Immunogenetics (2019) 71:437–443
https://doi.org/10.1007/s00251-019-01110-3

SHORT COMMUNICATION

Strong selection of the TLR2 coding region among the Lagomorpha suggests an evolutionary history that differs from other mammals

Fabiana Neves[1] · Ana Águeda-Pinto[1] · Ana Pinheiro[1] · Joana Abrantes[1] · Pedro J. Esteves[1,2,3]

Received: 8 January 2019 / Accepted: 15 February 2019 / Published online: 14 March 2019

Abstract

Toll-like receptors (TLRs) are one of the first lines of defense against pathogens and are crucial for triggering an appropriate immune response. Among *TLRs*, *TLR2* is functional in all vertebrates and has high ability in detecting bacterial and viral pathogen ligands. The mammals' phylogenetic tree of *TLR2* showed longer branches for the Lagomorpha clade, raising the hypothesis that lagomorphs experienced an acceleration of the mutation rate. This hypothesis was confirmed by (i) Tajima's test of neutrality that revealed different evolutionary rates between lagomorphs and the remaining mammals with lagomorphs presenting higher nucleotide diversity; (ii) genetic distances were similar among lagomorphs and between lagomorphs and other mammals; and (iii) branch models reinforced the existence of an acceleration of the mutation rate in lagomorphs. These results suggest that the lagomorph *TLR2* has been strongly involved in pathogen recognition, which probably caused a host-pathogen arms race that led to the observed acceleration of the mutation rate.

Keywords Lagomorphs · *TLR2* · Evolution · Mutation rate

Background

Recognition of invading pathogens by the host immune system is essential for the initiation of an immune response. The innate immune system is the first line of defense and is composed of cells that express several pattern recognition receptors (PRRs). PRRs are able to detect unique signature molecules (pathogen-associated molecular patterns—PAMPs) of invading organisms and promptly activate an appropriate immune response (Akira et al. 2006; Kumar et al. 2009). Among PRRs, the Toll-like receptors (TLRs) are the most widely studied. TLRs have a unique ability to distinguish between "self" and "non-self" and therefore recognize the first signs of infection, being also strong inducers of the inflammatory response (Mukherjee et al. 2016).

There are currently ten *TLRs* described in human and 13 in mouse with different PAMP recognition (Kawai and Akira 2011; Brennan and Gilmore 2018). From these, type I transmembrane glycoproteins TLR2 and TLR4 are the most studied due to their ability to identify the broadest range of pathogen ligands (Mukherjee et al. 2016). Notably, *TLR2* is present in all vertebrate species and is associated with immune response to bacterial lipoprotein products (Roach et al. 2005) and recognition of certain ssRNA viruses such as human cytomegalovirus, herpes simplex virus (Niedcwiedzka-Rystwej et al. 2013; Lester and Li 2014), and HIV-1 gp120 protein infection (Ding and Chang 2012; Hug et al. 2018).

The mammalian immune system genes tend to have faster evolutionary rates as a result of the continuous arms race between hosts and pathogens. Several studies have confirmed this by describing patterns of positive selection acting on immune system genes and, relevantly, on TLR sites/regions, mainly the extracellular

