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# Keeping the blood flowing—plasminogen activator genes and feeding behavior in vampire bats

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**Abstract** The blood feeding vampire bats emerged from New World leaf-nosed bats that fed on fruit and insects. Plasminogen activator, a serine protease that regulates blood coagulation, is known to be expressed in the saliva of *Desmodus rotundus* (common vampire bat) and is thought to be a key enzyme for the emergence of blood feeding in vampire bats. To better understand the evolution of this biological function, we studied the plasminogen activator (PA) genes from all vampire bat species in light of their feeding transition to bird and subsequently mammalian blood. We include the rare species *Diphylla ecaudata* and *Diaemus youngi*, where plasminogen activator had not previously been studied and demonstrate that PA gene duplication observed in *Desmodus* is not essential to the vampire phenotype, but relates to the emergence of predominant mammalian blood feeding in this species. Plasminogen activator has evolved through gene duplication, domain loss, and sequence evolution leading to change in fibrin-specificity and susceptibility to plasminogen activator inhibitor-1. Before undertaking this study, only the four plasminogen activator isoforms from *Desmodus* were known. The evolution of vampire bat plasminogen activators can now be linked phylogenetically to the transition in feeding behavior among vampire bat species from bird to mammalian blood.

**Keywords** Species adaptation · Positive selection · Domain evolution · Gene duplication · Ecological niche

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## Introduction

While vampire bats are legendary, little is known about their evolution and extraordinary feeding habits. Occurring exclusively in the New World, they concentrate mainly in the Neotropics. Three species, *Desmodus rotundus* (common vampire bat), *Diaemus youngi* (white-winged vampire bat), and *Diphylla ecaudata* (hairy-legged vampire bat), are

known. These species are closely related with blood-feeding activity thought to have arisen once (Wetterer et al. 2000).

Vampire bats are the only extant mammals whose nutrition depends entirely on blood, while closely related New World leaf-nosed bat species (such as *Carollia perspicillata*) eat insects or fruit. Among the vampire bats, *D. rotundus* feeds on mammals (Greenhall et al. 1983), *D. ecaudata* utilizes bird blood as a food source (Hoyt and Altenbach 1981), and *D. youngi* has been reported to feed on mammals while preferring bird blood (Sazima and Uieda 1980; Greenhall and Schutt 1996).

Blood coagulates after exposure to air and since this interferes with the ability to lap it from a wound, vampire bats make use of several hemostatic modulators to maintain its fluidity (Basanova et al. 2002). One of these modulators is a homolog of tissue type plasminogen activator (t-PA) (Hawkey 1966; Schleuning et al. 1992). Plasminogen activators are serine proteases that cleave the plasmin proenzyme to produce active plasmin. Plasmin is responsible for the degradation of blood clots.

Tissue-type plasminogen activator contains five domains, Finger (F), Epidermal Growth Factor (G), Kringle 1 (K1), Kringle 2 (K2), and Serine Protease (P). Both the F and K2 domains are involved in the competitive binding of fibrin, as well as its inhibitor, plasminogen activator inhibitor 1 (PAI-1), and DD(E), a degradation product of cross-linked fibrin. Additionally, there are fibrin-binding surface patches in the protease domain (Stewart et al. 1998). Plasminogen activator shows activity as a single chain form, but has substantially enhanced activity as a cleaved two chain form.

It was previously found that the common vampire bat expresses four plasminogen activators ($\alpha 1$, $\alpha 2$, $\beta$, $\gamma$) in its saliva (Kratzschmar et al. 1991). All four of these copies lack a plasmin-sensitive activation cleavage site and the K2 domain. The $\beta$ form additionally lacks the Finger domain, while the $\gamma$ copy also lacks both the Finger and Epidermal Growth Factor domains. Cleavage-site loss relegated these plasminogen activators to activity as single chain forms (Bode and Renatus 1997; Renatus et al. 1997). The K2 domain loss leads to decreased fibrinogen, PAI-1, and DD (E) sensitivity coupled with strong fibrin specificity for activation (Gardell et al. 1989). The $\alpha 1$ form (lacking the cleavage site and the K2 domain) is of clinical interest for treatment of stroke victims (Liberatore et al. 2003).

