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REVIEW

The Evolution of Reproduction-Related *NLRP* Genes

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Abstract NLRP proteins are important components of inflammasomes with a major role in innate immunity. A subset of *NLRP* genes, with unknown functions, are expressed in oocytes and early embryos. Mutations of *Nlrp5* in mice are associated with maternal-effect embryonic lethality and mutations of *NLRP7* in women are associated with conception of biparental complete hydatidiform moles (biCHMs), suggesting perturbed processes of genomic imprinting. Recessive mutations on *NLRP2/7* in humans are associated with reproductive disorders and appear to be induced by a demethylation of the maternal pronucleus. In this study, we find that radiation of *NLRP* genes occurred before the common ancestor of Afrotheria and Boreoeutheria, with the clade of oocyte-expressed genes originating before the divergence of marsupial and eutherian mammals. There have been multiple independent duplications of *NLRP2* genes one of which produced the *NLRP7* gene associated with biCHMs.

Keywords Mammals · Phylogeny · Genetics · Molecular evolution · Reproductive disorders

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Introduction

NLRP proteins (Nod-like receptors with a pyrin domain) have attracted recent attention because of their role in innate immunity and inflammation (Kufer and Sansonetti 2011; Strowig et al. 2012). A subset of *NLRP* genes are expressed in mammalian oocytes (Hamatami et al. 2004; Ponsuksili et al. 2006; Zhang et al. 2008) and maternal deficiency of some of these "reproduction-related" *NLRPs* (*rNLRPs*) have been shown to cause embryonic lethality in mice (Hamatami et al. 2004; Tong et al. 2000; Peng et al. 2012) and perturbations of genomic imprinting in human oocytes (Murdoch et al. 2006; Kou et al. 2008; Parry et al. 2011).

Tian et al. (2009) undertook a phylogenetic analysis of the *NLRP* genes from humans, chimpanzees, rats, mice, cattle, and dogs. These species had similar sets of *NLRP* genes, with the *rNLRP* genes forming a monophyletic group. Therefore, the major radiation of *NLRP* genes was already present in the most recent common ancestor of Boreoeutheria. These authors observed that *rNLRPs* were more evolutionarily labile, in both copy number and sequence, than other *NLRPs*.

Evidence that *rNLRPs* might play a role in the establishment or maintenance of genomic imprinting motivated us to re-investigate the evolutionary history and possible functions of *rNLRPs*. We extend the analysis of Tian et al. (2009) using *NLRP* sequences from an afrotherian (African elephant *Loxodonta africana*), marsupial (gray short-tailed opossum *Monodelphis domestica*), and monotreme (platypus *Ornithorhynchus anatinus*). These sequences allow inferences about the *NLRP* repertoire of the last common ancestor of eutherian mammals, the last common ancestor of marsupials and eutherians, and the last common ancestor of all extant mammals. Moreover, advances in knowledge

of the effects of *rNLRPs* allows us to speculate about the role of these genes in genomic imprinting and reproductive disorders of mice and humans.

Results

Mammalian *NLRP* Repertoires

NLRP genes occur at eight locations in the human genome: 11p15.5 (*NLRP6*); 11p15.4 (*NLRP10*); 11p15.4 (*NLRP14*, 1 Mb from *NLRP10*); 19q13.42 (*NLRP12*); 19q13.42 (*NLRP2*, *NLRP7*); 19q13.43 (*NLRP4*, *5, 8, 9, 11, 13*); 1q44 (*NLRP3*); and 17p13.2 (*NLRP1*). *NLRP* genes were found in the elephant genome at seven of these locations (based on conserved flanking markers). The exception was elephant *NLRP6* which occurs on an unassembled fragment without flanking markers. The elephant genome contains an additional *NLRP* gene between *MID2* and *VSIG1* that we have provisionally named *NLRPX* (currently annotated as *NLRP12-like* in elephant, macaque, and marmoset). An *NLRP* pseudogene is located between these markers on the human X chromosome (*NLRP3P*). *NLRPX*-related sequences are sister to a clade containing *NLRP10* genes in our phylogenetic tree.

