

## ORIGINAL ARTICLE

# *Leucoagaricus gongylophorus* uses leaf-cutting ants to vector proteolytic enzymes towards new plant substrate

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**The mutualism between leaf-cutting ants and their fungal symbionts revolves around processing and inoculation of fresh leaf pulp in underground fungus gardens, mediated by ant fecal fluid deposited on the newly added plant substrate. As herbivorous feeding often implies that growth is nitrogen limited, we cloned and sequenced six fungal proteases found in the fecal fluid of the leaf-cutting ant *Acromyrmex echinator* and identified them as two metalloendoproteases, two serine proteases and two aspartic proteases. The metalloendoproteases and serine proteases showed significant activity in fecal fluid at pH values of 5–7, but the aspartic proteases were inactive across a pH range of 3–10. Protease activity disappeared when the ants were kept on a sugar water diet without fungus. Relative to normal mycelium, both metalloendoproteases, both serine proteases and one aspartic protease were upregulated in the gongylidia, specialized hyphal tips whose only known function is to provide food to the ants. These combined results indicate that the enzymes are derived from the ingested fungal tissues. We infer that the five proteases are likely to accelerate protein extraction from plant cells in the leaf pulp that the ants add to the fungus garden, but regulatory functions such as activation of proenzymes are also possible, particularly for the aspartic proteases that were present but without showing activity. The proteases had high sequence similarities to proteolytic enzymes of phytopathogenic fungi, consistent with previous indications of convergent evolution of decomposition enzymes in attine ant fungal symbionts and phytopathogenic fungi.**

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## Introduction

Obligate symbioses are characterized by high degrees of partner commitment and functional complementarity (Janzen, 1985). Such interactions normally show only minor remnants of the potential reproductive conflicts that needed to be overcome when these partnerships evolved, allowing them to ultimately become single adaptive units with organismal properties (Queller and Strassmann, 2009; Boomsma, 2013). Such high levels of interdependency are most characteristic for endosymbioses where the symbiont lives in cells or tissues of the host, but may also occur in ectosymbioses like the

eusocial fungus-farming mutualisms of ants and termites where the fungus gardens are ectosymbionts for the individual ants but endosymbionts for the colony (Poulsen and Boomsma, 2005; Aanen *et al.*, 2009). We are intuitively inclined to consider the insect colony as the active farming host and the fungus gardens as passive crops, but the contention that fungal clones have contracted ant families to propagate them across generations appears to be equally valid, especially in the attine ants where the fungal symbiont is vertically transmitted (Poulsen and Boomsma, 2005).

In terms of biomass conversion efficiency, the functional complementarity between attine ants and their fungus garden symbionts achieved spectacular progression, moving from initial stages of ant-driven litter-based decomposition farming that evolved 50 million years ago (MYA) to advanced farming of irreversibly domesticated crop fungi 20 MYA (Mueller *et al.*, 1998; Schultz and Brady, 2008; Mehdiabadi and Schultz, 2010), culminating in

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aggressive herbivorous leaf-cutting farming 10 MYA (De Fine Licht *et al.*, 2010; Schiøtt *et al.*, 2010). Particularly, the latter transition allowed the ants to evolve much larger and complex societies (Mueller *et al.*, 1998; Villesen *et al.*, 2002; Schultz and Brady, 2008; Fernandez-Marin *et al.*, 2009; Mehdiabadi and Schultz, 2010) after they had managed to overcome a series of challenges that are associated with a herbivorous life-style (Schiøtt *et al.*, 2008, 2010; De Fine Licht *et al.*, 2010, 2013). Although depending on live vegetation rather than dead leaf litter undoubtedly implied better access to the proteins that normally limit insect growth, an almost exclusive fungal diet is generally poorer in protein than the carnivorous diets of many hunter-gatherer species in the Myrmicinae subfamily of ants (Davidson, 2004). Hosts and symbionts can thus be assumed to have been under consistent selection to maximize protein yield as this will determine colony growth rate and the production of winged virgin queens that transmit both their own genes and clonal copies of the fungal symbiont to future generations.

