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Resurrecting the Ancestral Steroid Receptor: Ancient Origin of Estrogen Signaling

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Receptors for sex and adrenal steroid hormones are absent from fully sequenced invertebrate genomes and have not been recovered from other invertebrates. Here we report the isolation of an estrogen receptor ortholog from the mollusk *Aplysia californica* and the reconstruction, synthesis, and experimental characterization of functional domains of the ancestral protein from which all extant steroid receptors (SRs) evolved. Our findings indicate that SRs are extremely ancient and widespread, having diversified from a primordial gene before the origin of bilaterally symmetric animals, and that this ancient receptor had estrogen receptor–like functionality. This gene was lost in the lineage leading to arthropods and nematodes and became independent of hormone regulation in the *Aplysia* lineage.

Vertebrate genomes contain six evolutionarily related nuclear receptors for steroid hormones: two for estrogens (ERα and ERβ) and one each for androgens (AR), progestins (PR), glucocorticoids (GR), and mineralocorticoids (MR). These ligand-activated transcription factors mediate the actions of hormones that direct sexual differentiation, reproduction, behavior, immunity, and stress response (*1*). There are no orthologs of these genes in the insect *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, or the urochordate *Ciona intestinalis* (*2*, *3*), and a polymerase chain reaction (PCR) screen (*4*) failed to identify any of these genes outside the vertebrates. The most closely related nuclear receptors are the estrogen-related receptors (ERRs), an ortholog of which is present in the fruit fly genome (*2*). Based on this gene distribution, steroid receptors (SRs) are thought to have evolved in the chordate lineage some 400 to 500 million years ago, due to a duplication of a more ancient ERR gene (*4*–*6*). Estrogens and other vertebrate-type steroids appear to be involved in the reproductive endocrinology of certain mollusks, however (*7*, *8*). Further, arthropods and nematodes are relatively closely related within the Ecdysozoan clade of molting organisms (*9*), suggesting the possibility that the SR family may have been lost in the lineage leading to both phyla. A previous analysis of SR sequences indicated that the ancient progenitor of this protein class was most similar to extant ERs (*10*).

We used degenerate PCR and rapid amplification of cDNA ends to isolate an ER sequence (figs. S1 and S2) from a mollusk, the sea hare *Aplysia californica*. Using primers derived from vertebrate ERs, we amplified an ER-like cDNA sequence from both adult neural tissue and ovotestes of *A. californica* (*11*). The protein sequence of the *Aplysia* receptor's DNA-binding domain (DBD) is highly similar to that of the vertebrate ERs but much less similar to those of other nuclear receptors, including the ERRs (Fig. 1A). Within the DBD, the P box, which mediates recognition of specific response elements by estrogen and other SRs (*12*), is identical only to that of the human ERs (Fig. 1B). The ligand-binding domain (LBD) of the *Aplysia* receptor is less conserved but is also most similar to that of the vertebrate ER. The *Aplysia* receptor's AF-2 activation domain—a small region in the LBD that mediates ligand-regulated interactions with coactivators (*13*)—is nearly identical to that of the human ERs but not to those of the ERRs or other SRs (Fig. 1B).

The true test of orthology is phylogeny, so we analyzed the relations among 74 steroid and related receptors, including the *Aplysia* ER, using maximum parsimony (MP) and Bayesian Markov Chain Monte Carlo (BMCMC) techniques (*11*). Both methods (Fig. 2A) strongly indicate that the *Aplysia* sequence is an ortholog of the vertebrate ERs. The node indicating orthology with the vertebrate ERs is well supported, with a BMCMC posterior probability of 100%, a bootstrap proportion of 90%, and a decay index of 6. Although BMCMC probabilities can sometimes overestimate statistical confidence (*14*), a 90% bootstrap normally indicates confidence well over 95% (*15*). Further, the maximum likelihood of this phylogeny is >100,000 times greater than that of the best phylogeny in which the *Aplysia* receptor is placed outside the clade of SRs. As this phylogeny shows, the gene duplication that produced the first SR preceded the ancient divergence of deuterostomes (the superphylum that includes chordates and echinoderms) from protostomes [mollusks, arthropods, nematodes, anne-