Four *NLRP* genes are currently annotated in the opossum genome. *NLRP10* is located on opossum chromosome 4 between *GVIN1* (corresponding to the *GVINP1* pseudogene at human 11p15.4) and *CSMD2* (human ortholog at 1p34). Two other opossum *NLRPs* also map to chromosome 4: one appears orthologous to *NLRP12* whereas the other (provisionally named *NLRPa*, currently annotated as *NLRP12*) appears in our phylogeny as sister to the *rNLRP* clade albeit with weak support. The fourth gene (provisionally named *NLRP5-like*, currently annotated as *NLRP14-like*) occurs next to *EPN1* on an unassembled fragment. Human *EPN1* neighbors the cluster of six *rNLRPs* at 19q13.43.

Five *NLRP* genes were found in the platypus genome. Four appear to be orthologs of human *NLRP3* (currently annotated as *NLRP12-like*), *NLRP6*, *NLRP10* and *NLRP12*. The fifth (provisionally named ψ *NLRP10*, currently annotated as *NLRP3-like*) occurs on the same fragment as *NLRP10* and is possibly a monotreme-specific duplicate of that gene. None of the platypus genes group with the *rNLRPs*.

Figure 2 summarizes the *NLRP* repertoires of the mammalian species used in this study. We represent the data as a table of species against *NLRP* genes with phylogenies depicted as a reference for both. The cells of the table are shaded according to the presence or absence of a given *NLRP* gene in a particular species.

Phylogenetic Tree Root

Our tree places *NLRP6* as sister to the other eutherian *NLRP* genes using chicken "*NLRP3*" to root the tree, similar to the analysis of Tian et al. (2009) who used the same root. A sister relation between *NLRP6* and other mammalian *NLRPs* has been found repeatedly in phylogenetic analyses. These include analyses of NACHT (Hughes 2006; Laing et al. 2008) and LRR domains (Ng et al. 2011) that rooted the *NLRP* clade using non-NLRP proteins. A slightly different root was proposed in a phylogenetic analysis of PYD domains that placed the root between a clade containing *NLRP6* and *NLRP10* and the other *NLRP* genes (Kersse et al. 2011).

Diversification of *NLRP2* and *NLRP7*

Figure 3 presents a Bayesian phylogenetic tree for current genes and pseudogenes labeled *NLRP2* or *NLRP7* in the databases as of May, 2012. The primate-specific duplication noted by Tian et al. (2009) is confirmed but independent duplications have also occurred in pigs, cattle, horses, and elephants. We suggest, as an interim measure, that use of *NLRP7* be restricted to genes of that name in the primate clade.

Syntenic Analyses

Online Resource 3 shows the probable orthologies of all *NLRP* genes from humans against the other species of mammals, as well as chicken. These figures use the latest genome assemblies (as of May, 2012), which is why they differ slightly from the data-set used for the phylogenetic tree in Fig. 1.

Discussion

Evolutionary History of *NLRP* Genes

The similarity of the *NLRP* repertoires of humans and elephants shows that the major radiation of *NLRP* genes, including *rNLRP* genes, had already occurred in the common ancestor of Afrotheria and Boreoeutheria.

Distal chromosome 4 of opossum contains genes with orthologs on human chromosomes 11p and 19q. Thus, the data are compatible with a scenario in which the opossum genome maintains ancestral linkage of *NLRP* genes that have been dispersed onto the equivalents of human 11p and 19q in eutherian mammals. Unfortunately, the evolutionary inference is weak because opossum, platypus, and chicken genomes are only partially assembled for these regions.

The existence of *NLRPa* and *NLRP5-like* in opossum suggests that *rNLRP* genes evolved before the last common ancestor of Metatherian and Eutherian mammals. Our phylogenetic analysis (Fig. 1) confirms that opossum *NLRP5-like* belongs to the *rNLRP* clade. Whether it is expressed in opossum oocytes is unknown. Conservation of synteny suggests *NLRP5-like* is probably located on opossum chromosome 4 with the other *NLRP* genes. Four out of four opossum *NLRP* genes plausibly map to a region of chromosome 4 with orthologs on human chromosomes 11p and 19q (where 12 out of 14 human *NLRP* genes are located).