Specialized foraging on live plant material as growth substrate for the fungal symbiont *Leucoagaricus gongylophorus* implies that leaf-cutting ants are major herbivores in the Neo(sub)tropics with substantial roles in recycling nitrogen and phosphorus (Fowler *et al.*, 1989). A recent study has confirmed that the leaf-cutting fungus-farming mutualism is likely to be nitrogen limited as *Atta* fungus gardens harbour substantial levels of nitrogen-fixing bacteria (Pinto-Tomás *et al.*, 2009). Studies on the other genus of leaf-cutting ants, *Acromyrmex*, have shown that the fungal symbiont uses the ants to vector pectinases and a laccase from the most productive middle layers of fungus gardens to the top where they are most useful for the symbiosis but where fungal biomass to produce these enzymes is in short supply (Schiøtt *et al.*, 2010; De Fine Licht *et al.*, 2013). The fungal gongyliidia, special hyphal tips produced only to feed the ants and their brood (Bass and Cherrett, 1995), are instrumental in this process as they upregulate the expression of genes producing these enzymes (Schiøtt *et al.*, 2010; De Fine Licht *et al.*, 2013). These studies indicate that the fungal symbiont uses the ants for vector enzymes for the neutralization of phenolic compounds (laccase) and for loosening up the cell wall matrix (pectinases) to new garden sections. However, whether these initial decomposition steps are followed by a similar boost in protease activity to decompose intracellular proteins has remained unknown.

The ants process freshly cut leaf fragments into a mass of leaf pulp while mixing it with droplets of fecal fluid and subsequently deposit the new substrate on fungal ridges in the top of the garden where they inoculate it with small tufts of mycelium from the older part of the garden (Weber, 1966). In the 1970s, leaf-cutting ant fecal fluid was shown to contain active proteases (Martin, 1970, 1974;

Martin and Martin, 1970a, b, 1971) with similar chemical properties as enzymes originating from the fungal symbiont (Boyd and Martin, 1975a, b), and recent work has shown that fungus garden endo-protease activity in evolutionarily derived leaf-cutting ants is much higher than in sister ant lineages that do not use fresh leaves to make their gardens grow (De Fine Licht *et al.*, 2010), activity that could subsequently be assigned to metalloproteases and serine proteases (Semenova *et al.*, 2011).

Recently, we obtained the proteome of *Acromyrmex echinator* fecal fluid and were able to identify 33 proteins. Among these were seven pectinases (Schiøtt *et al.*, 2010), one laccase (De Fine Licht *et al.*, 2013) and seven proteases. Only a single of these proteases is known to be produced by the ants, whereas the other six are contributed by the fungal symbiont. We hypothesized that a number of these proteases might serve to either process the intracellular proteins of freshly opened plant cells or to assist in the breakdown of secondary plant defences (Christeller *et al.*, 1992; Mercado-Flores *et al.*, 2003; Ievleva *et al.*, 2006). The latter is a distinct possibility as plants are known to use protease inhibitors to counteract the harmful effect of proteases produced by phytopathogenic fungi and to generally decrease the digestibility of their tissues to discourage herbivores (Habib and Fazili, 2007). The objectives of the present study were to: (1) measure the specific protease activity in fecal fluid of gardening *A. echinator* workers, (2) confirm that they are derived from the fungal symbiont and have different expression levels and pH optima, (3) assess the extent to which the expression of genes coding for these enzymes was enhanced in the fungal gongyliidia, as would be expected when vectoring these enzymes to the top of fungus gardens is a specific adaptation of the symbiosis and (4) discuss the implications of our results for understanding the co-adaptations between partners that has allowed this symbiosis to evolve its substantial ecological footprint in the Neo(sub)tropics.

## Materials and methods

### *Biological material*

We used seven colonies of *A. echinator* (Ae263, Ae280, Ae322, Ae332, Ae335, Ae349 and Ae370), collected in and around Gamboa, Panama, between 2004 and 2007 and kept in rearing facilities in Copenhagen under controlled conditions of ca 25 °C and ca 70% humidity, where they were fed twice a week with fresh bramble leaves, apple pieces and dry rice. Fecal fluid was obtained by gently squeezing the abdomen of large workers with a forceps on a microscope slide. Each fecal droplet was then mixed with 0.5 µl of demineralized water, collected with a micropipette and stored in an Eppendorf tube on ice. Sixty droplets from five colonies each (Ae263, Ae280, Ae322,

Ae332 and Ae349) were collected this way, pooled per colony and diluted with demineralized water to a final volume of 250  $\mu$ l.

For the gene expression measurements, gongyldia clusters (staphylae) and normal mycelium were collected separately under a stereomicroscope at  $\times 40$  magnification from each of five colonies (Ae263, Ae280, Ae322, Ae335 and Ae370) in 2 ml Eppendorf tubes floating in liquid nitrogen. After collecting approximately 100  $\mu$ l for each type of tissue, samples were stored at  $-80^{\circ}\text{C}$  for subsequent RNA extraction.