In this study, we sought to determine the number and gene structures of plasminogen activator genes in the three vampire bat species and the history of duplication coupled to sequence evolution and selection. Starting with a neutral null model of no selection for duplication and sequence evolution, we ask if there is evidence for selection on the retention and subsequent evolution of duplicate copies. We relatedly ask if the copy number and domain structures found in the common vampire bat are necessary for life as a vampire bat, or if alternatively these were neutral or selected for another aspect of the feeding behavior of the common vampire bat in a lineage-specific manner.

## Materials and methods

### DNA extraction

Genomic DNA was extracted from ethanol preserved liver tissue, hair, wing clippings, and muscle from several individuals of *D. rotundus, D. youngi, D. ecaudata*, and *C. perspicillata,* using both conventional phenol-chloroform extraction and the DNeasy animal tissue protocol (Qiagen, Germany), depending on the available amount of sample.

### Cloning and PCR

A first round of polymerase chain reactions (PCRs) was conducted using primer pairs developed from full-length cDNA sequence available in GenBank. In order to control for the presence of genotypic variation, and to identify duplicated copies, all successful first round PCRs were cloned using the TOPO TA® (pCR®2.1-TOPO®) kit (Invitrogen, California, USA) and grown on individual plates. Approximately 20 positive clones (identified by blue-white screening) per plate were picked and subjected to PCR screening using universal M13 primers.

### RNA extraction and RT-PCR

RNA was extracted from wing clippings and swabbed saliva cells, preserved in RNALater® (Ambion, Texas, USA). We used Trizol® (Invitrogen, California, USA), according to the manufacturer's specifications. First strand cDNA synthesis was carried out using the Superscript®III kit (Invitrogen, California, USA) according to manufacturer's specifications. This was used as a template for PCR. Primer pairs covered exon 3 through 6, and 7 through 10 to check for differential tissue expression patterns of exons 4 and 5, and 8 and 9, respectively.

### Sequencing

Sequencing was carried out using BigDye® Terminator v3.1 chemistry (Applied Biosystems, California, USA) at the Nucleic Acid Exploration Facility (NAEF) of the University of Wyoming, USA, and BigDye® Terminator v3.1 chemistry at the UiB Sequencing Facility, University of Bergen, Norway.

### Southern Blots

Southern Blots were carried out using genomic DNA extracts. Samples were digested using BamH1, Xba1 and Sma1 (NEB, Massachusetts, USA), after checking for restriction sites on full DSPA sequences from GenBank. Three enzymes were used to ensure consistency of results. Standard protocols were used for non-alkaline transfer to a nylon membrane (Hybond™N; GE Healthcare, England) (Frank 1997). Hybridization was carried out with a radioactive 32P labeled ss-probe, derived from the full length of exon 12 (which is present in all bat-PAs sequenced in this study). Hybridized probes were visualized on Kodak X-Ray film after 3 days exposure at −80°C.

### Phylogenetic analyses

#### *Sequence alignment*

Sequences were aligned using the amino acid translation in MAFFT 6, implementing the iterative refinement method and considering consistency scores (G-INS-I; Needleman-Wunsch), (Katoh et al. 2005). Alignments were then back-translated into respective nucleotide sequences, which were used to compute the tree topologies.

#### *Tree search*

Tree building was initially performed for the entire set of sequences and subsequently with a consensus sequence from each gene where multiple alleles were detected. Modeltest (Posada and Crandall 1998; Posada and Buckley 2004) was used to test for the best model of nucleotide substitution (GTR+I+Γ).

Trees were constructed using MrBayes (Ronquist and Huelsenbeck 2003) and maximum-likelihood as implemented in PHYML (GTR model, 100 bootstrap iterations) (Guindon and Gascuel 2003). Each Bayesian inference (three inferences), starting from a random tree and using four Metropolis-coupled Markov Monte Carlo chains, consisted of 1,000,000 generations with sampling every 100 generations. The average standard deviation of split frequencies was used to assess the convergence of the runs. Bayesian posterior probabilities were calculated from the majority rule consensus of the tree sampled after the initial burn-in period as determined by checking the convergence of likelihood values across generations.