Platypus *NLRP3* is located adjacent to *RPS5* and *SLC27A5*, genes whose orthologs are located near the telomere of human 19q (close to the major cluster of *rNLRPs*), whereas human *NLRP3* is located near the telomere of chromosome 1q as part of a small, recent addition to an otherwise ancient linkage group (Haig 2005). Therefore, *NLRP3* may have been linked to *rNLRP* genes in the most recent common ancestor of monotremes and therian mammals. This leaves *NLRP1* at human 17p13.2 as the only eutherian *NLRP* whose ortholog cannot be provisionally assigned to this ancestral linkage group.

However, a scenario that is consistent with our data is that *rNLRPs* were absent in the common ancestor of Monotremes and Metatherians. Then, a single *rNLRP* gene evolved in a common ancestor of Metatherians and Eutherians. This ancestral *rNLRP* is orthologous to possum *NLRPa* and it later diversified to give rise to the clusters *NLRP2/7* and *NLRP9/11/4/13/8/5*, as well as *NLRP14*. *NLRP2/7* may be still present in possum, but the current assembly does not contain them or their flanking markers (see Online Resource 3).

Lineage-Specific Duplications and Deletions

Our analysis provides evidence of lineage-specific duplications and deletions of *NLRP* genes in eutherian mammals. *NLRP11* was previously proposed to be restricted to primates and *NLRP4* to be restricted to Euarchontoglires (Tian et al. 2009) but we find orthologs of *NLRP11* in pig and of *NLRP4* in pig and horse (see Fig. 2 and Online Resource 4). Pig *NLRP11* represents an interesting example, because were it absent, parsimony would indicate a single loss of *NLRP11* in Laurasiatheria. Its presence indicates independent losses in mouse, dog, horse, and cattle.

The data also suggests an independent loss of *NLRP8* and *NLRP13* in marmoset, mouse, and elephant. In particular, the marmoset genome has *NLRP11* and *NLRP4* alone in an unplaced fragment, while conservation of synteny suggests these genes should be placed between *NLRP9* and *NLRP5*. A future assembly, however, might reconstruct this area and place the fragment inside it, potentially also reconstructing *NLRP8* and *NLRP13*.

A second phylogenetic reconstruction without any of the reconstructed pseudogenes is available in Online Resource 5. There are three minor differences between the phylogeny in Fig. 1 (which includes pseudogenes) and Online Resource 5. First, the reversal of the divergence of the *NLRP8-13* and *NLRP5-14* gene pairs. Second, the *NLRP10-X* gene pair changing from being a monophyletic group with *NLRP3* and *NLRP12*, to being its outgroup. Third and finally, possum *NLRP5-like* clustering with *NLRP8* instead of *NLRP5*.