#### Protein identification and gene cloning

SDS-polyacrylamide gel electrophoresis and mass spectrometry were performed as described previously (Schiøtt *et al.*, 2010; De Fine Licht *et al.*, 2013). RNA extraction from the fungal symbiont, followed by cDNA construction, was done as given in Schiøtt *et al.* (2010). *Aspartic protease 2 (AspII)*, *metallopeptidase 1 (MetI)*, *serine protease 1 (SerI)* and *serine protease 2 (SerII)* were initially identified by PCR amplification from cDNA using degenerate primers constructed from the mass spectrometry data using the PCR scheme: one cycle of  $95^{\circ}\text{C}$  for 5 min, 35 cycles of  $94^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 2 min, and ending with one cycle of  $72^{\circ}\text{C}$  for 7 min. *Aspartic protease 1 (AspI)* and *metallopeptidase 2 (MetII)* were identified at a later stage by a Blast search of the ca 100x coverage *A. echinator* genome (Nygaard *et al.*, 2011) and a low coverage genome sequence of the *A. echinator* fungal symbiont (De Fine Licht *et al.*, 2013) using the mass spectrometry data as queries. Sequencing of full-length gene transcripts was done using a RACE (Rapid Amplification of cDNA Ends) strategy. 3'- and 5'-RACE libraries were made from ca 1  $\mu$ g of purified RNA with the SMART RACE cDNA kit (Clontech, Mountain View, CA, USA), and gene sequences were PCR amplified from these libraries using specific primers designed from the PCR-amplified gene fragments (*AspII*, *MetI*, *SerI* and *SerII*) or BLAST search-identified sequence reads (*AspI* and *MetII*) along with the primers enclosed in the SMART RACE cDNA kit. In some cases, the 5'-RACE had to be done in several steps to get a full transcript sequence. The following PCR scheme was used in the RACE experiments: one cycle of  $95^{\circ}\text{C}$  for 5 min, 10 cycles of  $94^{\circ}\text{C}$  for 20 s,  $72^{\circ}\text{C}$  for 30 s (with a decrease in temperature of  $0.5^{\circ}\text{C}$  in every cycle) and  $72^{\circ}\text{C}$  for 2 min, followed by 35 cycles of  $94^{\circ}\text{C}$  for 20 s,  $67^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 2 min, and ending with one cycle of  $72^{\circ}\text{C}$  for 7 min. All PCR products were cloned in pCR4-TOPO using the TOPO TA cloning method (Invitrogen, Carlsbad, CA, USA) and sequenced at Eurofins MWG Operon (Ebersberg, Germany). Gene sequences are deposited in GenBank with accession numbers KF571927–KF571932. Primer sequences for cloning of the genes are provided in Supplementary Table 1.

#### Enzyme assays

Protease activity was measured for four different protease inhibitors and two controls (without inhibitors, one of which incubated and the other not incubated) at eight different pH levels. The inhibitors used in the experiment were 5 mM 1,10-phenanthroline to inhibit metalloendoproteases, 2  $\mu$ M pepstatin to inhibit aspartic proteases, 10 mM phenylmethylsulfonyl fluoride to inhibit serine proteases and 10  $\mu$ M *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane to inhibit cysteine proteases. In the assay, 4  $\mu$ l of the diluted fecal fluid was mixed with 1  $\mu$ l of inhibitor or distilled demineralized water and incubated on ice for 1 h. Next, 35  $\mu$ l of 0.1 M Britton Robinson buffer (Britton and Robinson, 1931) with 2% azoalbumin was added and the mix was incubated at  $30^{\circ}\text{C}$  for 1 h. For the blank control (not incubated), the azoalbumin mix was added at the last moment without incubation to prevent any protease activity to occur. To stop the activity of the proteases, 120  $\mu$ l of 10% trichloroacetic acid was added, after which the mix was vortexed and incubated for 15 min before centrifugation for 5 min at 8000 g. The supernatant (86  $\mu$ l) was added to 100  $\mu$ l of freshly prepared 1 M NaOH on a 96-well cell culture plate.

Absorbance was measured at 440 nm on a Versamax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA) and scored after subtracting the corresponding blank-control measurements to correct for background absorbance. To find the enzyme activity in each of the four protease classes, measurements obtained with specific inhibitors for each protease class were subtracted from the total protease activity in identical assays without addition of inhibitors. Protease activity was defined as the amount of enzyme required to cause a unit increase at 440 nm across a 1 cm path length (Sarath *et al.*, 2001). The number of units of protease activity data was plotted and analysed with R (R Core Team, 2013), using Linear Mixed-Effects Models ('lme'; Pinheiro *et al.*, 2013), with colony as random factor, and generating *P*-values with General Linear Hypotheses ('glht') in the Simultaneous Inference in General Parametric Models package 'multcomp' (Hothorn *et al.*, 2008).

For measuring protease activity in control ants, 100–200 workers of the colonies Ae322, Ae332 and Ae349 were transferred to subcolonies with no access to fungus garden material, but with an *ad libitum* diet of 10% sucrose and bramble leaves. After ca 2 weeks, protease activity was measured in pooled samples of fecal droplets from two ants for each colony at pH 6 in three replicates per colony.