A

	DBD	LBD
AplysiaER	100	100
humanERα	88	35
humanERβ	85	32
humanERR1	67	23
humanERR2	70	30
humanERR3	67	30
D.melanERR	67	25
humanAR	58	21
humanPR	55	20
humanGR	58	21
humanMR	56	23

B

	P-box	AF-2	CTE
AplysiaER	CEGCKA	DLLTEMLDAHNS	(+27)
humanERα	.....	...L.....RL	(+46)
humanERβ	.....	...L.....RL	(+30)
humanERR1	..A...	K.FL...E.MMD	*
humanERR2	..A...	K.FL...E.KV	*
humanERR3	..A...	K.FL...E.KV	*
D.melanERR	..A...	K.FL...EPLAR	*
humanAR	.GS..V	VDFP..MAEIIS	(+18)
humanPR	.GS..V	VEFP..MSEVIA	(+19)
humanGR	.GS..V	IEFP..LAEIIT	(+19)
humanMR	.GS..V	VEFP..LVEIIS	(+19)

Fig. 1. The *Aplysia* ER protein sequence is most similar to that of human ERs. (**A**) Percent similarity of the *Aplysia* ER to steroid and related receptors of vertebrates and insects in the DNA- and ligand-binding domains. (**B**) Detail of sequences in the P box of the DBD, which mediates recognition of the core response element on DNA; in the AF-2 activation function of the ligand-binding domain, which is essential for ligand-activated transcription; and in the C-terminal extension (CTE) of the protein, with the length of the CTE in amino acids indicated. Dots show residues identical to those of *Aplysia* ER; an asterisk indicates the end of the coding sequence.

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lids, and most other invertebrates (Fig. 2A)]. The SR genes are therefore far older than previously thought, with an origin earlier than ~600 to 1200 million years ago (*16*). Because the ER is present in deuterostomes and protostomes but not in *D. melanogaster* or *C. elegans*, it must have been lost in the Ecdysozoan lineage (Fig. 2B).

To characterize the molecular function of the *Aplysia* ER, we have separately analyzed the activity of the DBD and LBD by expressing them in fusion constructs in a cell culture system (*17*). To test the hypothesis that the *Aplysia* ER-DBD functions in ER-like fashion, we prepared a fusion of the *Aplysia* ER-DBD with a constitutive activation domain (AD) and cotransfected it with an estrogen response element (ERE)–luciferase reporter into CHO-K1 cells (*11*). The *Aplysia* ER-DBD fusion protein activated luciferase expression approximately 10-fold above control levels, which is slightly more than that produced by the human ERα-DBD fusion (Fig. 3A).

To characterize the functionality of the LBD, a fusion of the *Aplysia* ER-LBD with a Gal4-DBD was cotransfected with a luciferase reporter driven by an upstream activator sequence (UAS), the response element for Gal4-DBD (*11*). The *Aplysia* ER-LBD activates transcription constitutively: Expression of the reporter in the presence of the *Aplysia* ER-LBD, using charcoal-stripped serum and without any added ligand, was about 35-fold above control levels (Fig. 3B). In contrast, the human ERα-LBD activated expression only in the presence of estrogens. None of a diverse panel of vertebrate steroid hormones—estrogens, androgens, progestins, and corticoids—even at micromolar doses, further activated or repressed this constitutive activation by the *Aplysia* ER-LBD. Introduction of a radical mutation that replaces a critical residue in the conserved AF-2 activation function [E419Q (*18*)] abolished this ligand-independent trans-activation (Fig. 3B). Together, these experiments suggest that the *Aplysia* ER is a constitutive activator of gene expression from EREs. In a radioligand binding assay (*11*), the *Aplysia* ER-LBD fusion construct did not specifically bind estradiol (Fig. 3C), although we cannot rule out the possibility that the full-length receptor might bind estrogen or some other ligand in its native context.