### dN/dS ratios and selection

We tested for positive selection along branches and at sites, by using a variety of models implemented in PAML v3.14 (Yang 2007), and explicit probabilistic ancestral sequence reconstruction coupled to "along-branch" counting (Liberles 2001). We first checked for positive selection along specific lineages (ω (dN/dS) ratio allowed to vary among branches) (Yang 1998). It is however possible that positive selection is restricted to specific lineages and few amino acid sites within a protein, so we also examined a set of models that allow to variation among codon sites and lineages within a phylogeny (Zhang et al. 2005). In this model (Model A), the branch of interest (foreground) can have sites with an ω-value larger than one and all other branches (background) are restricted to ω-values below or equal to one (tests 1 and 2; test 1 reported). A Bayes empirical Bayes (BEB) procedure (Yang et al. 2005) was used to identify the sites evolving under potential positive selection (M1/M2). All results are presented in Tables 1 (Yang 2007) and 2 (Liberles 2001).

Confirmatory evidence for positive selection at the codon level was also sought using HyPhy (Pond et al. 2005). The REL approach allows the synonymous substitution rate to vary among codons under positive or negative selection along a branch. Since the dataset is relatively small and type I errors might be more common, random effects likelihood (Bayes Factor=50) was used. Consistent with the PAML results, the same sites were pinpointed with REL, together with some additional sites that achieved no significant values in PAML.

## Results

Consistent with previous research, we isolated four t-PA sequences from *D. rotundus*, corresponding to the known plasminogen activators. Southern Blots (Fig. 1) confirmed that each of these sequences represents a distinct gene. Intriguingly, while a fifth gene corresponding to the activities of t-PA from other mammalian species might have been expected (with activity as a dimer and regulation by fibrinogen and PAI-1), this was not observed. This is the first report of *Desmodus* t-PA gene structures. It has previously been found that mice with tissue-type plasminogen activator knocked out are viable (Carmeliet et al. 1994) and the common vampire bat may be a natural example of this. It may be that a combination of residual t-PA activity and overlapping activity with urokinase-type plasminogen activator may enable the viability of this genotype in the common vampire bat. This is discussed further in the context of the *Diaemus* gene below.

From the Southern Blot, the *Diaemus* and *Diphylla* genomes are found to contain only a single t-PA. Sequencing of the gene from several *Diaemus* and *Diphylla* individuals identified three divergent alleles with shared exon structures in *Diaemus* and four such alleles in *Diphylla*. The level of divergence and phylogenetic pattern

**Table 1** Results from testing nested models in PAML to detect positive selection along individual branches and sites are shown

| | -lnL | Omega values | LRT *P* value |
|---|---|---|---|
| Null model | 3,800.37 | ω=0.41 | N/A |
| Model (1a) | 3,742.37 | ω=0.34 (*p*=0.58) | N/A |
| Branch Model | Q: What branches are under positive selection? | | |
| 1—*Desmodus*+*Diaemus* | 3,792.89 | ω=2.23; background ω=0.36 | 0.0001 |
| 2—*Diaemus* | 3,795.77 | ω=1.41; background ω=0.37 | 0.0024 |
| 3—*Desmodus* α1 | 3,797.05 | ω=1.29; background ω=0.38 | 0.01 |
| 4—*Desmodus* | 3,792.67 | ω=7.79; background ω=0.37 | 0.0001 |
| 5—*Desmodus* α2,β,γ | 3,797.85 | ω=2.01; background ω=0.39 | 0.0248 |
| 6—*Desmodus* α2,β | 3,797.73 | ω=2.38; background ω=0.39 | 0.0216 |
| Branch–site models *Model A versus Model 1a* | Q: Are there site classes on particular lineages across the tree that have significantly different dN/dS from the rest of the tree, where dN/dS>1? | | |
| 1—*Desmodus*+*Diaemus* | 3,733.82 | w=0.27 (*p*=0.53); w=10.49 (pa=0.07; pb=0.05) | <0.0001 |
| 2—*Diaemus* | 3,740.95 | w=0.27 (*p*=0.56); w=16.79 (pa=0.01; pb=0.01) | <0.05 |
| 3—*Desmodus* α1 | 3,740.73 | w=0.26 (p=0.50); w=2.34 (pa=0.08; pb=0.06) | <0.05 |
| 4—*Desmodus* | 3,739.44 | w=0.03 (*p*=0.55); w=23.41 (pa=0.03; pb=0.02) | <0.05 |
| Bayes empirical Bayes | Q: Which of those sites have a dN/dS >1 when compared with dN/dS=1 rather than background selection? | | |
| | **Sites** | | |
| 1—*Desmodus*+*Diaemus* | 9, 127, 165, 168, 172, 178, 265, 270, 273, 276, 301, 370, 428, 432, 437, 467, 470, 484, 499, 502 | | |
| 3—*Desmodus* α1 | 113, 114, 151, 328, 354, 356 | | |
| 4—*Desmodus* | 278, 352, 394, 395, 401, 419, 420, 445, 468, 469, 493 | | |