#### Quantitative real-time PCR

Primers for the six different genes (Supplementary Table 2) were designed by matching the obtained cDNA sequences to a database of a partially sequenced genome of the *A. echinator* fungal

symbiont (De Fine Licht *et al.*, 2013) using BLASTn to identify intron and exon sequences. Primers were designed to span an intron to avoid amplification of genomic DNA. The quantitative PCRs (qPCRs) were run on an Mx3000P QPCR System (Agilent, Santa Clara, CA, USA) in 20  $\mu$ l reactions (0.5  $\mu$ l cDNA, 10  $\mu$ l 2  $\times$  SYBR Premix Ex Taq (TaKaRa Bio Inc., Otsu, Japan) and 0.4  $\mu$ l of each primer (10  $\mu$ M)) with the following PCR conditions: one cycle of 95  $^{\circ}$ C for 2 min, followed by 40 cycles of 95  $^{\circ}$ C for 30 s, 55  $^{\circ}$ C (*AspI*, *AspII*, *MetII*, *SerI*, *SerII* and *elongation factor 1- $\alpha$*  (*EF1 $\alpha$* )) or 57  $^{\circ}$ C (*MetI*, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *ubiquitin* (*Ubc*)) for 30 s and 72  $^{\circ}$ C for 30 s, and ending with a melting curve cycle of 95  $^{\circ}$ C for 30 s, 55  $^{\circ}$ C or 57  $^{\circ}$ C for 30 s and 95  $^{\circ}$ C for 30 s.

For each of the 10 samples (one gongylidia and one mycelium sample from five colonies), three technical qPCR replicates were done for nine genes: three housekeeping genes (*GAPDH*, *Ubc* and *EF1 $\alpha$* ) and six target genes (*MetI*, *MetII*, *AspI*, *AspII*, *SerI* and *SerII*). All Ct values from the reverse transcription qPCR analysis were analysed in R with packages 'ReadqPCR' (Perkins and Kohl, 2011) and 'NormqPCR' (Kohl and Perkins, 2011) using averages across the three technical replicates. Stability of housekeeping genes was assessed across the entire data set, which showed that *GAPDH* and *Ubc* were more stable than *EF1 $\alpha$* , so the latter gene was discarded in the further analyses. *GAPDH* and *Ubc* were then used to calculate the normalized expression ( $2^{\Delta Ct}$ ) of the target genes in gongylidia and mycelium. Values of  $2^{-\Delta\Delta Ct}$  (Livak and Schmittgen, 2001) for each gene subsequently produced estimates of fold changes in relative gene expression between gongylidia and mycelium. This sequence of procedures allowed us to identify genes with significantly different expression levels and to obtain estimates of their normalized and relative expression.

## Results

The mass spectrometry data of fecal fluid proteins (Table 1) described previously (Schiøtt *et al.*, 2010) allowed us to clone and sequence six protease genes encoded by the fungal symbiont genome, and to identify an ant-encoded protease (EGI65848) belonging to a family of M14 carboxypeptidases, which is significantly expanded in the genome of *A. echinator* (Nygaard *et al.*, 2011). Comparison with the MEROPS database release 9.8 (<http://merops.sanger.ac.uk/>; Rawlings *et al.*, 2012; Supplementary Table 3) showed that all six proteases (*AspI*, *AspII*, *MetI*, *MetII*, *SerI* and *SerII*) were similar to peptidases known from other fungi; *AspI* with *saccharopepsin* (A01.018) from *Laccaria bicolor* (78.57%,  $E$ -value =  $1.10e^{-142}$ ); *AspII* with *polyporopepsin* (A01.019) from *L. bicolor* (71.60%,  $E$ -value =  $1.80e^{-125}$ ); *MetI* with *peptidyl-Lys metallopeptidase*

(M35.004) from *Pleurotus ostreatus* (56.55%,  $E$ -value =  $1.50e^{-53}$ ); *MetII* with *peptidyl-Lys metallopeptidase* (M35.004) from *P. ostreatus* (69.23%,  $E$ -value =  $2.60e^{-42}$ ); *SerI* with an unassigned peptidase from the S8A subfamily from *L. bicolor* (61.28%,  $E$ -value =  $6.90e^{-111}$ ); *SerII* with *grifolisin* (S53.010) from *L. bicolor* (66.67%,  $E$ -value =  $4.40e^{-136}$ ).

To test the overall protease activity in the ant fecal fluid and to determine whether the fungus garden is responsible for this activity, protease activity was measured in fecal droplets of worker ants taken directly from their colonies and from purged ants that had been kept on sugar water and bramble leaves for ca 2 weeks with no access to fungal garden material. This showed that protease activity is present in fecal fluid when the ants have their normal diet, but disappears almost completely when the ants are deprived of fungus garden material ( $t_{17} = 14.8972$ ,  $P < 0.0001$ ), indicating that the protease activity in the ant fecal fluid is caused by ingested proteases from the fungal symbiont and not by proteases produced by the ants themselves (Figure 1).