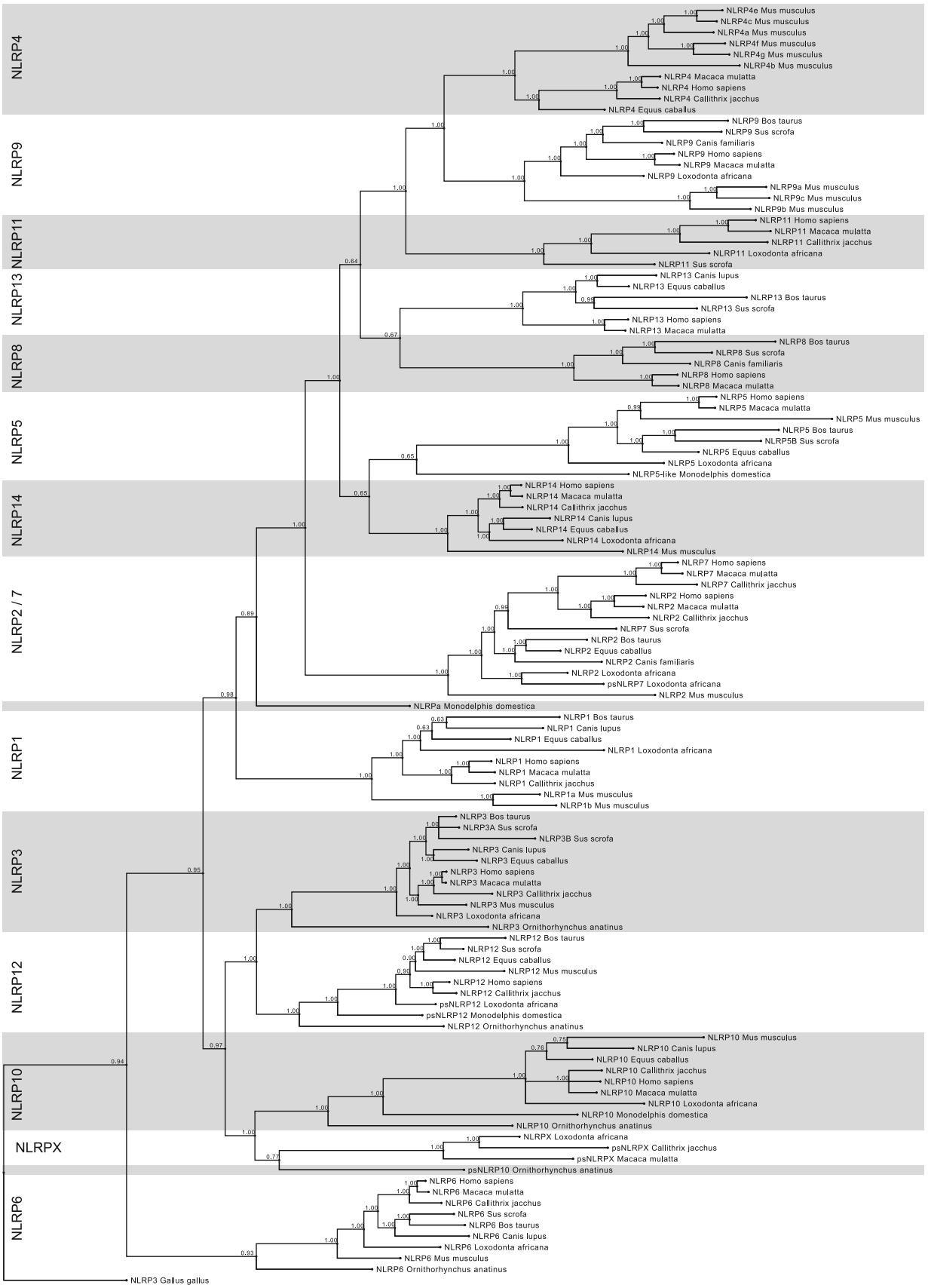
Maternal-Effect Lethality in Mice

Inactivation of *Nlrp5* (also known as *Mater*) in mouse mothers causes arrested development of embryos at the two-cell stage whether or not an embryo inherits a functional copy of *Nlrp5* from its father (Tong et al. 2000). Nlrp5 protein is associated with the cytoplasmic lattice of mouse oocytes and appears to be essential for the formation and/or stability of the lattice (Kim et al. 2010). Mitochondria of *Nlrp5*-deficient oocytes are scattered throughout the cytoplasm, rather than concentrated in the subcortical layer (Fernandes et al. 2012). Knockdown of *Nlrp2* or *Nlrp14* mRNA in mouse oocytes similarly causes arrested development of embryos during early cleavage (Hamatami et al. 2004; Peng et al. 2012).

Nlrp5 protein associates with Ecat1/Filia protein in mouse oocytes and early embryos (Ohsugi et al. 2008; Zheng and Dean 2009). Mutations in *Filia* cause maternal-effect embryonic lethality with apparent defects in the assembly of mitotic spindles (Zheng and Dean 2009). *Filia* belongs to a family of genes expressed in oocytes and early embryos (Pierre et al. 2007) that includes *C6orf221* (see below).