The existence of a ligand-independent ER raises questions about SR evolution. To explain the existence of both estrogen-activated and estrogen-insensitive receptors, steroid binding must have been lost in the lineage leading to *Aplysia* or gained twice independently, once in the lineage leading to the vertebrate ERs and once in that leading to the AR, PR, GR, and MR (Fig. 2B). To resolve this question, we physically synthesized and functionally characterized the conserved functional domains of the ancestral steroid receptor (AncSR1) from which all extant SRs evolved (Fig. 2A). We phylogenetically inferred the maximum likelihood reconstruction (MLR) of the protein sequences of the AncSR1 DBD and LBD, using the sequence matrix described above, an empirically based model of protein evolution, a gamma distribution of evolutionary rates across sites, and the MP tree (*11*). Although support for the AncSR1 node is not strong in a parsimony context, it has 100% posterior probability, and the best tree with this node has a likelihood 4.7 million times greater than the best tree without this node, indicating high confidence in a model-based framework. To facilitate robust ancestral state reconstruction, the matrix includes broadly sampled representatives of both ligand-regulated and ligand-independent receptors. The MLR sequence for the AncSR1-DBD has a mean probability of 81% per site, whereas that for the AncSR1-LBD has a mean probability of 62% (table S2).

DNA sequences coding for the inferred AncSR1 DBD and LBD peptides were synthesized by serial overlap PCR and subcloned into fusion constructs (*11*). When AncSR1 domains were expressed in CHO-K1 cells, their activity corroborated the prediction that the ancestral receptor would function like an ER (*10*). The AncSR1-DBD fusion increased transcription from an ERE ~4-fold, which is slightly less than the human ERα-DBD but significantly above control levels (Fig. 3A); other extant SRs do not activate effectively on EREs (*12*). The AncSR1-LBD activated transcription in a dose-dependent fashion in the presence of three estrogens (Fig. 3D), although the magnitude of hormone-induced activation was smaller than that effected by the human ERα-LBD. When treated with the preferred ligands for other steroid receptors (androgens, progestins, or corticoids), maximal activation of the AncSR1 ranged from just 1 to 45% of that associated with estradiol. Further, dose-response analysis shows that AncSR1 is from 107 to