Next, we tested the activity of the four main types of proteases using different inhibitors specifically targeted at each category of proteases: metalloendopeptidases, serine proteases, aspartic proteases and cysteine proteases. Only the metalloendopeptidases and serine proteases had significant activities, with respective peaks at pH 6 ( $z = -10.824$ ,  $P < 0.0001$ ) and pH 7 ( $z = 4.654$ ,  $P < 0.0001$ ) and serine protease activity also being significantly enhanced at pH 6 ( $z = 2.004$ ,  $P < 0.05$ ), consistent with fecal fluid also having pH 6 (Figure 2). In contrast to serine proteases, metalloendopeptidases were active at a very broad range of pH values and retained activities up to pH 10. We found no activity for aspartic proteases (present in fecal fluid), and cysteine proteases (absent in fecal fluid).

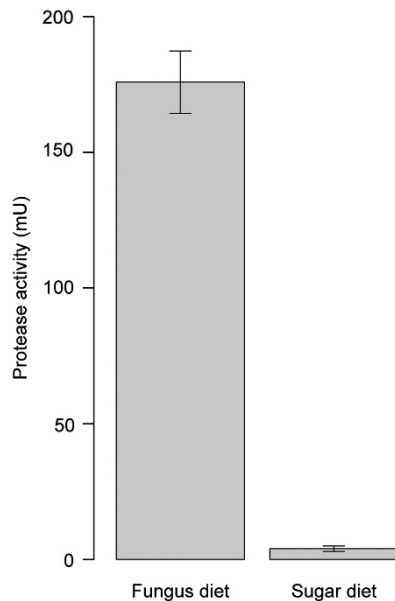
Relative changes in gene expression in gongylidia versus mycelium (fold-changes:  $2^{-\Delta\Delta Ct}$ ) showed that the expression of five out of the six fungal protease genes was upregulated in the gongylidia compared with the mycelium (Figure 3a): *peptidyl-Lys metallopeptidase I* and *II* (*MetI* and *MetII*;  $z = -3.979$ ,  $P = 0.00004$ ;  $z = -20.010$ ,  $P = 0.00001$ ), *saccharopepsin* (*AspI*;  $z = -3.946$ ,  $P = 0.00004$ ), *subtilisin* (*SerI*;  $z = -8.927$ ,  $P = 0.00001$ ) and *grifolisin* (*SerII*;  $z = -9.041$ ,  $P = 0.00001$ ), whereas the expression of *polyporopepsin* (*AspII*) remained unchanged ( $z = 2.580$ ,  $P = 0.99492$ ). This is consistent with all but one of these fungal proteases having their main function after being ingested by the ants, that is, after they pass the ant guts unharmed to be deposited with the fecal fluid.

Plotting relative gene expression (fold change in gongylidia versus mycelium) as a function of normalized gene expression in the gongylidia (that is, expression relative to housekeeping genes; Figure 3b) showed proportionality for four of the

**Table 1** Peptide sequences of proteases in fecal fluid of *Aechinator* and corresponding molecular weights (Da) of full-length proteins

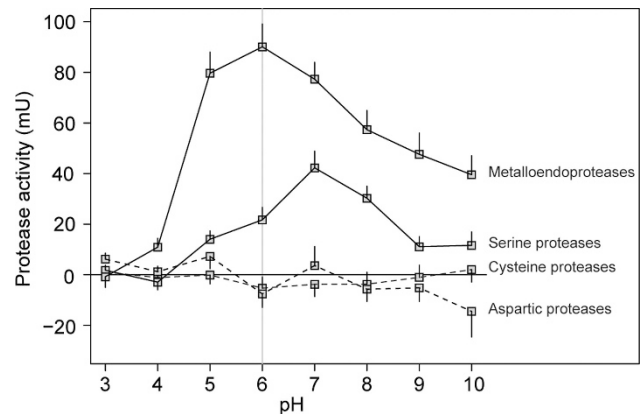
Protein	Mass spectrometry data	Sequence data	MW (Da)
Aspartic protease 1	DENDGGEATFGGJNPSSYR NAYWEV YFTVYDJGR	DENDGGEATFGGINPSSYR NAYWEV YFTVYDLGR	44 788
Aspartic protease 2	FTGAJNFTPR SSGFEGTDGJGJGPVDJTR GTJSPAVNSJVPVTDNJFSSGR TSTSPSSJFWGJNQSVR VJDNNTGJJR	FTGALNFTPR SSGFEGTDGILGIGPVDLTR GTLSPAVNSLVPTVTDNLFSSGR TSTSPSSLFWGLNQSVR VLDNNTGLLR	43 158
Metallopeptidase 1	TYASNAATYJSSHSSSTR YTTWFGTYTSAR	TYASNAATYLSSHSSSTR YTTWFGTYTSAR	37 207
Metallopeptidase 2	TYASNAJJYJR	TYASNALIYLR	37 511
Serine protease 1	AWAGJHFAVAAGNDNR GSDGSGTSDVJAGVQWAAR	AADAGLHFAVAAGNDNR GSDGSGTSDVIAGVQWAAR	50 114
Serine protease 2	ADJQTFFR AGYJDEFANR AJYNTVNYVPSQTSR DGJGVAGYJDEFANR GVAGYJDEFANR GSVGGTSASSPTVAGVFAJJNDFR FRPDAAGSSFTTVR YFSTPSYQSAAVSR	ADLQTFFR AGYLDEFANR ALYNTVNYVPSQTSR NGLGVAGYLDEFANR GVAGYLDEFANR GSVGGTSASSPTVAGVFALLNDFR FRPDAAGSSFTTVR YFSTPSYQSAAVSR	63 388
Carboxypeptidase A (EGI65848)	QNNPGVFFESGJHAR	QNNPGVFFESGIHAR	46 943