Perturbations of Genomic Imprinting in Humans

During normal embryonic development, the maternal allele is methylated and paternal allele unmethylated at most imprinting control regions (ICRs). The *H19* ICR is an exception to this generalization, with an unmethylated maternal allele and methylated paternal allele (Reik and Walter 2001; Schulz et al. 2010). DNA in the sperm pronucleus of recently fertilized mammalian oocytes undergoes conversion of most 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) whereas 5mC is protected from this activity in the egg pronucleus (Iqbal et al. 2011; Wossidlo et al. 2011). Paternally derived chromosomes then progressively lose 5hmC during early embryonic development (Inoue and Zhang 2011). Taken together these results suggest that most ICRs are methylated in the


NLRP4
NLRP4e Mus musculus
NLRP4c Mus musculus
NLRP4a Mus musculus
NLRP4f Mus musculus
NLRP4g Mus musculus
NLRP4b Mus musculus
NLRP4 Macaca mulatta
NLRP4 Homo sapiens
NLRP4 Callithrix jacchus
NLRP4 Equus caballus
NLRP9
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NLRP9 Sus scrofa
NLRP9 Canis familiaris
NLRP9 Homo sapiens
NLRP9 Macaca mulatta
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NLRP5B Sus scrofa
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NLRP5 Loxodonta africana
NLRP5-like Monodelphis domestica
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NLRP14 Macaca mulatta
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NLRP7 Homo sapiens
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NLRP6 Callithrix jacchus
NLRP6 Sus scrofa
NLRP6 Bos taurus
NLRP6 Canis lupus
NLRP6 Loxodonta africana
NLRP6 Mus musculus
NLRP6 Ornithorhynchus anatinus
NLRP3 Gallus gallus

◀**Fig. 1** Consensus phylogenetic tree of the *NLRP* gene family. The phylogenetic tree was inferred using MrBayes for 10,000,000 time steps. We used 115 amino-acid sequences of *NLRP* genes and "pseudogenes" from 11 mammalian species (human, macaque, marmoset, mouse, pig, cattle, horse, dog, elephant, possum, and platypus) as well as one sequence from chicken as an outgroup

maternal germ line with paternal ICRs unmethylated because of demethylation of the sperm pronucleus after fertilization, with the methylated paternal *H19* ICR somehow protected from this process.

Women homozygous for mutations of *NLRP7* exhibit maternal-effect embryonic lethality in the form of the repeated conception of biparental complete hydatidiform moles (biCHMs) (Murdoch et al. 2006; Kou et al. 2008; Parry et al. 2011). These "embryos" exhibit biparental non-methylation of most imprinting control regions (ICRs), with the notable exception of the *H19* ICR (Kou et al. 2008; El-Maarri et al. 2003). Women with mutations of both alleles of *C6orf221* also produce biCHMs with loss of differential methylation at most ICRs except *H19* (Parry et al. 2011; Judson et al. 2002). Thus, loss-of-function of *NLRP7* and *C6orf221* cause similar perturbations of imprinting. *C6orf221* belongs to the *Ecat*/*Filia* gene family whose members are expressed in oocytes and early embryos and are known to interact with NLRP proteins (Zheng and Dean 2009).

The methylation pattern of biCHMs would be explained if maternal ICRs become demethylated in embryos because of a failure to protect the maternal pronucleus from conversion of 5mC to 5hmC. One possibility is that maternal deficiency of *NLRP7* or *C6orf221* results in a disruption of