l*n*L=log likelihood value for the model given the tree, dataset, and parameter estimates. The numbering of sites corresponds to AA counting beginning in the multiple sequence alignment (not correspondent to chymotrypsin numbering)

were consistent with intra-specific variation (see S1, S2). Interestingly, in *Desmodus*, we found a previously undetected remnant of an unexpressed exon 8 in the genomic sequences of *Desmodus* α1, α2, and β (Fig. 2). Similar to *Desmodus*, the *Diaemus* PA sequences also have a partial exon 8, and a missing exon 9 (K2 domain) in their gene structure (Fig. 2). Reverse transcription (RT)-PCR analysis of *Diaemus* salivary gland samples shows that the gene is expressed in saliva and that the cDNA resembles the exon composition of *Desmodus* α1. In both, *Diphylla* and *Carollia* we identified only one t-PA gene that was consistent with the typical t-PA, with all exons being present in the genomic sequence (Fig. 2). We were unable due to lack of sample availability to test for salivary expression and expressed domain structure of the *Diphylla* t-PA. Therefore, we cannot rule out an alternatively spliced isoform expressed in the saliva.

We were unable to detect expression of PA in *Diaemus* wing clippings. We cannot rule out that this negative result was due to technical barriers rather than truly reflecting a lack of expression. However, if the *Diaemus* gene is widely expressed and functioning as a t-PA, it is unclear what the phenotypic consequences of the missing K2 domain and the binding sites it contains are.

Phylogenetic analysis using both Bayesian (Ronquist and Huelsenbeck 2003) and maximum likelihood (Guindon and Gascuel 2003) methods with mouse t-PA as the outgroup resulted in identical tree topologies reflecting the known species relationships and three rounds of gene duplication on the *Desmodus* lineage (Fig. 3a). *Desmodus* salivary type PAs are derived from tissue type PAs, and inferring three rounds of gene duplication, are specific to the *Desmodus* lineage. The most parsimonious mapping of exon structure on the ML topology shows a pattern of progressive exon loss, beginning with the loss of exon 9 and part of exon 8 (K2) at the base of *Desmodus* and *Diaemus*, and culminating in the additional reduction of *Desmodus* t-PA γ by exon 8, 4 (F), and 5 (G), plus the independent loss of exon 4 (F) in *Desmodus* t-PA β. The loss of K2 predated the modification of the activation cleavage site, which is unique to the *Desmodus* lineage. This is summarized in Fig. 3b.

At the molecular level, gene duplication and subsequent functional divergence has long been recognized as a potential source of adaptive evolutionary novelty (Ohno 1970). However, neutral processes are also known to generate faster nonsynonymous nucleotide substitution rates and can lead to the retention of duplicate genes as well (Lynch and Conery 2003; Roth et al. 2007; Hughes and Liberles 2007). The ratio of nonsymonymous to synonymous nucleotide substitution rates (dN/dS) was used to test for positive diversifying selection along all lineages of the