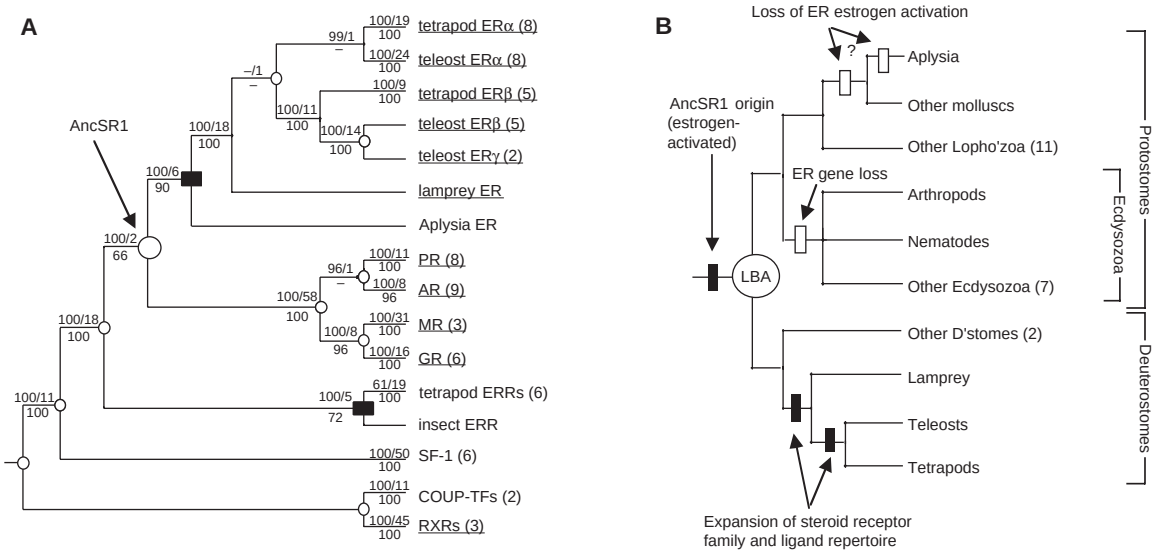

Fig. 2. (**A**) Gene family phylogeny indicates an ancient SR origin. The phylogeny of 74 steroid and related receptors was inferred by MP and BMCMC methods. A reduced version of the tree found by both methods is shown, with the number of sequences per group in parentheses. (For complete tree and accessions, see fig. S3 and table S1.) Underlined receptors are known to be ligand-activated. Open circles mark gene duplications. The protostome-deuterostome divergence is labeled with black squares. Node labels above a branch show Bayesian posterior probabilities (>50% only), followed by decay indices. Below the branch are MP bootstrap percentages (>50% only). SF-1, steroidogenic factor 1; COUP-TFs, chicken ovalbumin upstream promoter transcription factors; RXRs, retinoid X receptors. (**B**) SR diversification and functional evolution. Gene duplications, losses, and changes of SR function are plotted on a phylogeny of the animals based on combined morphology and sequences (*9*). The state for ligand binding for AncSR1 was determined experimentally (Fig. 3). LBA, last bilaterian ancestor; numbers in parentheses indicate the number of extant phyla in terminal clades.