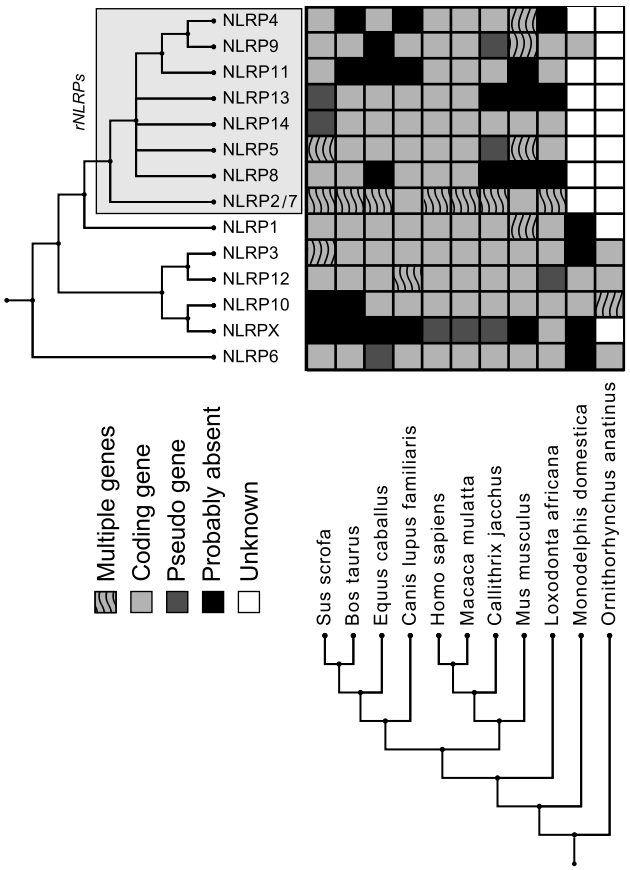

Fig. 2 Presence and absence of *NLRP* genes in mammalian species. This table summarizes the known information about the distribution of *NLRP* genes across the mammalian species included in our analyses. The cells of the table are shaded according to the presence or absence of a given *NLRP* gene in a particular species. *Light gray* is used to indicate the presence of a single *NLRP* gene identified in the databases as "protein coding." *Light gray with a wave pattern* is used if multiple copies of the gene are present, either identified as "protein coding" or "pseudogene." *Dark gray* indicates that a single gene is present and identified as "pseudogene." *Black* is used to denote the probable absence of the gene; in general, we consider a gene absent if it is not currently annotated in the species, absent from *BLAST* searches, and syntenic examination on the other mammalian species. We use *white* to denote that there is not sufficient evidence based on flanking markers to consider a gene absent

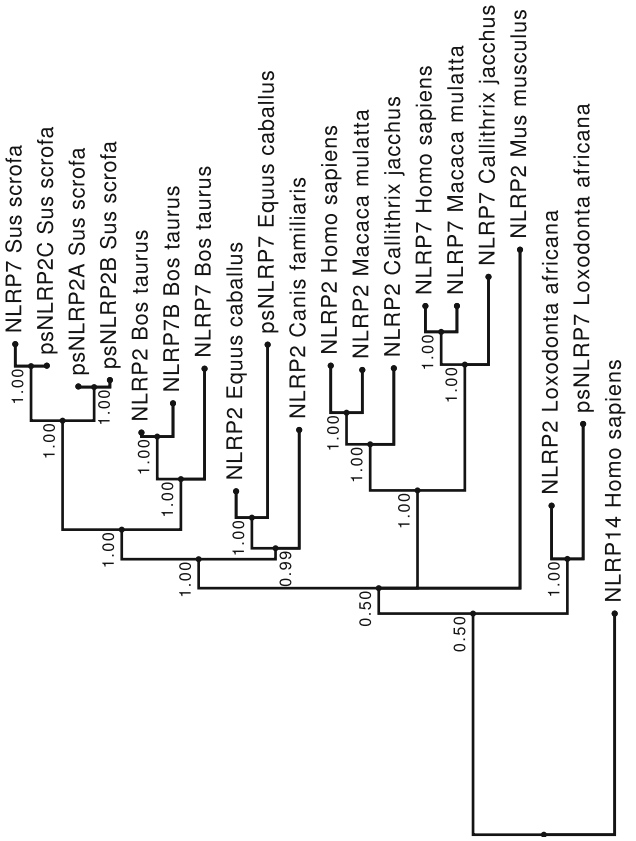

Fig. 3 Consensus phylogenetic tree of *NLRP2* and *NLRP7*. The phylogenetic tree was inferred using MrBayes for 1,000,000 time steps. We used 19 amino-acid sequences of all available *NLRP* genes and "pseudogenes" denoted as *NLRP2* or *NLRP7* in the mammalian species used in our other analyses, as well as human *NLRP14* as an outgroup. Potentially independent duplications of *NLRP2/7* can be seen in Laurasiatheria, primates, and elephant

spatial relations in the fertilized oocyte causes the egg pronucleus to be subject to the same epigenetic modifications as the sperm pronucleus.