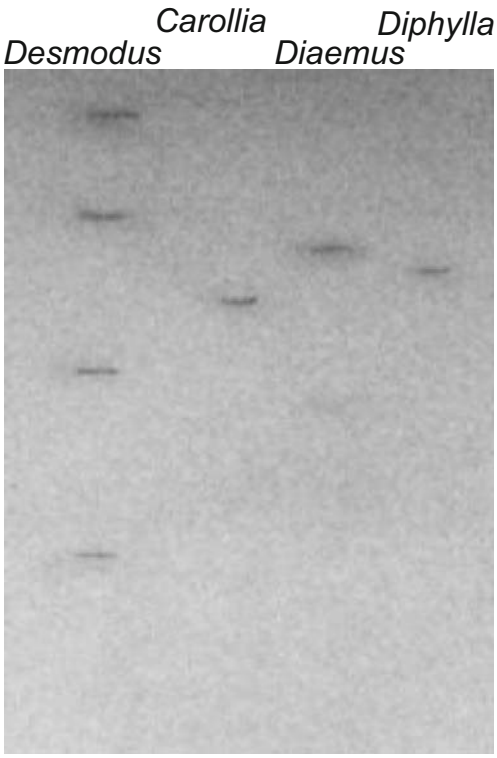


**Fig. 1** A Southern Blot shows the number of genes found in *Desmodus rotundus*, *Carollia perspicillata*, *Diaemus youngi*, and *Diphylla ecaudata*, respectively. This particular blot was generated from genomic DNA digested with BamHI and probed with an oligonucleotide specific for exon 12. The result was consistent with cleavage using additional restriction enzymes as described in the methods section as well as with phylogenetic characterization of the sequenced genes

gene family tree using "branch" and "branch-site" models in PAML (Yang 2007) as well as explicit probabilistic ancestral sequence reconstruction coupled to counting changes along branches (Liberles 2001). Results from PAML were confirmed with results from HyPhy (Pond et al. 2005). HyPhy detected the same sites as PAML plus additional sites (not reported). All results were broadly consistent across methods, as shown in Fig. 3a and in Tables 1 and 2.

Interestingly, the branch preceding the divergence of *Diaemus* and *Desmodus* was under positive diversifying selection. Likewise, all branches following the three *Desmodus* duplication events, as well as the branch leading to the *Diaemus* gene showed evidence of lineage-specific positive selection. With the exception of the branch leading to *Diaemus*, all of these branches were correlated with either duplication events and/or changes in exon structure. This suggests the possibility of intra-molecular co-evolution of residues generating an increase in nonsynonymous substitution, driving the fine-tuning of function or structure associated with exon loss.

To characterize the specific changes that occurred along the branches previously identified with dN/dS>1, we evaluated positive selection at individual codons. In this test, branches were divided a priori into foreground and background lineages, and the branch-site test allowed for sites under positive selection along the foreground branch only. The branch-site test as constructed found significant support for specific codons along the three branches leading to *Diaemus* plus *Desmodus*, to the *Desmodus* duplicates, and to *Desmodus* $\alpha$1.

## Discussion

Following the phylogenetic characterization of exon loss and three rounds of gene duplication in the *Desmodus* lineage, positively selected sites were identified. These positively selected sites along the three branches are depicted on a representation of the protein structure (Fig. 4). In total, we identified 37 sites on three branches under positive selection. The active site residues (corresponding to His57, Asp102, Ser195 in chymotrypsin) were absolutely conserved. Most of the positively selected residues clustered in the K1 and serine protease domains, although the specific residues varied in a lineage-specific manner.

Residue 16 was detected as under positive selection in the lineage preceding *Desmodus* duplication events, where Ile changed to Ser and was subsequently conserved. This change precluded the plasmin-dependent switch that triggers an active conformation in other mammalian t-PAs and renders the PA fibrin specific in its activation (Bode and Renatus 1997). The conservation of Ile in other vampire bat species suggests that these enzymes can be activated through cleavage in a fibrin-sensitive manner. *Diaemus* PA is expected to be intermediate in its fibrin specificity in that it lacks the K2 domain, but bears the plasmin-sensitive cleavage site.

Other positively selected sites in the *Desmodus* lineage include parts of the activation domain. In particular, Glu145, which is part of the autolysis loop, changed to Lys145, thus significantly altering the environment of the activation loop, with which it directly interacts (Renatus et al. 1997). Additionally, sites Gln 184 and Arg 185 (186-loop) are in the immediate vicinity of, or part of, the fibrin attachment site (Fig. 4) (Bode and Renatus 1997). It has been shown, that a chimeric human t-PA containing the homologous *Desmodus* 186-loop was four times more fibrin specific than endogenous human t-PA (Dong et al. 2004).

