nigroviridis*), an echinoderm (*Strongylocentrotus purpuratus*) and a cnidarian (*Nematostella vectensis*). The location of the fourteen repeats detected in humans (Marín 2006) are indicated. *N. vectensis* LRRK2 lacks repeats 11-13.

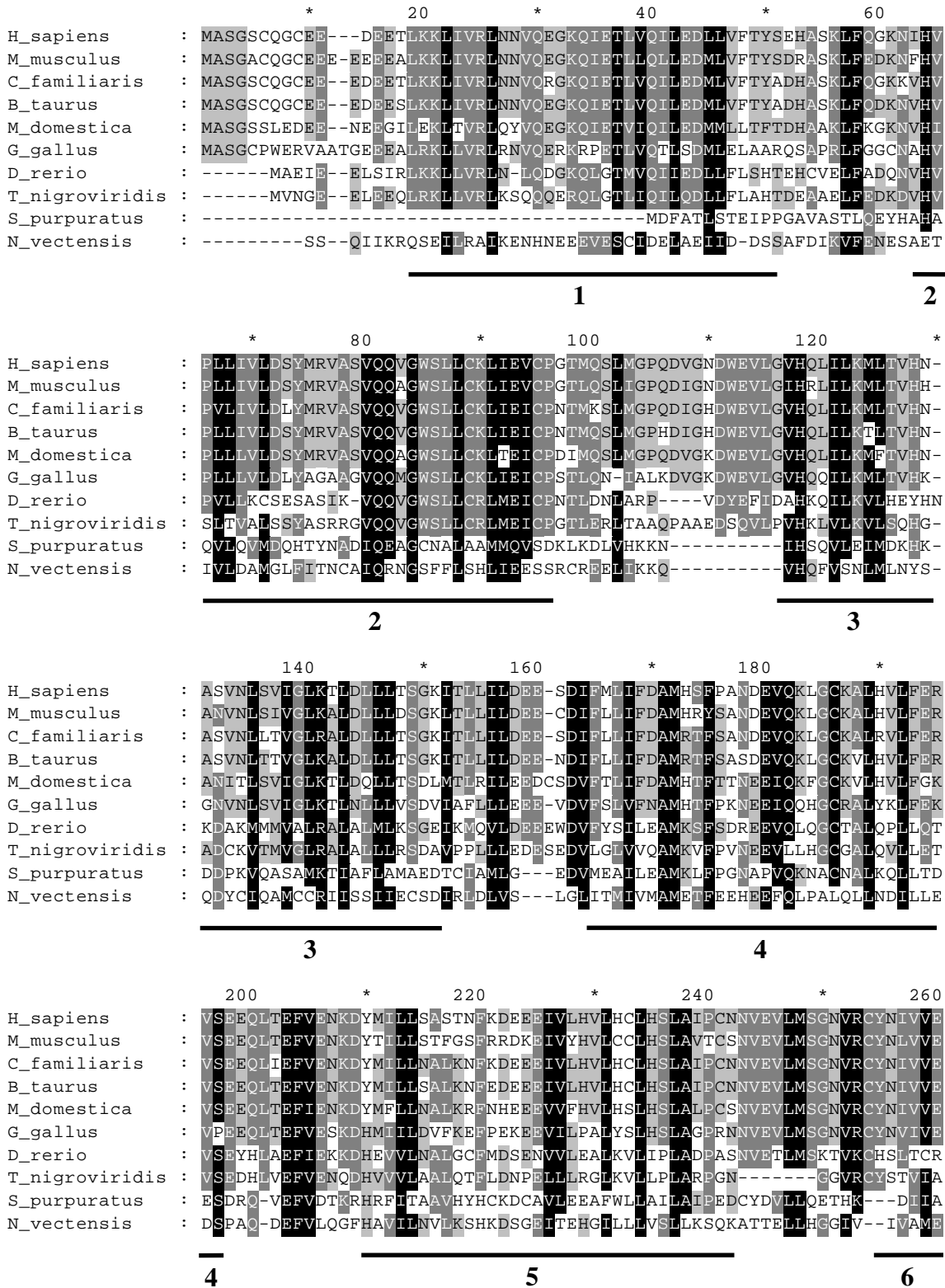
**Figure 3.** Dendrogram obtained when COR domain sequences are analyzed. The topology shown was the one deduced using NJ. Those generated with MP and ML are, however, so similar to that one that all results can be shown in a single tree. Numbers refer to percentage of bootstrap replicates that support the corresponding branches. The three numbers correspond, respectively, to NJ/MP/ML analyses (see Methods for the details). Only branches supported by the three methods and in which bootstrap support was higher than 50% in at least two of them are shown. Significant values for some outer branches, which group vertebrates or insects, have been omitted for simplicity.