Human *NLRP7* is the product of a recent duplication of *NLRP2*. Maternal homozygosity for a frame-shift mutation in *NLRP2* has been associated with loss of "maternal" methylation at 11p15.5 and a clinical diagnosis of Beckwith–Wiedemann syndrome (BWS) in offspring (Meyer et al. 2009). Finally, two individuals with phenotypic features of BWS have been reported with independent breakpoints in *ZNF215* (*ZKSCAN11*), a gene immediately adjacent to *NLRP14* (Alders et al. 2000).

Conclusions

Our analysis shows that all the major *NLRP* genes are present in eutherian mammals and that *rNLRP*-related genes are present in marsupials. The *rNLRPs* have a complex history of independent duplications in several eutherian lineages. While strong inferences about the origin of *rNLRP* genes remain elusive, the following scenario is consistent with the data. The appearance and diversification of *rNLRP* genes in the common ancestor of Metatherians (where opossum chromosome 4 maintains ancestral linkage of *NLRP* genes) and Eutherians, followed by breakage of linkage and consequent dispersal to their present locations in eutherian mammals.

Mutations in *NLRP2* and *NLRP7* in humans have been associated with disorders of genomic imprinting, Beckwith–Wiedemann syndrome and biparental complete hydatidiform moles. A plausible explanation is that deficiencies of *NLRP2* and *NLRP7* result in the maternal pronucleus being subject to the programmed demethylation that is normally restricted to the paternal pronucleus. Because human *NLRP2* and *NLRP7* are the product of a primate-specific duplication, mice are perhaps not the best model organisms to study these reproductive disorders.

Methods

Data-Set and Syntenic Analyses

A total of 116 amino-acid sequences for NLRP proteins were obtained from GenBank (Benson et al. 2011) and used in the phylogenetic analyses. We used a combination of genes annotated as NLRP or NLRP-like, identified as protein coding or as pseudogenes. Online Resource 2 contains the accession numbers of the sequences used in our analyses.

We focused on genes from human (*Homo sapiens*), macaque (*Macaca mulatta*), marmoset (*Callithrix jacchus*), cattle (*Bos taurus*), pig (*Sus scrofa*), dog (*Canis lupus familiaris*), horse (*Equus caballus*), mouse (*Mus musculus*), elephant (*Loxodonta africana*), opossum (*Monodelphis domestica*), platypus (*Ornithorhyunchus anatinus*), and chicken (*Gallus gallus*). Blastp (Altschul et al. 1997) was used with the 14 human NLRPs as queries to the non-redundant protein sequences (NCBI: nr) to assure no other NLRP proteins were undetected from these taxa. The sequence data used in the main phylogeny (Fig. 1) was collected on or before September 2011, using the current genome assemblies at the time. While new assemblies for dog, cattle, and pig are available at the time of this writing, there are no new assemblies for elephant, possum, or platypus. Therefore, we chose not to redo our analyses, for the new data do not pertain the initial diversification of *NLRP* genes.

A total of 112 protein sequences were available for *NLRP* genes from the species listed above and identified as protein coding. A gene annotated as *NLRP1*-like in chicken [GenBank:XP_422818.3] and one of the two *NLRP5s* annotated in pig [GenBank:NP_001156879.1] were outliers in our preliminary alignments and phylogenetic trees and were excluded from further analyses. The *NLRP1*-like gene of chicken is on chromosome 9 in a region of conserved synteny with human chromosome 3 from which no *NLRP* or *NLRP*-like has been reported. The results of blastp using chicken *NLRP1*-like as query shows that the most likely ortholog is an uncharacterized protein with *IFT80* and *IL12A* as flanking markers in mammalian species. The characterization of this protein as NLRP1-like is likely due to the presence of FIIND and DEATH domains, which are also present in mammalian *NLRP1* genes.

Six "pseudogenes" were included in our analysis: two from elephant, and one each from macaque, marmoset, opossum, and platypus. Pseudogene sequences were transformed into amino acid sequences by identifying exon boundaries and removing frame shifts. These reconstructed "proteins" aligned well with proteins encoded by orthologous *NLRP* genes.