In addition to the fibrin specificity, the loss of the K2 domain reduces sensitivity to PAI-1. PAI-1 is bound in the Finger domain, the K2 domain, and the protease domain, with the K2 domain binding site playing the more

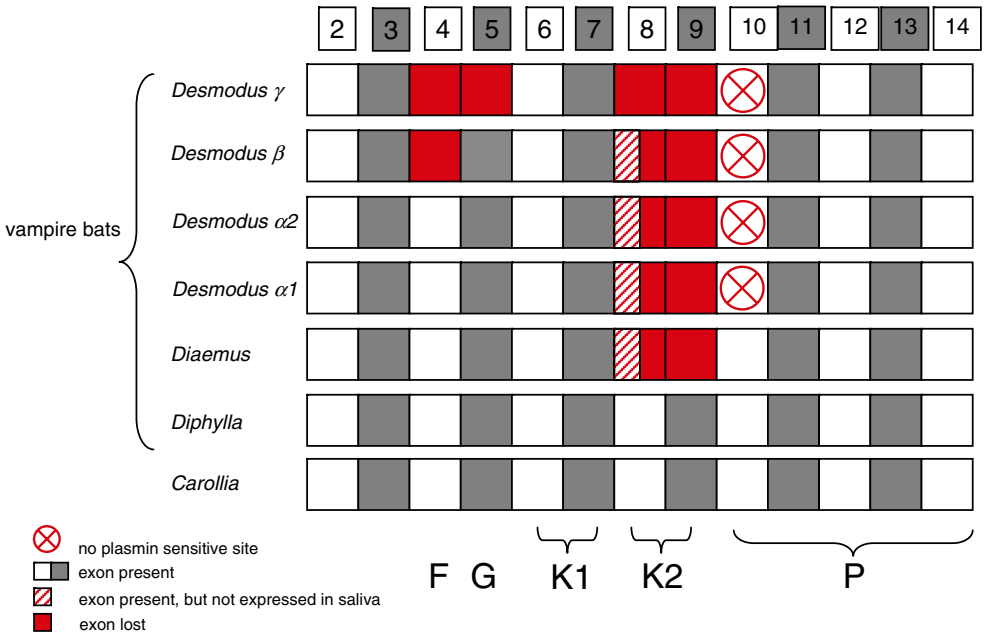


**Fig. 2** The exon and domain structures of the genes that were sequenced are shown. The following abbreviations are used: *F* Finger domain, *G* Epidermal Growth Factor domain, *K1* Kringle domain 1, *K2* Kringle domain 2, *P* Serine Protease domain

important role in the single chain forms found in *Desmodus* (Bennett et al. 1991; Kaneko et al. 1992; Thelwell and Longstaff 2007). The loss of the K2 domain reduces PAI-1 inhibition both through the loss of the K2 binding site and through reduced inhibition by fibrinogen, known to also effect PAI-1 inhibition (Thelwell and Longstaff 2007). PAI-1 is found in mammalian but not bird genomes and typically appears in freshly formed clots (Dellas and Loskutoff 2005). Removal of this inhibition may have been important to feeding on mammalian blood.

When tracing vampire bat feeding behavior in an evolutionary context, a clear shift from arboreal bird feeding to terrestrial feeding in *Desmodus* emerges (Fig. 3a). Associated with this shift are multiple adaptive challenges to the bat, not only because mammals occupy different niches than birds, but also because there are differences in physiological blood parameters. It is known that vampire bats have undergone morphological adaptation, such as the development of specially adapted stomachs and salivary glands, or flight initiating jumps in the terrestrial *Desmodus* (Schutt et al. 1997).

*C. perspicillata*, which feeds on fruit, and *Diphylla*, a vampire bat feeding exclusively on birds have single copies of plasminogen activators that are similar to those found in other mammals. *Diaemus*, a vampire bat feeding on birds and occasionally mammals has lost a K2 domain enabling increased fibrin specificity for enhanced ability to feed on mammalian blood. In *Desmodus*, through three rounds of gene duplication coupled to mutation and further domain

