Adaptive Functional Diversification of Lysozyme in Insectivorous Bats

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Abstract

The role of gene duplication in generating new genes and novel functions is well recognized and is exemplified by the digestion-related protein lysozyme. In ruminants, duplicated chicken-type lysozymes facilitate the degradation of symbiotic bacteria in the foregut. Chicken-type lysozyme has also been reported to show chitinase-like activity, yet no study has examined the molecular evolution of lysozymes in species that specialize on eating insects. Insectivorous bats number over 900 species, and lysozyme expression in the mouths of some of these species is associated with the ingestion of insect cuticle, suggesting a chitinase role. Here, we show that chicken-type lysozyme has undergone multiple duplication events in a major family of insect-eating bats (Vespertilionidae) and that new duplicates have undergone molecular adaptation. Examination of duplicates from two insectivorous bats—Pipistrellus abramus and Scotothillus kuhlii—indicated that the new copy was highly expressed in the tongue, whereas the other one was less tissue-specific. Functional assays applied to pipistrelle lysozymes confirmed that, of the two copies, the tongue duplicate was more efficient at breaking down glycol chitin, a chitin derivative. These results suggest that the evolution of lysozymes in vespertilionid bats has likely been driven in part by natural selection for insectivory.

Key words: Chiroptera, lysozyme, diet, gene duplication, adaptive evolution.

Introduction

Gene duplication is a widespread evolutionary mechanism that can lead to the generation of new genetic material as well as new biological functions (Ohno 1970; Zhang 2003; Innan and Kondrashov 2010). Following duplication, new genes may be lost, functionally conserved, or, in some cases, may undergo functional modifications or innovations (Zhang 2003). The enzyme lysozyme is considered a classic example of adaptive gene duplication, with duplicates detected in a wide range of animals (Cámara and Priour 1984; Hammer et al. 1987; Irwin and Wilson 1989; Kornegay 1996; Regel et al. 1998).

The chicken-type lysozyme—which has been particularly well-studied—appears to function primarily in breaking down the peptidoglycan of bacterial cell walls, and is thus considered part of the immune response (Callewaert and Michiels 2010). In some taxa such as artiodactyl ruminants, the lysozyme also plays a role in facilitating digestion (Callewaert and Michiels 2010). The guts of these mammals play host to mutualistic symbiotic bacteria, which synthesize cellulases and thus allow nutrients to be obtained from otherwise indigestible plant material. In turn, the host expresses lysozymes to break down the dead bacteria and release further nutrients (Dobson et al. 1984). As an adaptation for this so-called foregut fermentation, the cow appears to have evolved ten lysozyme duplicates, four of which are expressed in stomach (Irwin and Wilson 1989) where they are well-adapted to the highly acidic environment (Dobson et al. 1984). Molecular evolutionary analyses show that these stomach-specific copies of ruminant lysozymes have been subject to concerted evolution, a process that probably acts to maintain and reinforce this specialized function (Irwin and Wilson 1990; Irwin et al. 1993; Wen and Irwin 1999). Such patterns strongly contrast with those seen in the related yet omnivorous pig, which possesses just a single copy of the lysozyme gene (Yu and Irwin 1996).

Chicken-type lysozyme has been shown to have chitinase activity (Berger and Weiser 1957; Amano and Ito 1978; Li et al. 2013) and, therefore, it is possible that apart from foregut fermenters, adaptive gene duplication might also have occurred in species that ingest large amounts of chitin. Bats (order Chiroptera) number over 1,100 species and, while showing unparalleled dietary diversification among mammals, over 900 extant bat species are exclusively insectivorous (Simmons 2005), which is also considered the ancestral state of bats (Gunnell and Simmons 2005). Insectivorous bats appear to most commonly prey on insects from the orders Lepidoptera (moths and butterflies), Coleoptera (beetles) and Diptera (flies) (Hill and Smith 1984), and may consume...
around three quarters of their body mass per night (Kunz et al. 1995). Given that bats must process their insect prey quickly, and that some prey possess chitinous exoskeletons that are hard to be chewed (Evans and Sanson 2005), it follows that any biochemical breakdown of chitin to access its carbohydrate content could be highly adaptive. Supporting this idea, lysozyme expression in bat salivary glands correlates well with the extent of cuticle intake, suggesting that it may have been recruited for its chitinase activity in these taxa (Phillips et al. 1998). Furthermore, chitinase has been detected in the intestines of vespertilionid bats (Whitaker et al. 2004) and has also been shown to be active in the stomachs of several European bat species from the same family (Strobel et al. 2013).