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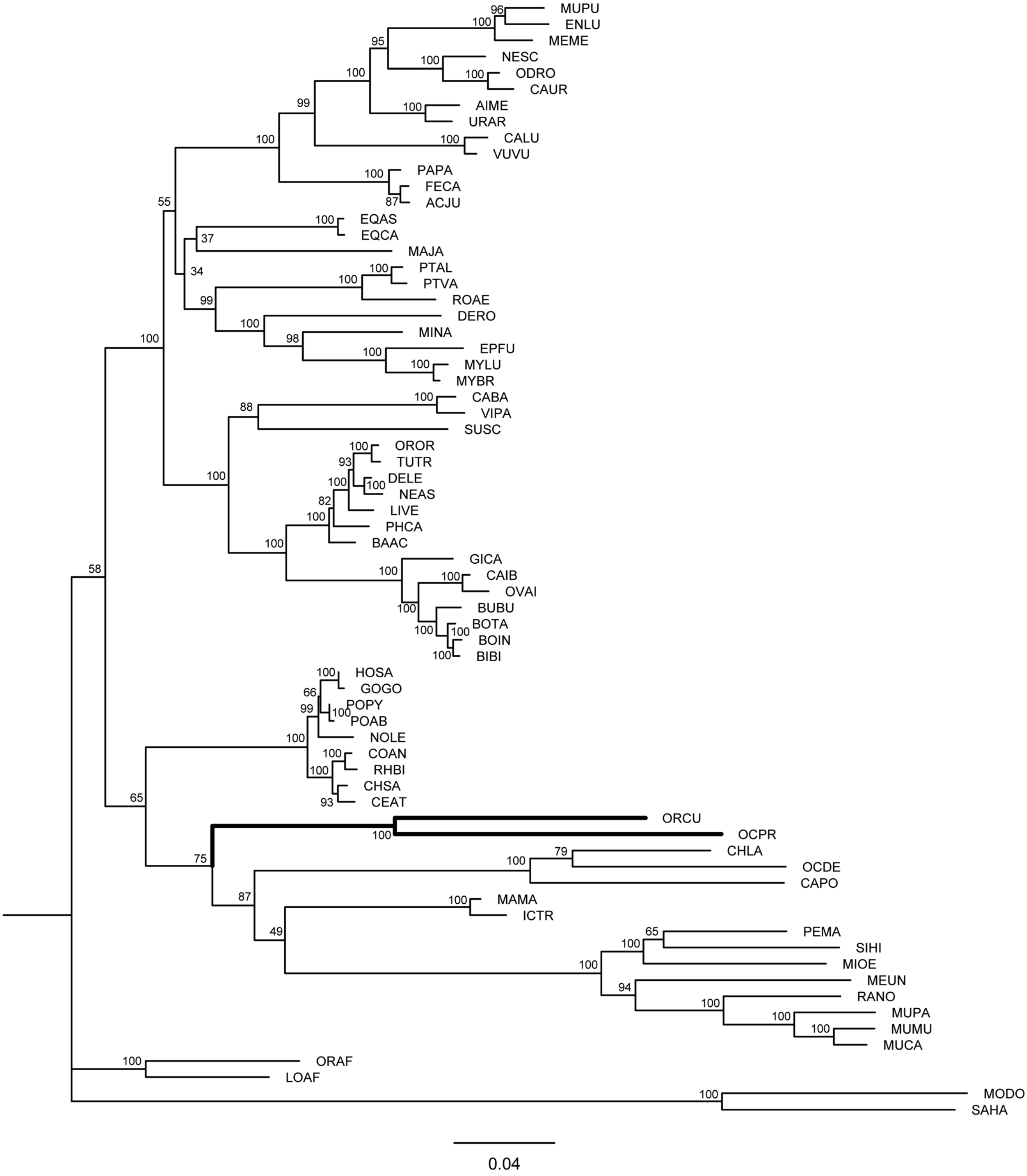
Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00251-019-01110-3) contains supplementary material, which is available to authorized users.

✉ Pedro J. Esteves
pjesteves@cibio.up.pt

[1] CIBIO-UP, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO, Laboratório Associado, Universidade do Porto, Campus Agrário de Vairão, Rua Padre Armando Quintas, nr.7, 4485-661 Vairão, Portugal

[2] Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto, Portugal

[3] CITS - Centro de Investigação em Tecnologias de Saúde, CESPU, Gandra, Portugal



domain, related with pathogen detection (Areal et al. 2011; Lewis and Obbard 2014; Smith et al. 2014; Darfour-Oduro et al. 2015).

The order Lagomorpha is composed by two families (Ochotonidae and Leporidae) that diverged approximately 30 million years ago (mya) (Matthee et al. 2004), presenting a widespread distribution, from deserts to the arctic (Chapman and Flux 2008). The Ochotonidae family comprises a single genus (*Ochotona*) while the Leporidae family includes 11 genera of rabbits and hares (Matthee et al. 2004; Melo-Ferreira et al. 2015; Fontanesi et al. 2016). The importance of the European