The letter J symbolizes the amino acids leucin and isoleucin, which have the same molecular mass and are therefore indiscernible by mass spectrometry. The single discrepancy between mass spectrometry data and sequence data (letters D and N in bold) may have been caused by deamination of asparagine into aspartic acid.



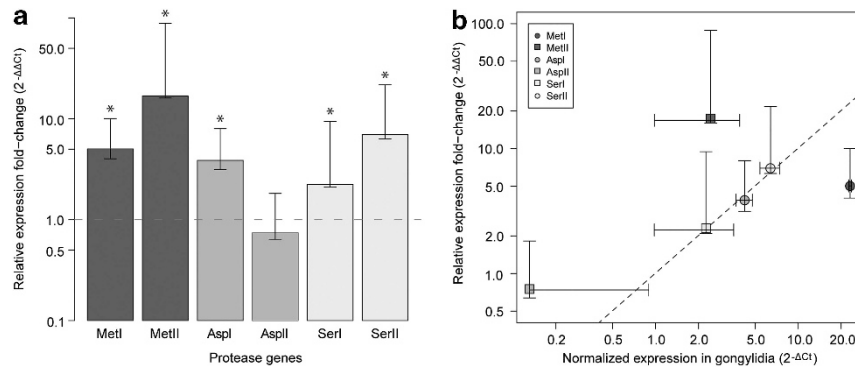
**Figure 1** Enzyme activity in milliunits of proteases (mU) in fecal fluid of workers of the leaf-cutting ant *A. echinator* kept on natural fungus-garden-diet and on a diet of only sugar water and possibly plant sap as control ( $t_{17} = 14.8972$ ,  $P < 0.0001$ ). Whiskers are s.e. of the mean.

proteases (*saccharopepsin*, *subtilisin*, *grifolisin* and *polyporopepsin*). However, the two metalloendoproteases deviated from the 1:1 proportionality represented by the diagonal in Figure 3b. One of them (*MetIII*) combined a fairly low normalized expression (Supplementary Table 4) with a 17x upregulation in



**Figure 2** Activity of metalloendoproteases, serine proteases, aspartic proteases and cysteine proteases in milliunits (mU). Both metalloendoproteases ( $z = 10.824$ ,  $P < 0.0001$ ) and serine proteases ( $z = 2.004$ ,  $P < 0.05$ ) showed significantly enhanced activity at pH 6 relative to the zero baseline. Serine proteases had their peak activity at pH 7 ( $z = 4.654$ ,  $P < 0.0001$ ) and activities of metalloendoproteases at pH levels  $> 7$  remained equally significant as those at pH 6. Whiskers are s.e. of the mean.

gongylidia, and the other (*MetI*) combined a high normalized expression with a relatively low 5x fold upregulation in gongylidia. This implies that the expression of *MetII* is much more gongylidia specific than the expression of *MetI*, which is apparently also needed in high quantities in the undifferentiated mycelium. It also appeared that the single non-differentially expressed protease (*AspII*) had a low normalized expression level (Figure 3b), indicating that it may not have a very important role