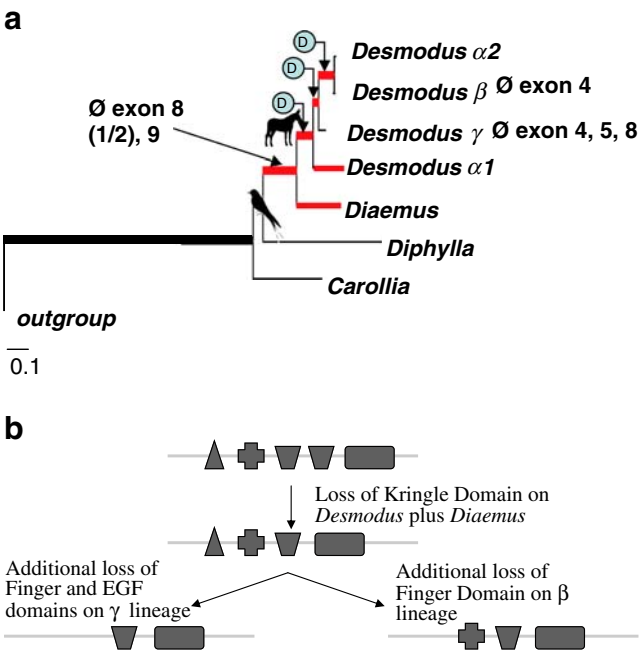


**Fig. 3** **a** Phylogenetic characterization of gene sequences that were obtained is shown. Branches where positive selection was detected are depicted in *red*. Nodes corresponding to gene duplication events are indicated as are the nodes and leaves following domain and exon loss events. Several alleles were obtained from *Carollia perspicillata*, *Diphylla* and *Diaemus* and consensus sequences were used for the generation of this tree. The outgroup sequence was mouse t-PA. Ø indicates exon loss events with *arrows* indicating the lineage exons were lost from. **b** The changes of domain arrangement across species and gene copies, as implied by Fig. 3a are shown. In this figure, the *triangle* represents the Finger domain, the cross represents the EGF domain, the *trapezoids* represent Kringle domains, and the *rectangle* represents the Protease domain

**Table 2** The branch-specific results of dN/dS ratio estimation using a combination of ancestral sequence reconstruction and counting are shown

| Lineage | dN/dS |
|---|---|
| Vampire bats | 0.50 |
| *Desmodus+Diaemus* | 1.42 |
| *Diphylla* | 0.82 |
| *Desmodus* | 2.78 |
| *Diaemus* | 1.23 |
| *Desmodus* α1 | 2.24 |
| *Desmodus* α2+β+γ | 1.81 |
| *Desmodus* γ | 0.76 |
| *Desmodus* α2+β | 2.50 |
| *Desmodus* α2 | 0.00 |
| *Desmodus* β | 0.42 |

loss, an active single chain form with reduced sensitivity to PAI-1 was generated. These forms enable enhanced ability to feed on mammalian blood coupled to the species-level emergence of this feeding preference. But can we reject neutral processes for these evolutionary events? Positive selection has been demonstrated for substitutions leading to the single chain form in both *Desmodus* gene copies with the loss of a single K2 domain. The positive selection observed after each duplication event in the *Desmodus* lineage would support positive selection and neofunctionalization as the preservation mechanism for those duplication events. However, the critical K2 domain loss in the lineage leading to *Diaemus* and *Desmodus* was a single event. While this lineage also demonstrated positive selection at the substitution level, it can not be firmly