◀ **Fig. 1** Phylogenetic tree of the mammalian *TLR2* gene. A maximum likelihood approach was used with Tamura-Nei as the substitution model and partial deletion to gaps/missing data treatment and 1000 bootstrap replicates. The foreground branches used for branch analyses are highlighted in bold. Angola colobus (COAN), black snub-nosed monkey (RHBI), green monkey (CHSA), sooty mangabey (CEAT), northern white-cheeked gibbon (NOLE), Bornean orangutan (POPY), Sumatran orangutan (POAB), western lowland gorilla (GOGO), human (HOSA), European rabbit (ORCU), American pika (OCPR), North American deer mouse (PEMA), hispid cotton rat (SIHI), tundra vole (MIOE), Mongolian gerbil (MEUN), Gairdner's shrewmouse (MUPA), house mouse (MUMU), Ryukyu mouse (MUCA), brown rat (RANO), alpine marmot (MAMA), thirteen-lined ground squirrel (ICTR), guinea pig (CAPO), long-tailed chinchilla (CHLA), common degu (OCDE), leopard (PAPA), cat (FECA), cheetah (ACJU), red fox (VUVU), dog (CALU), grizzly bear (URAR), giant panda (AIME), Hawaiian monk seal (NESC), northern fur seal (CAUR), walrus (ODRO), European badger (MEME), ferret (MUPU), sea otter (ENLU), Sunda pangolin (MAJA), large flying fox (PTVA), black fruit bat (PTAL), Egyptian fruit bat (ROAE), common vampire bat (DERO), Natal long-fingered bat (MINA), big brown bat (EPFU), Brandt's bat (MYBR), little brown bat (MYLU), donkey (EQAS), horse, (EQCA), alpaca (VIPA), Bactrian camel (CABA), wild boar (SUSC), Alpine ibex (CAIB), domestic sheep (OVAI), water buffalo (BUBU), European cattle (BOTA), zebu (BOIN), plains bison (BIBI), giraffe (GICA), sperm whale (PHCA), minke whale (BAAC), baiji (LIVE), killer whale (OROR), common bottlenose dolphin (TUTR), white whale (DELE), narrow-ridged finless porpoise (NEAS), aardvark (ORAF), African bush elephant (LOAF), gray short-tailed opossum (MODO), and Tasmanian devil (SAHA)

rabbit (*Oryctolagus cuniculus cuniculus*) as a biological model to study human diseases (Esteves et al. 2018) reinforces the need of studying the lagomorphs' immune system.

Despite the increasing number of studies focusing on the lagomorphs' immune system (Neves et al. 2015a, b; Pinheiro et al. 2016; Esteves et al. 2018), knowledge on TLRs is still scarce (Abrantes et al. 2013; Chen et al. 2014; Zhang et al. 2014; Awadi et al. 2018), mainly if compared with human or mouse. In this work, we studied the *TLR2* evolution with particular focus on the Lagomorpha order. Lagomorphs are affected by a broad range of diseases and TLRs are likely to be involved. Our goal with this study was to test if the *TLR2* gene experienced an acceleration of the mutation rate in the Lagomorpha lineage in comparison with the other mammals.

Materials and methods

Publicly available mammalian *TLR2* coding sequences, representative of Artiodactyla, Perissodactyla, Carnivora, Pholidota, Chiroptera, Primates, Rodentia, Lagomorpha, Afrotheria and Marsupialia, were retrieved from the National Center for Biotechnology Information (NCBI) (accession numbers are given in Online Resource 1). Sequences were aligned using multiple sequence comparison by log-expectation (MUSCLE) available at http://www.ebi.ac.uk/ (Edgar 2004) and adjusted manually with BioEdit (Hall 1999). A total of 69 sequences were used for this study.

A maximum likelihood method was used to reconstruct the *TLR2* phylogenetic tree (Fig. 1) in MEGA-X (Kumar et al. 2018) with options Tamura-Nei as the substitution model and partial deletion to gaps/missing data treatment. The consistency of the clusters was tested by applying a bootstrap method with 1000 bootstrap replicates. The estimated gene tree did not reflect the true species tree. Thus, we constrained the *TLR2* gene tree in order to best recover the mammalian relationships according to an accepted phylogeny (Esselstyn et al. 2017).

In order to understand the evolutionary rate and the statistical significance, we performed a Tajima's relative rate test (Tajima 1993) in the Lagomorpha *TLR2* coding sequences using MEGA-X (Kumar et al. 2018). We have also estimated the number of nucleotide differences per site between sequences in MEGA-X with the following options: bootstrap method (1000 replicates), *p*-distance, and pairwise deletion for gaps/missing data treatment.

To test whether there was a difference in selection acting on *TLR2* in the Lagomorpha lineage (labeled as foreground branch) vs. the remaining mammalian sequences (background branches), we used the branch models available in CODEML program of PAML 4.9 package (Yang 2007) and performed the RELAX analysis (Wertheim et al. 2015) available in the Data Monkey web server (http://www.datamonkey.org/relax). These approaches allow the ratio (ω) of nonsynonymous (dN) to synonymous (dS) substitutions to vary among branches and compare them through a likelihood ratio test (LRT). In CODEML, our goal was to test if *TLR2* experienced different selective forces in the Lagomorpha lineage. For this, we tested a null one-ratio model (M0—one ω ratio for all branches—options F3X4 as codon frequency model; model = 0; NSsites = 0) against the two-ratio model (M2—ω values are allowed to vary between branches—options F3X4 as codon frequency model; model = 2; NSsites = 0) by using a LRT and calculating twice the difference in the natural logs of the likelihood (2ΔlnL). The significance test was obtained by using a χ^2 distribution with 1 degree of freedom (df = 1), allowing the rejection of the null hypothesis ($p < 0.05$). We compared the Lagomorpha lineage (branch leading to lagomorphs and both European rabbit and American pika branches) labeled as foreground branch vs. the remaining mammals (background branches) and ran multiple initial values of the parameter ω (0.1, 1.0, 2.0) in order to ensure the convergence of parameter optimization. Additionally, and in order to test the strength of natural selection acting on the Lagomorpha lineage, we performed the RELAX analysis. The RELAX analysis is a powerful tool that calculates ω for each branch, identifying trends or changes in the evolution of natural selection on a gene, and determining if a specific clade goes under relaxed ($\omega = 1$) or intensified ($\omega > 1$) selection. This is achieved by comparing the null model (background