**Figure 3** Average relative gene expression (fold change) across gongyliidia collected from fungus gardens of five colonies of *A. echinator*: dark grey = metalloendoproteases, grey = aspartic proteases, light grey = serine proteases. (a) Relative expression fold changes (mean  $\pm$  s.e.) in gongyliidia compared with mycelium, with the dashed horizontal line representing no change in expression. Target genes were significantly upregulated in the gongyliidia ( $P=0.00004$ ,  $0.00001$ ,  $0.00004$ ,  $0.99492$ ,  $0.00001$  and  $0.00001$  for *MetI*, *MetII*, *AspI*, *AspII*, *SerI* and *SerII*, respectively, marked with asterisk (\*)), except for *polyporopepsin (AspII)*. (b) Relative expression fold-change plotted against gene expression normalized for the housekeeping genes *GAPDH* and *Ubc* (means  $\pm$  s.e.). The three upregulated serine (*SerI* and *SerII*) and aspartic (*AspI*) proteases had fold-changes proportional to normalized gene expression (being very close to the dashed line diagonal), but *peptidyl-Lys metallopeptidase I (MetI)* combined a high normalized gene expression with a relatively low gene fold-change, and *peptidyl-Lys metallopeptidase II (MetII)* combined a low normalized gene expression with a relatively high gene fold-change.

under normal conditions, but might have an unknown adaptive function under specific field conditions that did not apply to our laboratory colonies.

## Discussion

In the present study, we used mass spectrometry to identify seven proteases in the fecal fluid of *A. echinator* leaf-cutting ants. For all but one of these we could exclude that they are produced by the ants, because we have a high-quality reference genome for the ants (Nygaard *et al.*, 2011), to which only one protease matched, and a poorly assembled genome for the fungal symbiont (De Fine Licht *et al.*, 2013) to which the remaining six sequences matched. Our protease assays showed both metalloendoprotease activity and serine protease activity in the ant fecal fluid, but when we deprived the ants of their fungal symbiont by giving them a diet of sugar water and bramble leaves, essentially all protease activity disappeared. We further showed that five out of the six protease genes were overexpressed in the fungal gongyliidia that the ants eat. In combination, the results strongly suggest that these proteases mostly belong to a distinct but restricted group of proteins that have been selected to be ingested by the ants for the sole purpose of being passed on to the fecal fluid where they provide functional mutualistic benefits for the farming symbiosis. The most parsimonious hypothesis to explain this remarkable phenomenon is that the stationary fungus uses the ants to vector crucial enzymes produced in the central parts of the garden, where the ratio between fungal biomass and remaining substrate allows high gongyliidia production, to the upper parts of the garden, where an abundance of new substrate is available but as yet without much

hyphal growth. These findings and interpretations are consistent with earlier results obtained for seven pectinases (Schiøtt *et al.*, 2010) and a laccase enzyme (De Fine Licht *et al.*, 2013). It remains possible that the ingested fungal enzymes also interact with unknown other compounds in the ant gut, produced either by the ants or their microbiome, but such additional complexities appear unnecessary to explain our current or previous results obtained for other enzymes in the gongyliidia and fecal fluid (Rønhede *et al.*, 2004; Schiøtt *et al.*, 2010; De Fine Licht *et al.*, 2013).

It remains to be seen whether the proteases that we identified may also have functions that are restricted to the gongyliidia. If that would be the case, the non-adaptive null hypothesis would be that finding them in the fecal fluid may merely imply that digestion was incomplete, but this seems unlikely given that SDS-polyacrylamide gel electrophoresis-banding patterns of fecal fluid were always consistent, showing a very specific subset of proteins that escape digestion (Schiøtt *et al.*, 2010). Together with our previous work (Schiøtt *et al.*, 2010; De Fine Licht *et al.*, 2013), we have now shown that 12 of the 14 identified fungal pectinase, laccase and protease enzymes in the *A. echinator* fecal fluid have upregulated gene expression in the gongyliidia, which we believe underlines the key significance of these special hyphal tips as fungal organs mediating a series of symbiotic adaptations.

As it appears, the main function of the fecal fluid proteases is enhanced digestion of the most valuable nitrogen-containing molecules of the fresh plant material harvested by the leaf-cutting ants. *Peptidyl-Lys metallopeptidases (MetI and MetII)* and *serine carboxyl-peptidases (SerII)* have been suggested to be involved in the extraction of nutrients from extracellular proteins (Heck, 2012; Oda, 2012). As both *MetI*, *MetII* and *SerII* were active in the fecal

fluid (Figure 2) and upregulated in the gongylidia (Figure 3), it seems reasonable to hypothesize that these enzymes increase nitrogen extraction from the chewed-up leaf pulp after other enzymes such as the pectinases (Schiøtt *et al.*, 2010) have partially broken down the cell walls. The other active serine protease, subtilisin, is structurally similar to several unassigned fungal proteases in the subfamily S8A (Supplementary Table 3). Most of the matches in protein databases for this enzyme were proteases from general pathogenic or phytopathogenic fungi. This finding is intriguing, because most of the subtilisins in subfamily S8A are part of a large gene-family expansion in Angiosperms with functional roles in seed or fruit development or manipulating cell walls in response to changes in biotic and abiotic factors (Schaller, 2012; Schaller *et al.*, 2012).