**Figure 4.** Dendrogram obtained analyzing kinase domain sequences. Conventions are as in Figure 3. Again, some significant results have been omitted for simplicity.

Figure 1. Marín

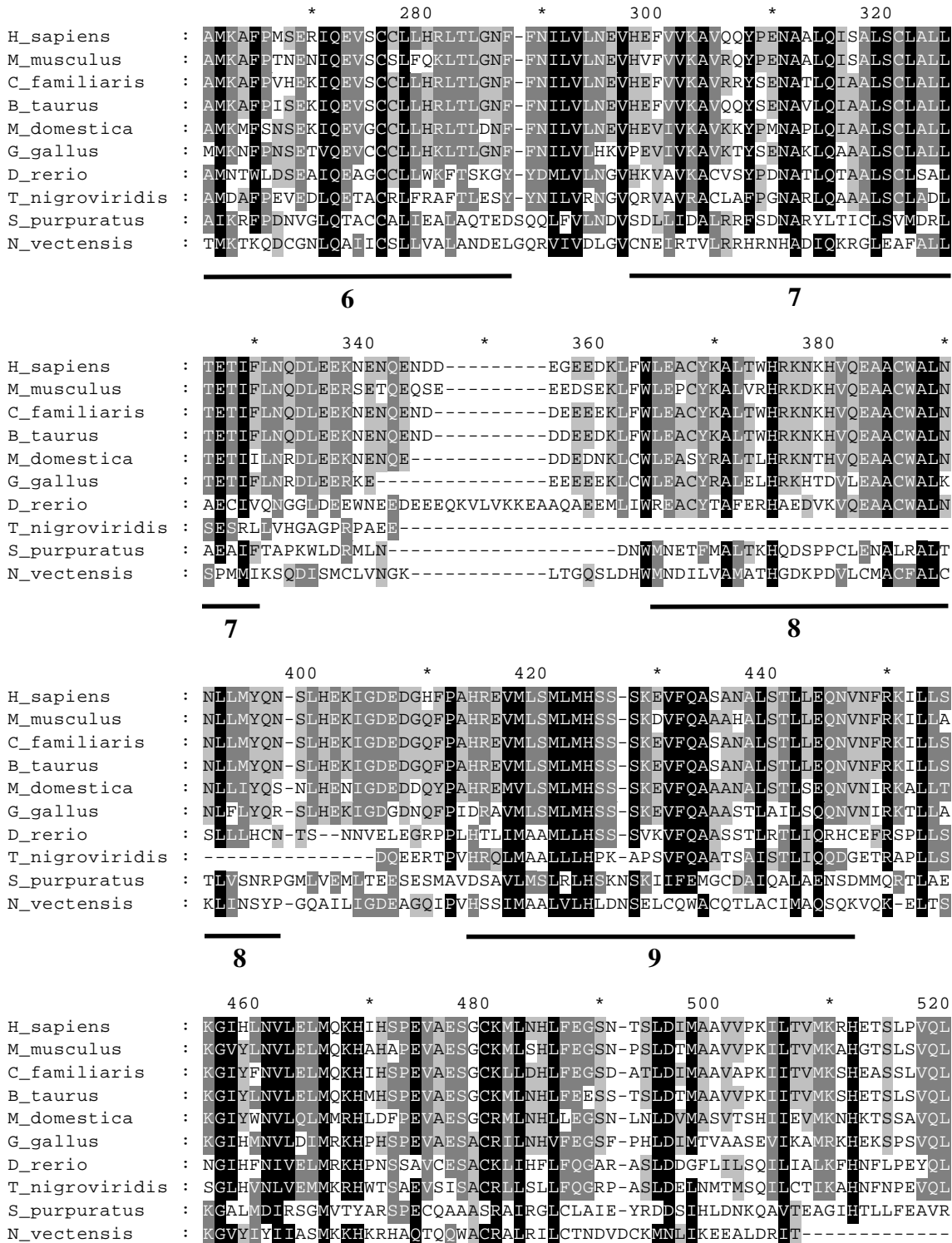


**Figure 2. Marín**





**Figure 2. Marín (continued)**



**Figure 