Table 1 TLR2 evolutionary distances between lagomorphs and other mammals

	Nucleotide distances		Amino acid distances	
	European rabbit	American pika	European rabbit	American pika
European rabbit	–	0.1957	–	0.2097
American pika	0.1957	–	0.2097	–
Human	0.2143	0.2217	0.2372	0.2391
Brown rat	0.2730	0.2860	0.2959	0.3222
Large flying fox	0.2359	0.2552	0.2835	0.3022
Horse	0.2248	0.2300	0.2554	0.2625
Water buffalo	0.2384	0.2569	0.2950	0.3009
Killer whale	0.2313	0.2413	0.2730	0.2890

branches present $\omega = 1$) against the alternative model (foreground branches share a single intensity parameter—k). A LRT analyzes the two models trough χ^2 distribution with df = 1. The null hypothesis is rejected if the foreground branches are intensified ($k > 1$) or relaxed ($K < 1$) (Wertheim et al. 2015).

Results and discussion

For this study, a total of 69 mammalian *TLR2* coding sequences, representative of most mammalian orders, (Online Resource 1) were retrieved from NCBI database.

The ability of TLRs to recognize a broad range of pathogens makes them a key factor when hosts set up an immune response. As several other multigene families, *TLRs* arose by gene duplication, which usually follows three pathways: pseudogenization, neofunctionalization, or subfunctionalization (Hughes and Piontkivska 2008; Ding et al. 2012). The newly originated genes might evolve so that the encoding proteins interact with other proteins and hence adapt to the environment (Andersson et al. 2015; Guthrie et al. 2018). Positive selection may play an important role for their maintenance rather than for their disappearance or inactivation (Nei and Rooney 2005; Beisswanger and Stephan 2008). TLRs seem to be evolving under positive selection in mammals (Jann et al. 2008; Wlasiuk and Nachman 2010; Areal et al. 2011; Tschirren et al. 2011; Fornuskova et al. 2013; Smith et al. 2014; Darfour-Oduro et al. 2015), reptiles (Shang et al. 2018), fishes (Zhu et al. 2013), and birds (Alcaide and Edwards 2011; Grueber et al. 2014; Vinkler et al. 2014), and the TLR2 family has more species-specific adaptations than other TLR families (Roach et al. 2005).

The observation that, in the *TLR2* phylogeny (Fig. 1), lagomorphs' coding sequences presented longer branches when compared to the remaining mammals' *TLR2* genes prompted us to investigate the possibility of an acceleration of the mutation rate in that specific lineage. For this, we first performed a Tajima's relative rate test which counts the number of alterations that occurred along two species that split from a common ancestral, the outgroup. The Tajima's relative rate uses a LRT to compare two hypotheses: the null hypothesis, which assumes equal rates among lineages, and the alternative hypothesis, which allows one of the lineages to be evolving at different rates. We tested the differences between taxon A (lagomorphs; both European rabbit and American pika) and taxon B (different species that diverged from Lagomorpha between 80 and 102 mya (Kumar et al. 2017)) and used the most basal eutherian mammal, the African bush elephant, *TLR2* sequence as outgroup (Online Resource 2). All the analyses produced statistically significant P values ($p \ll 0.05$), allowing the rejection of the null hypothesis, i.e., equal rates among lineages.

TLRs originated more than 600 million years ago (Leulier and Lemaitre 2008). Usually, longer divergence times are synonymous of higher evolutionary distances (Christin et al. 2014). However, taking into account the nucleotide and amino acid distances shown in Table 1, the European rabbit and the American pika, that diverged ~ 30 mya (Matthee et al. 2004; Melo-Ferreira et al. 2015), are as distant as lagomorphs and humans that diverged ~ 90 mya (Kumar et al. 2017) (~ 0.20 and ~ 0.22, respectively). Moreover, the distances between the European rabbit and Chiroptera, Perissodactyla, or Artiodactyla, that split more than 95 mya (Kumar et al. 2017), are also between 0.22 and 0.23.