Although subtilisins likely have defensive functions in plants, other studies have shown that subtilisins have important aggressive functions in saprotrophs, phytopathogens and other infectious pathogens (Sreedhar *et al.*, 1999; Dunaevskii *et al.*, 2006; Ievleva *et al.*, 2006; Bryant *et al.*, 2009; Li *et al.*, 2010). One explanation for this could be that using proteins very similar to those present in the hosts may allow pathogens to avoid detection by immune defences, a phenomenon referred to as molecular mimicry (Elde and Malik, 2009; Armijos Jaramillo *et al.*, 2013). Although the fungal symbionts of leaf-cutting ants hardly face any active immune defence in the severed plant material that the ants bring in, they will be challenged by substantial amounts of secondary (defensive) compounds, similar to any functional insect herbivores whose digestive systems need to cope with plant defences that decrease digestibility (Chen, 2008). Gongylidial subtilisin might therefore have some role in the penetration and digestion of newly harvested plant material, consistent with serine protease activity being also high in the gardens of other higher attine ants. Panamanian representatives of the genera *Trachymyrmex* and *Sericomyrmex* that readily use soft fresh leaves as substrate in the laboratory tend to have very high levels of serine protease activity in their fungus gardens, whereas these levels tend to be low in attine ant species that do not or only reluctantly use fresh leaves as fungal substrate (Semenova *et al.*, 2011, P. Kooij and M. Schiøtt, unpublished observations).

We found two aspartic proteases in the fecal fluid, but none of them showed activity (Figure 2), even though one of them, saccharopepsin, was upregulated in the gongylidia when compared with mycelium (Figure 3). This suggests that this protein has a function, either for the gongylidia or after ingestion by the ants. Saccharopepsin is known to be important in the activation of yeast vacuolar hydrolases (Parr *et al.*, 2007). This could explain the abundance of this enzyme in the gongylidia as they

always have a large vacuole, but it does not explain why this protease is passed on to the fecal fluid. As non-upregulated polyporopepsin is also found in fecal fluid, it might be that these proteases cleave very specific target sequences so they would not show activity in our general activity assays. Possible target sequences could be other fungal enzymes that are produced as inactive precursors (zymogens or proenzymes), which are subsequently activated when regulatory proteases cleave off part of the precursor amino-acid chain. Such an activation mechanism would be particularly appropriate for abundant plant-degrading enzymes that are also harmful to the fungus garden but not to the ants. These enzymes could then be produced in large quantities in the gongylidia, but only be activated in the ant gut to be mixed with the plant substrate via fecal fluid before the fungal hyphae are inoculated.

The possibility of leaf-cutting ant fungi having independently evolved adaptations reminiscent of phytopathogens is intriguing, so it is of interest to briefly evaluate recent studies addressing evolutionary arms-races between phytopathogens and their host plants (Misas-Villamil and van der Hoorn, 2008). Also here, metalloproteases appear to have a role in the breakdown of cell wall-stabilizing proteins (Rauscher *et al.*, 1995) and in targeting specific plant resistance proteins (Xia, 2004). Among serine proteases, trypsins appear to be most prevalent in phytopathogens, whereas subtilisins are more important in saprotrophs (Dunaevskii *et al.*, 2006, 2008). However, when they occur, subtilisins may have an important role in the breakdown of cell wall structural proteins (Murphy and Walton, 1996), as in infections of Kentucky bluegrass, *Poa pratensis* (Sreedhar *et al.*, 1999), and in *Colletotricum* fungi that apparently acquired a subtilisin gene by horizontal transfer from their host plant, encoding a protease which is active during infection (Armijos Jaramillo *et al.*, 2013).

The possible similarities in attine fungal proteases with those of phytopathogens (St Leger *et al.*, 1997) has interesting parallels to our earlier studies on pectinases (Schiøtt *et al.*, 2010), and a laccase (De Fine Licht *et al.*, 2013) in the fecal fluid of *A. echinator* ants. Also here, indications of convergent evolution were found, although the evidence remained largely indirect as in the present evaluation of our proteases results. Although many of the molecular mechanisms remain unknown (but see De Fine Licht *et al.*, 2013), it seems beyond doubt that selection pressure on herbivorous leaf-cutting ant fungi must have been largely in the same direction as in unrelated lineages of necrotrophic fungi. The relatively simple fecal fluid proteome (a total of ca 33 identified proteins so far in *A. echinator*) appears to be a valuable and relatively transparent window through which the molecular adaptations that have shaped fungus farming in the monophyletic clade of the attine ants can be unravelled.

## Conflict of Interest

The authors declare no conflict of interest.

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