2. Marín (continued)**

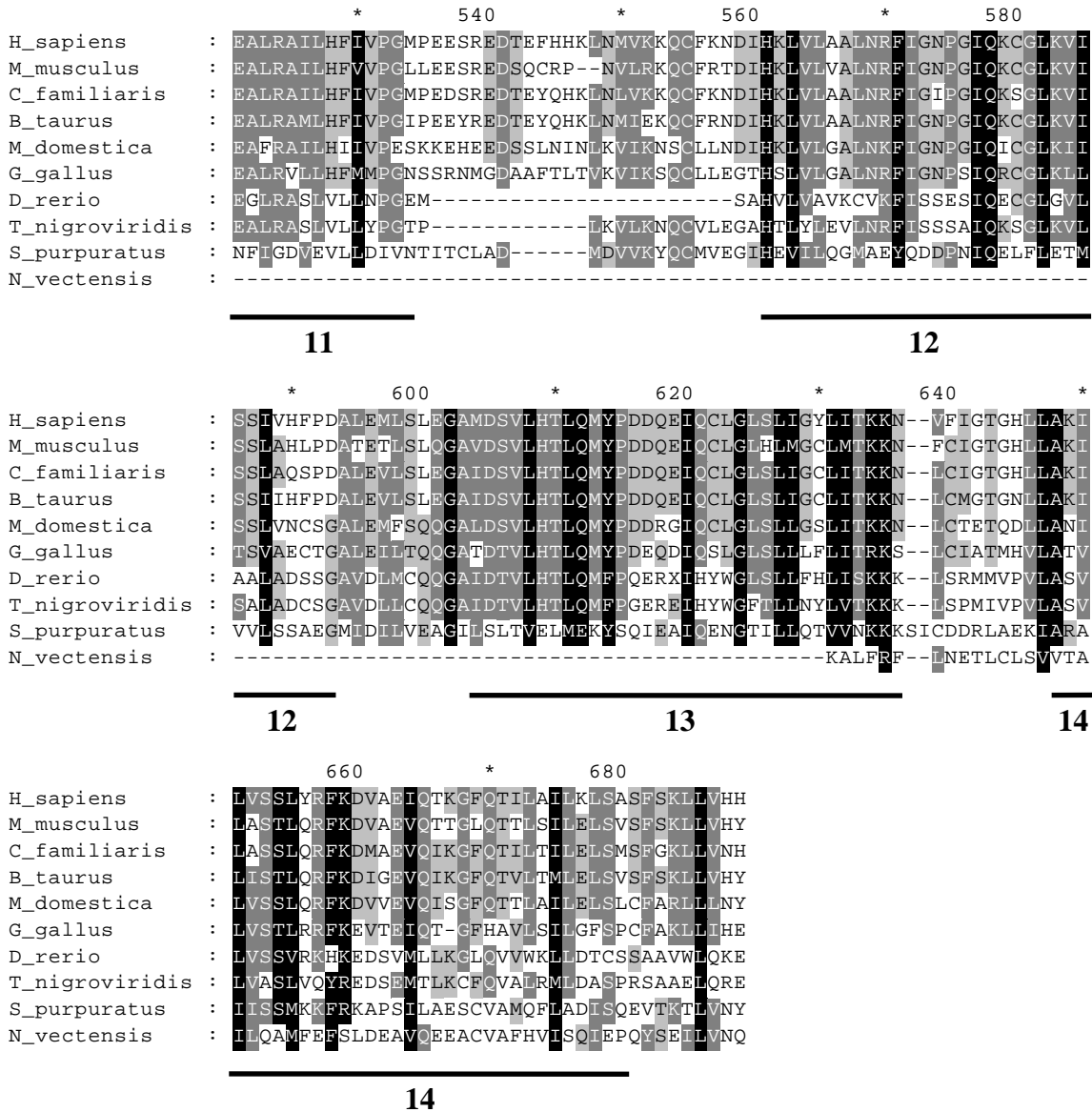
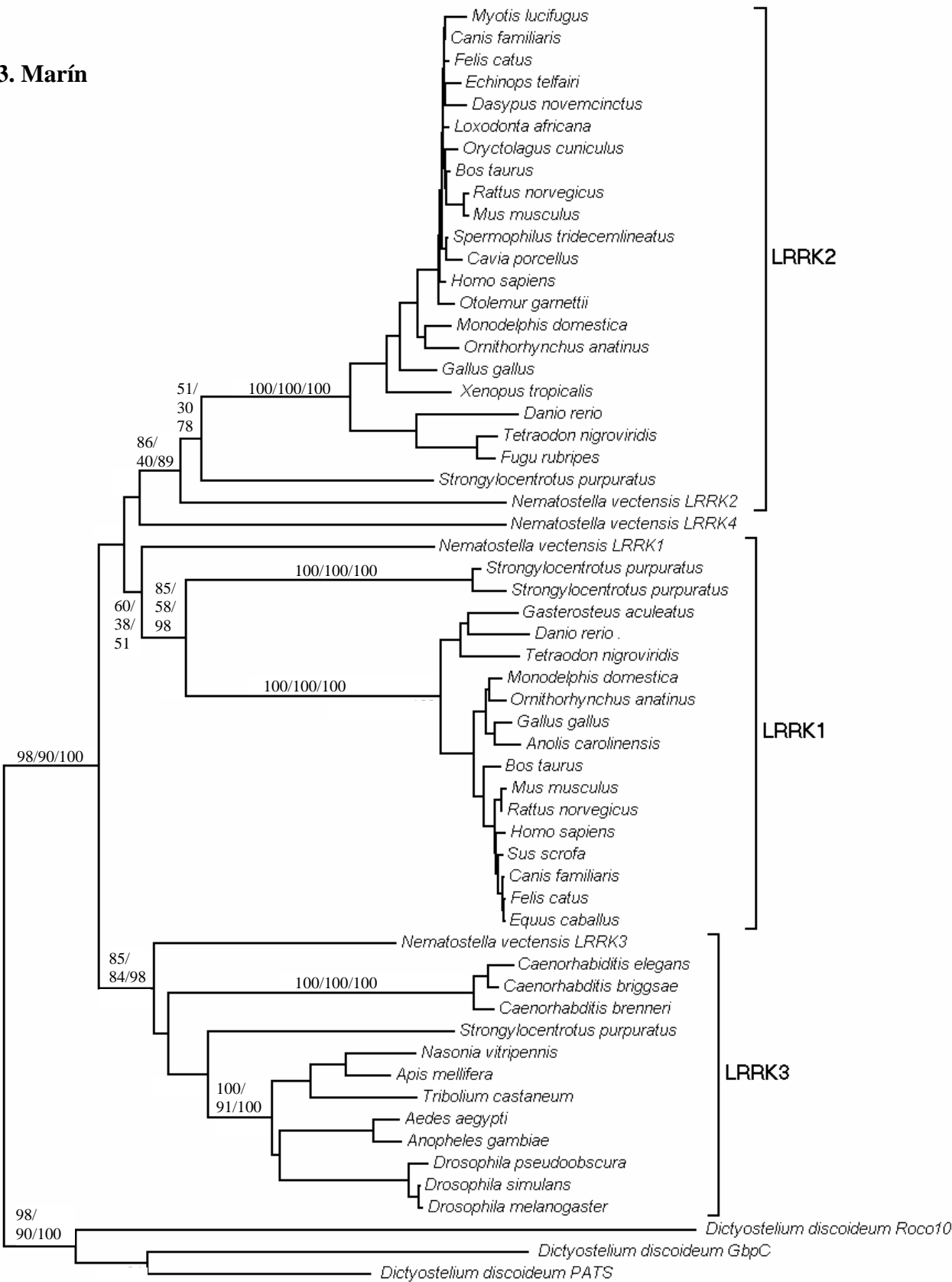


Figure 3. Marín



**Figure 4. Marín**