Blastn was performed to look for other pseudo-genes or gene traces in the genomes of possum and platypus, but no significant hits were found. Additionally, orthologous and paralogous relationships were established by inspecting flanking markers of all *NLRP* genes in the genome assemblies for all species used. We considered enough evidence for the absence of an *NLRP* gene/pseudogene when neither Blast nor syntenic analyses could find suitable candidates (see Online Resource 3).

When a gene was suspected absent, we performed a tblastn search with the corresponding human *NLRP* gene(s) as query. A cut-off E-value of 10^{-10} was used throughout. The only non-*NLRP* hits in non-human mammals were

CARD4, *CARD15*, *CIITA*, *NOD1*, *NOD2*, *NOD4*, *NLRC3*, *NLRC5*, *NLRX1*, and *RNH1*. All of these hits are due to the presence of shared domains with *NLRP* genes. Furthermore, all these non-human hits were found in synteny with the corresponding human genes and their flanking genes.

Sequence Alignment and Phylogenetic Analyses

We used UGENE as our working platform (Okonechnikov et al. 2012). Multiple sequence alignments were performed with T-Coffee (Notredame et al. 2000) and are available upon request. The phylogenetic trees were reconstructed with Maximum Likelihood (ML) methods using Garli (Zwickl 2006) and Bayesian estimation (BA) using MrBayes (Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2001).

For the Bayesian reconstructions we used 2 runs, each with 4 chains and a burn-in fraction of 0.25. The runs were carried for an initial one million iterations, with increments of one million iterations until convergence. We declared a run to converge when the differences in the Log-Likelihood were less than 0.1 % for the last million iterations and the *Potential Scale Reduction Factor* convergence criterion was within 1 % of 1. For the Maximum Likelihood reconstruction we used 2 search repetitions running for 5 million generations with 100 bootstrap iterations from an initially random tree. The JTT+G+F model of amino-acid evolution using the observed aminoacid frequencies (Jones et al. 1992) was selected based on the best Log-Likelihood score provided by ProtTest (Darriba et al. 2011).

The consensus tree (Online Resource 1) was constructed by merging the results from ML and BA using DendroPy (Sukumaran and Holder 2010). We chose to present only the Bayesian tree in Fig. 1 because it is more resolved.

In contrast to Tian et al. (2009), we included all amino-acids available from all sequences into the analysis, which included several partial and low-quality sequences. It is generally accepted that using partial sequences diminishes the accuracy of the inferred phylogeny. This detrimental effect is, however, still not well understood, with researchers suggesting detrimental effects ranging from insignificant to severe Wiens (2003); Hartmann and Vision (2008); Burleigh et al. (2009); Kück et al. (2010). Several approaches have been suggested to increase phylogenetic inference accuracy, ranging from masking (i.e., removal) of problematic data to the statistical simulation of missing data Hartmann and Vision (2008); Kück et al. (2010). However, Bayesian and, to a lesser extent, Maximum Likelihood approaches seem to be particularly resilient to missing data, with consistently high accuracy being achieved even in the absence of up to 95 % of some of the samples (Wiens and Moen 2008; Wiens and Morrill 2011). In our case, only 11 out of the 116 sequences (less than 10%) are partial (*NLRP6* of elephant and platypus, *NLRPb* of possum, *NLRP3* and *NLRP11* of marmoset) or low quality (*NLRP5* and *NLRP10* of elephant and horse, respectively, *NLRP1* of elephant, and *NLRP11* of pig). Also, the missing data of partial sequences is presumably small and is therefore unlikely it has a strong effect on accuracy. In particular, the shortest partial sequence corresponds to the *NLRP6* of platypus which has a length of 417 amino-acids. The longest *NLRP6* is in macaques and has 1045 amino-acids, while the longest *NLRP* sequence overall is *NLRP1*, also in macaques, which has 1475 amino-acids. Therefore, we can conservatively estimate less than 30 % missing data in less than 10 % of the sequences.

In Online Resource 5, we performed a phylogenetic recontructions of the *NLRP* genes in Fig. 1 leaving out all reconstructed pseudogene sequences.

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