In order to test if there were significant differences in the selective pressures acting between the Lagomorpha clade (European rabbit and American pika) and the remaining mammals, we tested two models (M0 vs M2) in PAML. Our results

Table 2 Branch model results obtained in PAML

Model	lnL	2ΔlnL	df	p value[1]	ω_2/ω_1 estimates[2]
One-ratio	− 40562.490683				0.3489
Two-ratio	− 40558.863572	7.25	1	< 0.01	0.2048/0.3526

[1] P value < 0.05 is used to reject the null hypothesis of equal rates between lineages

[2] ω_1 and ω_2 are ω values for foreground and background branches, respectively

Table 3 Test for selection relaxation on the lagomorphs' branch

Model	ω_1	ω_2	ω_3	lnL	P	AICc	Branch set
General descriptive	0.00 (65.82%)	0.99 (33.55%)	10.93 (0.62%)	− 39897.5	289	80376.0	Shared
RELAX alternative	0.00 (58.78%)	0.58 (40.90%)	9798619569.67 (0.32%)	− 39976.0	155	80263.0	Test
	0.00 (58.78%)	0.93 (40.90%)	23.84 (0.32%)				Reference
RELAX null	0.00 (60.76%)	0.95 (38.86%)	20.27 (0.38%)	− 39987.2	154	80283.3	Test
	0.00 (60.76%)	0.95 (38.86%)	20.27 (0.38%)				Reference
RELAX partitioned descriptive	0.03 (64.02%)	0.61 (35.98%)	17.03 (0.00%)	− 39973.3	159	80265.6	Test
	0.01 (62.02%)	1.00 (37.54%)	21.04 (0.37%)				Reference

Log likelihood values and parameter estimates for the RELAX analysis. ω_1, first omega rate class; ω_2, second omega rate class; ω_3, third omega rate class; *p*, number of parameters in the model; *lnL*, log likelihood value under the model; *AICc*, small-sample correction Akaike information criterion; Branch set, indicates which branch set each parameter belongs to. Test for selection intensification ($K = 7.25$) was highly significant ($P \ll 0.000$, LR = 22.35)

(Table 2) indicate that the null hypothesis (M0—allows a single ω ratio for all the branches) is rejected and consequently selective pressure acts differently between Lagomorpha and other mammals. Next, we used RELAX to test the intensity of selection acting on the Lagomorpha clade when compared with the remaining mammals. The results of this analysis (Online Resource 3, Table 3) support an intensification of selection on the Lagomorpha lineage ($k = 7.25$; $p = 0.000$, LRT = 22.35).

Overall, our results support an acceleration of the mutation rate on both the European rabbit and American pika lineages. Lagomorphs are affected by a wide range of diseases caused by several pathogens, from viruses to bacteria, fungi, and parasites (Wobeser et al. 2009; Oliveira-Nascimento et al. 2012). TLRs are in direct contact with pathogens, leading to the selection of pathogens that are able to evade the host immune response. In turn, evolution of the pathogens leads to the evolution of the host immune system so that advantageous mutations are selected and fixed in the population, leading to a cyclical process of adaptation and counter-adaptation between hosts and pathogens. This process might ultimately lead to an acceleration of the fixation of advantageous mutations as observed for *TLR2* in lagomorphs. While the role of *TLR2* in lagomorphs' diseases has been slightly disregarded, it is considered an important candidate gene for resistance against infectious diseases. Indeed, some studies have described that alterations in *TLR2* signaling allow *Francisella tularensis*, the etiologic agent of the zoonotic disease tularemia that affects rabbit and hares, to escape from early inflammasome and consequently replicate successfully (Kingry and Petersen 2014). In addition, *TLR2* is overexpressed in rabbit lungs when infected by *Escherichia coli* pneumonia (Kajikawa et al. 2005), and some polymorphisms in rabbit *TLR2* had been identified (Zhang et al. 2014), although their implications are unknown.

TLRs are crucial for mounting an appropriate immune response. In general, *TLRs* evolve under positive selection, mainly in sites important for pathogen recognition. We show that in lagomorphs, *TLR2* experienced an intensification of selection that might be due to the number of different pathogens that affect these species.

Funding information This article is a result of the project AGRIGEN – NORTE-01-0145-FEDER-000007, supported by Norte Portugal Regional Operational Programme (NORTE2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). Fundação para a Ciência e Tecnologia (FCT) supported the FCT Investigator grants of P.J. Esteves (IF/00376/2015) and J. Abrantes (IF/01396/2013).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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