Here we present the first in-depth study of the molecular evolution of chicken-type lysozymes in bats, combining sequencing with analyses of gene expression profiles and enzyme function assays. We test the following three hypotheses: 1) Chicken-type lysozyme will occur as multiple copies in insect-eating bats due to duplication events, 2) new duplicate gene copies of lysozyme will show evidence of Darwinian selection consistent with molecular adaptation for new roles in processing chitin, and 3) these new copies will show greater activity for breaking down chitin.

**Results**

**Lysozyme Gene Duplications in Bats**

We sequenced the complete coding region of chicken-type lysozyme genes from 19 bat species. The coding part of bat lysozyme genes spanned 447 bp, except for two copies detected in the genus Myotis (MricLyz-4 and MadvLyz-4), both of which shared a 3-bp deletion. Available lysozyme gene sequences of two bat species, *Myotis lucifugus* (*n* = 3) and *Pteropus vampyrus* (*n* = 1), were retrieved from the Ensembl genome database (www.ensembl.org, last accessed August 20, 2014). In total, taxonomic coverage included nine bat families, comprising eight families of insectivorous bats as well as the Pteropodidae (Old World fruit bats). In seven of the families surveyed, cloning yielded a single lysozyme gene copy. In contrast, multiple duplicated genes were found in the insectivorous family Vespertilionidae, with potential duplications detected in the genera *Ia*, *Myotis*, *Murina*, *Nyctalus*, *Pipistrellus*, *Scotophilus*, and *Scotomanes*. Additionally, one duplication event was also uncovered in the pteropodid *Cynopterus sphinx* (supplementary table S1, Supplementary Material online).

**Molecular Evolution of Bat Lysozyme Genes**

Gene trees generated by Bayesian, maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) methods revealed several independent duplication events in bats (fig. 1 and supplementary fig. S1, Supplementary Material online). Topological differences were observed among gene trees produced by the different tree-building methods. Overall, the gene copies clustered together in their respective subordinal groups with the exception of the two yinpterochiropterans *Taphozous melanopogon* and *Tadarida plicata*. Both of these species were correctly recovered in the Yinpterochiroptera in the NJ tree (supplementary fig. S1B, Supplementary Material online), but either one or both of them were in the Yinpterochiroptera in the Bayesian, ML and MP trees (with low statistical support), suggesting inconsistencies arise from methodological artifacts (fig. 1 and supplementary fig. S1, Supplementary Material online).

Within the Yangochiroptera, all genes from vespertilionid bats grouped in a well-supported clade across all methods, and, within the genus *Myotis*, we detected at least three gene duplications that have given rise to four main subgroups, which we refer to as *Lyz-1*, *Lyz-2*, *Lyz-3*, and *Lyz-4*. Further duplication events were detected in *Pipistrellus* and *Nyctalus*, occurring before the split of the two genera, and in the clade containing *Ia io* and *Scotomanes ornatus*. Independent gene duplications were also observed in either *Scotophilus kuhlii* or *Murina leucogaster* (fig. 1 and supplementary fig. S1, Supplementary Material online). No gene conversion was detected among paralogs from one species.
We tested whether the duplicated genes in bats were associated with molecular adaptation, which would be consistent with the recruitment of derived genes to perform new roles, such as in digestion. By estimating the ratio of the rate of nonsynonymous substitutions to the rate of synonymous ones (termed omega, \( \omega \)) we found several episodes of positive selection in the putative new bat genes, mainly in the family Vespertilionidae (\( 2\Delta \ell = 151.78, df = 74, P < 0.001 \)). In the majority of these cases we observed that one gene copy (e.g., Lyz-1 in this study) was found to be under purifying selection (\( \omega < 1 \)) with few nucleotide substitutions, whereas the other gene(s) (e.g., Lyz-2, Lyz-3, or Lyz-4) had undergone positive selection (\( \omega > 1 \)). Overall, our selection tests showed functional conservation of one gene copy and adaptation in the other duplicate(s) (supplementary fig. S2 and supplementary table S2, Supplementary Material online).