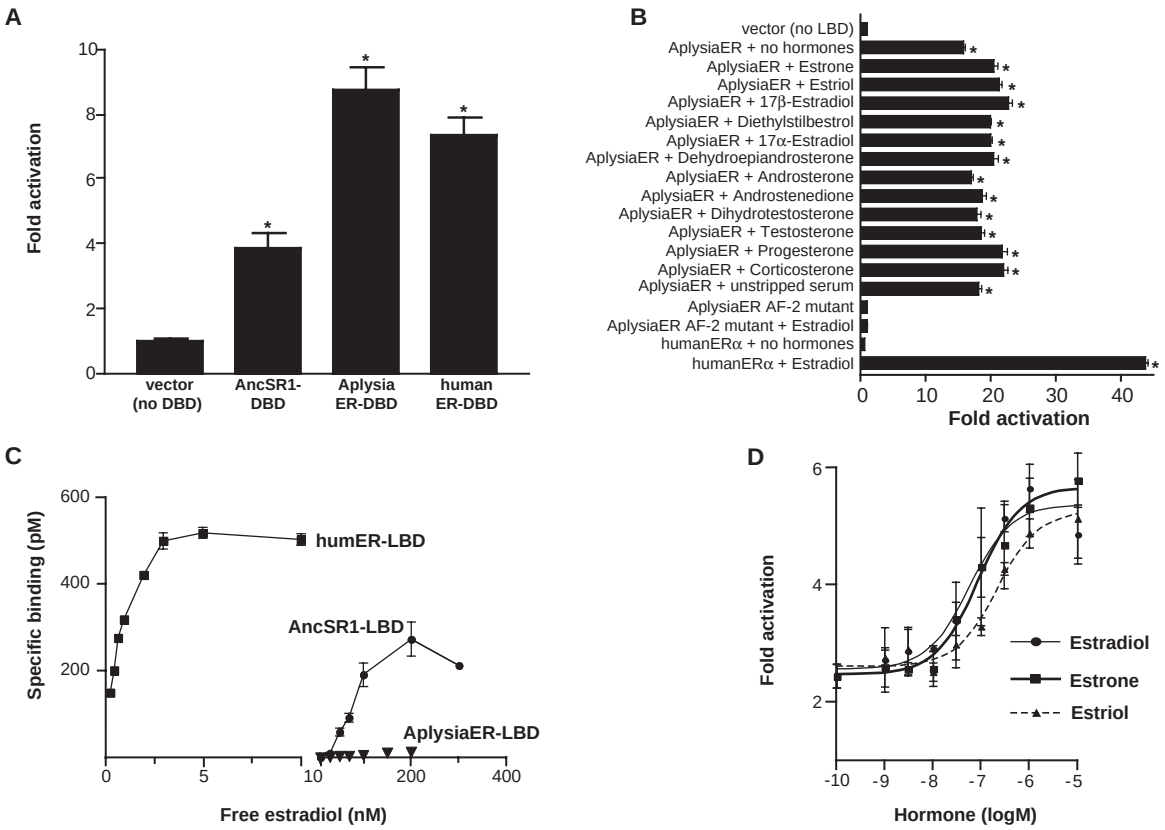

Fig. 3. Functional characterization of mollusk and ancestral SRs. (**A**) AncSR1 and *Aplysia* ER DBDs activate transcription from EREs. Fusions of each DBD were constructed with a constitutive nuclear factor–κB activation domain (AD) and expressed in CHO-K1 cells with an ERE-driven luciferase (luc) reporter and a normalization plasmid. Fold activation indicates normalized luc activity relative to control (AD alone), ± SEM. *, different from control, $P < 0.01$. (**B**) *Aplysia* ER-LBD is a constitutive transactivator. Fusions of the *Aplysia* ER or human ERα LBDs with a Gal4-DBD were expressed with a UAS-luc reporter and treated with 1 μM hormone. Fold activation shows normalized luc activity relative to control (Gal4-DBD alone), ± SEM. (**C**) Saturable specific estrogen binding by ancestral and human ERα LBDs. Homogenates from cells expressing LBD fusion constructs were incubated with 3H-estradiol in a competitive binding assay. Note the split *x*-axis scales for high- and low-affinity receptors. Dissociation constants (binding affinities) were as follows: human ERα = 0.8 nM, AncSR1= 198 nM. (**D**) AncSR1 LBD specifically activates transcription in the presence of estrogens. A fusion plasmid of the AncSR1 LBD with the Gal4-DBD was cotransfected with a UAS-luc reporter, then treated with increasing concentrations of hormones.

Table 1. AncSR1-LBD is an estrogen-activated transcriptional regulator. AncSR1-LBD/Gal4-DBD fusion protein was expressed in CHO-K1 cells with UAS-luciferase reporter and treated with increasing hormone concentrations. EC_{50} indicates the ligand concentration that causes a half-maximal response. RAE (relative activation efficacy) indicates the maximum fold increase in reporter expression, divided by the maximum fold increase for estradiol.

Hormone	EC_{50} (nM)	RAE
Estriol	323	1.40
Estradiol	37	1.00
Estrone	416	0.58
Progesterone	>1,000,000	0.45
Testosterone	3981	0.37
Dihydrotestosterone	9772	0.17
Androstenedione	-	0.01
Corticosterone	-	0.04
Cortisol	-	0.03

more than 30,000 times less sensitive to these ligands than to estradiol (Table 1), indicating a high level of estrogen specificity. In a radioligand binding assay, the AncSR1-LBD specifically bound estradiol, though with affinity lower than the human ERα-LBD (Fig. 3C). It is unlikely that the specificity of estrogen activation is an artifact of error in the reconstruction algorithm. Of the 26 sites in the ligand-binding pocket, 22 were identical to the human ERα and/or ERβ; the mean probability over these sites was >80%, whereas the mean probability at the four sites not shared with vertebrate ERs was 56%. Because random mutation impairs function more frequently than enhancing it, error in the reconstruction is more likely to reduce the efficiency of steroid binding and activation than to create it de novo.

These findings provide empirical support for the hypothesis that the ancient ancestral SR functioned as an ER. The most parsimonious reconstruction of SR evolution (Fig. 2B) is that ligand regulation was inherited relatively unchanged from the ancestral SR by the vertebrate ERs and with modification by other SRs after gene duplication. Within the protostomes, this ancient SR gene was lost entirely from the genome during Ecdysozoan evolution; in the lineage leading to the *Aplysia* ER, ligand regulation was lost. This scenario suggests a previously unrecognized degree of functional and genomic lability in SRs over deep evolutionary time. The existence of a mollusk ER inherited from the last common ancestor of all bilaterally symmetric animals suggests that many other non-Ecdysozoan invertebrates, such as echinoderms, annelids, platyhelminthes, and other mollusks (Fig. 2B), will also have SRs. Because there is evidence for a reproductive role of steroid hormones in cephalopod and gastropod mollusks (*7*, *8*, *19*), we predict that the loss of estrogen-dependent activation is recent and unique to the ERs of the Opisthobranchs. The fact that AncSR1 is specifically activated by estrogen, the terminal hormones in the steroid biosynthesis pathway, corroborates the ligand exploitation model for endocrine evolution: New hormones emerged when duplicated receptors evolved increased affinity for biochemical intermediates such as testosterone and progesterone, conferring on these steroids bona fide signaling functions (*10*).