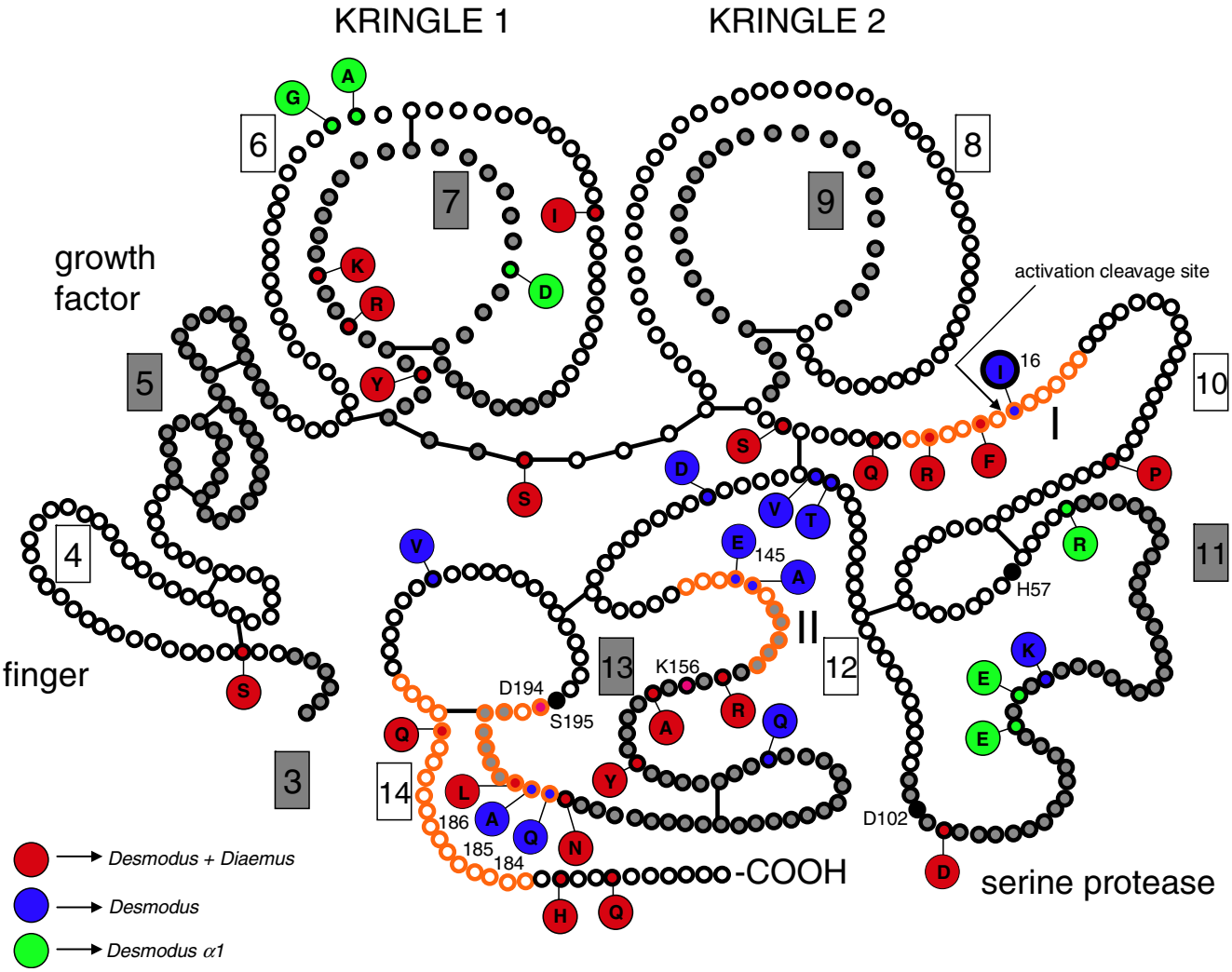


**Fig. 4** A representation of the structure of plasminogen activator is shown. The various domains and exons are depicted. From a branches and sites analysis using PAML (Yang 2007) to detect positive selection along three lineages, the detected sites that were most significant together with the derived amino acids at those locations are indicated. Clustering of such sites in the protease domain is observed. The *orange* in the figure represents the regions that fold together to form the activation loop. The *circled amino acids* represent the amino acid that emerged under positive selection on the *color-indicated* lineage. The domains are labeled indicating the sequence regions where they are encoded

established whether this domain loss was driven by positive selection or was a neutral pre-adaptation that was subsequently taken advantage of. The positive selection on this lineage may also be partly explained by structural accommodation of the domain loss event. The lineage leading to the α1 copy was continuously under positive selection. The lineages leading to the α2 copy were under positive selection continuously until the divergence with the β copy, after which the gene was under strong negative selection. However, the lineages leading to the β and γ copies were under elevated negative selection that cannot statistically be differentiated from neutrality. It may be that these copies are drifting and while expressed, do not play the important functional roles that the copies with just the K2 loss do. It is, however, informative that the genes are clearly not pseudogenes.

Clearly, the duplication events in the *Desmodus* lineage predated the introduction of domesticated cattle and the spread of additional mammalian food sources through the New World range of vampire bats. However, the ability of *Desmodus* and *Diaemus* to utilize mammalian blood, partly driven through plasminogen activator gene evolution, enabled these species to take advantage of the subsequent availability of domesticated cattle. This potentially facilitated the rise of *Desmodus* as the most common vampire bat species (Trajano 1996) with its derived feeding behavior.

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