Expression Profiles of Duplicated Genes

We characterized lysozyme expression patterns for two insectivorous bats, Pipistrellus abramus and S. kuhlii (Vespertilionidae), and one frugivorous bat, C. sphinx (Pteropodidae). The Lyz-1 gene was expressed in most organs examined in all three species, whereas Lyz-2 showed more tissue-specific expression profiles. In P. abramus, the PabrLyz-2 gene expression was detected in the spleen, the stomach, the intestine, and the tongue, with the peak expression value in the tongue, in contrast to PabrLyz-1 in which there was no detected expression in the tongue (fig. 2A). In S. kuhlii, SkuhLyz-2 also showed highest expression in the tongue, although this gene was also more widely expressed in other organs compared with PabrLyz-2 (fig. 2B). In C. sphinx, the CspLyz-2 gene showed extremely low expression level (0.0075 ± 0.0073) in the tongue and was not detected in any other organ types examined (fig. 2C).

Enzymatic Activities of Pipistrelle Lysozymes

Recombinant lysozymes based on PabrLyz-1 and PabrLyz-2 genes were synthesized individually and their enzymatic activities were measured. To determine the assumed original antibacterial function of the gene, we used Micrococcus lyso-deikticus as a substrate. We found that the enzymes differed in their enzymatic ability to break down bacteria (one-way analysis of variance [ANOVA], \( P = 0.015 \)), with PabrLyz-2 lysozyme showing significantly lower activity than hen egg-white lysozyme (HEWL) (Holm-Sidak, \( P = 0.017 \)). No significant differences were found between PabrLyz-1 and PabrLyz-2 (Holm-Sidak, \( P = 0.507 \)) or between PabrLyz-1 and HEWL (Holm-Sidak, \( P = 0.054 \)) (fig. 3A).

To test whether bat lysozymes, especially the PabrLyz-2, showed a chitinolytic activity consistent with a hypothesized chitinase role, we quantified the performance of the protein at breaking down the substrate glycol chitin. The results again revealed significant overall differences in hydrolytic activity (one-way ANOVA, \( P < 0.001 \)). PabrLyz-2 exhibited significantly higher lytic efficiency than PabrLyz-1 (Holm-Sidak, \( P < 0.001 \)), the efficiency of PabrLyz-1 was significantly higher than chitinase (Holm-Sidak, \( P = 0.005 \)), and the efficiency of PabrLyz-2 was significantly higher than chitinase (Holm-Sidak, \( P < 0.001 \)) (fig. 3B). Details of one-way ANOVAs are listed in supplementary table S3, Supplementary Material online.

Discussion

We undertook the first survey of lysozyme genes in bats and found evidence of widespread duplications having occurred in a major clade of insectivorous species (Vespertilionidae). In this group, which represents the most speciose family of bats (Simmons 2005), nine of the ten species examined possessed two or more chicken-type lysozyme copies, thus supporting our initial hypothesis. The only exception was Barbastella beijingensis, which appeared to have only one gene copy. In contrast, bats from all other families examined, from both the Yinpterochiroptera and Yangochiroptera suborders (Teeling et al. 2005), possessed just a single lysozyme gene, again with one exception: Two copies were detected in the Old World fruit bat C. sphinx.
In phylogenetic reconstructions based on bat lysozyme sequences, duplicated genes in bats did not always group together within taxa, in contrast to the situation seen in ruminants, where coding regions of lysozyme genes from the same species have been subject to concerted evolution (Irwin and Wilson 1990). In vespertilionid bats, lysozyme gene trees showed conflicts with known species relationships; to reconcile this discordance we conclude that independent gene duplications predated the split between the genera Pipistrellus and Nyctalus and also between the genera Scotomanes and Ia. Further gene duplications also occurred independently in the genera Scotophilus, Murina, and Myotis. Due to the low support for several nodes in the gene trees, it is not possible to estimate the exact number of gene duplications in this family. At the same time, however, we cannot completely rule out the potential for some differential representation of particular copies due to biases in the gene amplification and cloning process. Previous studies have shown that duplicated lysozymes perform a digestive function in the stomachs of foregut fermenting animals (Irwin and Wilson 1989; Kornegay 1996), and that gene conversion has acted to constrain this role in ruminants (Irwin and Wilson 1990).

Despite this we found no evidence of gene conversion in species with multiple lysozyme genes.

The finding that branches leading to duplicated gene copies in the vespertilionid clade (Lyz-2, Lyz-3, or Lyz-4 genes) were mostly characterized by positive selection, whereas those leading to the Lyz-1 genes were under purifying selection, supports our second hypothesis that the observed lysozyme duplicates in bats are adaptive. We suggest that conserved sequence variation in Lyz-1 genes could point to a function in host defense (Callewaert and Michiels 2010). On the other hand, the other derived gene copies in insectivorous bats have likely been recruited to perform new biological functions probably in digestion. Previous studies have reported new digestive functions of lysozymes in foregut-fermenting animals (Dobson et al. 1984; Kornegay et al. 1994). Similar functional shifts are also found in some invertebrate species, including goose- and invertebrate-types of lysozyme too (Regel et al. 1998; Xue et al. 2010; Wang et al. 2012), although no such evidence has yet been reported in bats.

Expression patterns of lysozyme genes support the expectation that duplicated lysozymes in bats are possibly related to chitin digestion activity (Phillips et al. 1998). Although based on small sample sizes, we found that the Lyz-2 genes from both Pipistrellus and Scotophilus showed exceptionally high expression in the tongue, compared with less tissue-specific expression patterns in the Lyz-1 copies that are more consistent with a general function in immunity. Ideally larger sample sizes are desirable to verify these results, and also to determine whether expression occurs throughout the tongue or whether it is concentrated in the exocrine von Ebner’s gland (Reitamo et al. 1977). Although we recorded low Lyz-2 expression levels in the stomach (fig. 2A and B), functional assays revealed the duplicate copy continued to be active at low pH (fig. 3B). Consequently it is possible that Lyz-2 from the tongue continues to function after being swallowed, where its role would be augmented by stomach chitinase (Strobel et al. 2013). Our results from experimental assays also add weight to a role of Lyz-2 in digestion; in particular, the Lyz-2 in P. abramus displayed significantly greater lytic efficiency for glycol chitin than did PabrLyz-1, whereas antibacterial activity in PabrLyz-2 was similar to that of PabrLyz-1. A degree of functional redundancy in the derived copy, which has been reported in RNase1 (Zhang et al. 2002), could provide more favorable conditions for natural selection to promote a modified role.

Given our results from insect-eating bats, it is noteworthy that we observed markedly different expression patterns for the two duplicated lysozyme genes observed within the frugivorous bat C. sphinx. Specifically, the CspHLyz-1 gene was seen to be expressed widely across different tissue types, whereas the CspHLyz-2 showed very weak expression in the tongue. The low expression level of CspHLyz-2 could point to nonfunctionality, although we cannot rule out the possibility it is expressed elsewhere. If the former case is correct, then high \( \omega \) value (2.67) in the fruit bat lineage could be the consequence of relaxed selective constraint (supplementary table S2, Supplementary Material online). Although C. sphinx, like...
the flying fox *Pteropus giganteus*, may host symbiotic bacteria to assist with the digestion of cellulose and xylan in leaves (Ruby et al. 2000; Prem Anand and Sripathi 2004), we found no evidence that the duplicated lysozyme in *C. sphinx* plays a similar function to that found in the foreguts of some ungulates (Dobson et al. 1984).

Chitin is a major component of insect cuticle (Andersen 1979) and poses challenges to insect-eating species such as bats that must consume large numbers of insects each night (Kunz 1974; Anthony and Kunz 1977; Encarnação and Dietz 2006). The cuticles of some insects such as beetles present particular difficulties to process and digest (Evans and Sanson 2006). Some bats are known to remove chitinous parts of insects such as wings and legs before eating the softer body parts (Barclay et al. 1991), whereas other species are thought to break down chitin using chitinases, which may be either synthesized by symbiotic bacteria in the intestine (Whitaker et al. 2004) or expressed endogenously in the stomach (Strobel et al. 2013). Although the harder parts of insect exoskeletons might not necessarily be the most chitin-rich (Richards 1951), Phillips et al. (1998) found a relationship between lysozyme-reactivity in bat salivary glands and feeding on hard-bodied insects.

Our results from vespertilionid bats provide the best evidence to date for a chitinase-like function of some lysozymes in bats. Beetles are frequently eaten by *Pipistrellus, Nyctalus, Murina*, and 11 species (Jones 1995; Lee and Lee 2005; Thabah et al. 2007; Ma et al. 2008), whereas *Barbastella*—in which gene duplication was absent—is known to specialize on softer bodied insects such as moths (Rydell et al. 1996). Apart from fish, beetles are also the main prey of *M. lucifugus* (Ma et al. 2006), whereas the congeners *M. silioresensis* and *M. lucifugus* are thought not to specialize on beetles (Wei et al. 2006; Burles et al. 2008). Our integrated results suggest that gene duplication with subsequent functional modification of lysozymes in vespertilionid bats is likely to have contributed to the success and diversification of insect-eating bats. At the same time however, a chitinolytic function for lysozyme might not solely be an adaptation for diet. Indeed it is noteworthy that chitin is also a major component of the cell walls of some fungi (Bartricki-Garcia 1968). Because some fungi species, including *Geomyces destructans* that causes white-nose syndrome (Blehert et al. 2009), represent potential threats to bats, we cannot rule out the possibility that duplications of lysozyme genes have in part been driven for immune function.

**Materials and Methods**

**Molecular Cloning and Gene Sequencing**

To study the molecular evolution of bat lysozymes, we undertook polymerase chain reactions (PCR) and cloned the amplified products for 19 species of bat. Using RNAiso reagent (TaKaRa), we isolated total RNA from the following archived bat organs in −80 °C refrigerator: Liver (from 18 species), intestine (18 species), tongue (16 species), kidney (three species), stomach (three species), spleen (two species), and lung (two species). First-strand cDNA was synthesized with reverse transcriptase using the SuperScript III kit (Invitrogen). PCR primers used were Lyz-F (5′-GTT CGT GCT TCG CAG TCA AC-3′) and Lyz-R (5′-GAG AGA AAA GGA GCT GAA GAA GG-3′). The PCR protocol was: 5 min at 95 °C, then 30 cycles of 30 s at 95 °C, 30 s at 54 °C and 40 s at 72 °C, and with a 10 min extension at 72 °C. For cloning, we used the pMD19-T vector (TaKaRa) and all clones were sequenced on an ABI 3730 (Applied Biosystems). Repeated PCR were carried out in each species to screen and verify the duplicated genes cloned. In addition, lysozyme sequences from *P. vampyrus* and *M. lucifugus* were obtained by BLAST searches of the Ensembl database (www.ensembl.org, last accessed August 20, 2014). Detailed species and gene information is given in supplementary table S1, Supplementary Material online.

**Phylogenetic Reconstruction and Selection Test**

For phylogenetic reconstruction, the TVM+Γ nucleotide substitution model was selected based on Akaike information criterion in ModelTest Server (Posada 2006). We undertook Bayesian phylogenetic inference using MrBayes 3.1.2 software (Ronquist and Huelsenbeck 2003), ML inference using RAxML 7.0.4 (Stamatakis 2006), NJ and MP inferences using MEGA 5 (Ronquist and Huelsenbeck 2003). For Bayesian inference, 5 million Markov chain Monte Carlo generations were performed with the first 2 million discarded as burn-in. For ML, 200 topologies were searched and 1,000 bootstrap replications were performed, and for NJ and MP, 2,000 bootstrap replications were performed. We tested for evidence of gene conversion using the software GENECONV (Sawyer 1989).

To test for positive selection on bat lineages, we estimated the ratio of the rate of nonsynonymous substitutions to the rate of synonymous substitutions (ω). Values of ω that exceed 1 indicate positive selection. We implemented the free-ratio model in PAML version 4 in which ω is estimated independently for each branch in the tree (Yang 2007). A likelihood ratio test was used to compare the fit of the free-ratio model with that of one-ratio model in which ω was the same across all branches (Yang 1998). The gene tree from figure 1 was used as the given topology in selection analyses.

**Quantitative Real-Time PCR**

To determine the expression profiles of the duplicated genes, we performed quantitative real-time PCR (qPCR) experiments on an ABI 7300 (Applied Biosystems) for two insectivorous bats (*P. abramus* and *S. kuhlii*) and one fruit bat (*C. sphinx*). Two adult and nonpregnant individuals per species were captured in the field. A total of nine tissue types (heart, liver, spleen, lung, kidney, muscle, intestine, stomach, and tongue) were sampled and immediately flash frozen in liquid nitrogen before being transferred to −80 °C refrigerator in lab. The qPCR primers and probes were designed using the software Primer Express (Applied Biosystems) and the detailed information is given in supplementary table S4, Supplementary Material online. For qPCR, the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a control. The 5′-ends of the TaqMan probes were labeled
with reporter dye (6-carboxyfluorescein; FAM) and the 3'-end with quenching dye (6-carboxytetramethylrhodamine; TAMRA). Note the 3'-ends of the two Scotophilus probes were labeled with a minor groove binder and a nonfluorescent quencher rather than TAMRA.

The amplification efficiencies of lysozyme and GAPDH genes were determined by qPCR using a serial dilution of cDNA samples (Livak and Schmittgen 2001). The efficiencies of the two genes were close to 100% and thus relative gene expression analysis could be applied using qPCR. The PCR was carried out in 20 µl volume containing 10 µl TaqMan master mix, 0.4 µl Rox Reference Dye, 900 nM primers, 100–200 nM TaqMan probe, and 1 µl cDNA sample. The PCR protocol was: 20 s at 95°C, then 40 cycles of 8 s at 95°C, and 31 s at 60.5°C. Each cDNA sample was amplified by three independent PCRs. All data were analyzed using Sequence Detector Systems software (Applied Biosystems). The average threshold cycle (ΔCt) values of Lyz-1 genes in muscle were used for calibration. To compare tissue-specific gene expression levels, we used the 2−ΔΔCt method in which all ΔCt values were compared with the value obtained for muscle (Livak and Schmittgen 2001).

Functional Assay on Recombinant Protein
To determine the putative function of bat lysozyme, we expressed both the PabrLyz-1 and PabrLyz-2 genes from the insectivorous bat P. abramus, the latter of which showed high expression in the tongue. The two pipistrelle genes were ligated into the Ncol and XhoI sites of a pET-28a (+) vector (Novagen), respectively. The recombinant plasmids were then transformed into the Escherichia coli strain Rosetta (DE3). Recombinant proteins were purified and quantified following published methods (Baneyx 1999; Supungul et al. 2010).

We quantified the bacteriolytic activity of the recombinant proteins using a modified version of the protocol described in Kikuchi et al. (1988). Here, we used 180 µl of 0.2 µg/µl M. lysodeikticus (Sigma-Aldrich) in phosphate-citrate buffer with pH values ranging from 4.2 to 8.0 (0.2 M disodium hydrogen phosphate and 0.1 M citric acid). Then, 4 µg lysozyme was mixed with the solution quickly to a final volume of 200 µl. The initial absorbance value at 450 nm was measured in an iMark microplate reader (Bio-Rad). The solution was then incubated at 37°C for 30 min with a final ice bath to stop the reaction. The absorbance value was measured immediately using an iMark microplate reader (Bio-Rad). The absorbance value was measured again immediately after reaction termination. One unit of lysozyme activity (U) is defined as the amount of enzyme causing a 0.001 decrease of absorbance value at 450 nm per minute (Kikuchi et al. 1988). HEWL (Sigma-Aldrich) was used as a positive control and the well contained all the reagents except the enzyme was used as a blank control.

To assess the chitinolytic activity of the bat lysozymes, we undertook assays using the chitin-derived substrate glycol chitin (~90% degree of acetylation, Polyscitech), which is commonly used as a substitute for nonsoluble chitin. In the assay, we added 4 µg of purified lysozyme to 100 µl of 0.05% substrate with 0.1 M acetic acid-sodium acetate buffer (pH values at 5.0, 6.0, 7.0, and 8.0). The solution was incubated for 30 min at 37°C. Two hundred microliters of color reagent (0.5 g of potassium ferricyanide dissolved in 1 L of 0.5 M sodium carbonate) was added and boiled for 15 min. To determine the activity of chitinolytic hydrolysis we examined the absorbance at 420 nm, which reflects the quantity of the product N-acetylglucosamine (Imoto and Yagishita 1971). We included a positive control that contained chitinase (Sigma-Aldrich) and a negative control that contained no enzyme but all other reagents. One unit of lysozyme activity is defined as the amount of enzyme that causes a decrease in absorbance of 0.01 at 420 nm per minute.

Supplementary Material
Supplementary figures S1 and S2 and tables S1–S4 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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