Our finding that SRs are not restricted to the small corner of the animal kingdom represented by vertebrates suggests a possible mechanism for the apparent role of gonadal steroid hormones in invertebrates. Most environmental regulations and testing programs for endocrine disrupters are focused solely on vertebrates (*20*), but our findings suggest that a much broader range of animal taxa could be subject to endocrine disruption by steroid-mimicking or steroid-blocking compounds in the environ-

ment. Finally, the complete loss of SRs from the genomes of our two major invertebrate models shows that reliable inferences about the evolution, distribution, and function of genes cannot be made on the basis of two relatively closely related species. Other ancient genes may also have been lost from the Ecdysozoans, resulting in considerably fewer vertebrate-specific gene families than currently believed.

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Island Biology and Ecosystem Functioning in Epiphytic Soil Communities

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Although island attributes such as size and accessibility to colonizing organisms can influence community structure, the consequences of these for ecosystem functioning are little understood. A study of the suspended soils of spatially discrete epiphytes or treetop "islands" in the canopies of New Zealand rainforest trees revealed that different components of the decomposer community responded either positively or negatively to island size, as well as to the tree species that the islands occurred in. This in turn led to important differences between islands in the rates of ecosystem processes driven by the decomposer biota. This system serves as a model for better understanding how attributes of both real and habitat islands may affect key ecosystem functions through determining the community structure of organisms that drive these functions.

Over the past decade there has been a rapidly growing interest in how community-level attributes such as biodiversity and community composition may influence the functioning of ecosystems (*1–4*). Although several recent studies have adopted experimental approaches to investigate these effects, much remains unknown about their importance in real ecosystems, especially when considered against the natural background variability caused by extrinsic driving variables such as climate and the availability of resources (*2–5*). Island attributes such as island size and accessibility of islands to colonizing organisms have long been recognized by ecologists as important determinants of community composition and diversity (*6–9*), as well as the nature of interactions among component organisms (*10*, *11*). Island systems therefore have considerable potential for investigating how differences in community composition among habitats may influence processes and properties at the ecosystem level of resolution (*12*, *13*), although few studies have used islands for explicitly addressing this type of question (*14–16*).

Our study focused on the decomposer communities in the organic matter or "suspended soils" produced by spatially separated epiphytes or treetop "islands" in the crowns of canopy tree species in a fragment of old-growth, warm-temperate rainforest in the Northland region of New Zealand (35°20′S, 173°52′E). Individual epiphytes in rainforest canopies function as insular communities with regard to the decomposer biota that they support (*17–19*). We used these epiphytes as model systems for assessing whether and how organism community structure determines ecosystem properties relevant to carbon and nutrient cycling. If decomposer community structure were to operate as an important driver of ecosystem functioning in this system, then we would expect ecosystem properties to be responsive to those island attributes that affect community structure.

The study had two parts. The first was a descriptive (observational) investigation, in which we sampled spatially discrete individuals of the epiphytic perching lily *Collospermum hastatum* (Liliaceae). Individual treetop islands (consisting of epiphytes plus associated litter and soil) were sampled from the canopies of each of three tree species [*Vitex lucens* (Verbenaceae), *Beilschmiedia tarairi* (Lauraceae), and *Podocarpus totara* (Podocarpaceae)], at a height between 2.1 and 10.2 m above the ground. The soil of each was assessed for decomposer community and ecosystem attributes (*20*). Different tree species were used because it is known that New Zealand rainforest trees differ in the composition of invertebrate communities that travel along their trunks and branches (*21*), and epiphytes of a given species growing on branches of different species should therefore differ in the invertebrate taxa that colonize their humus. Harvested treetop islands varied in dry mass by 4.5 orders of magnitude and were classified as small, medium, and large on the basis of the mass of suspended soil that they contained (*20*). The second part was an experimental investigation, aimed at assessing the effects of tree species and treetop island size on community and ecosystem properties, but with greater control over other extrinsic factors. Artificial treetop islands were created by filling baskets of three different sizes with defaunated humus and strapping them to the upper sides of branches in the canopies of each of the three tree species at a height between 2.6 and 7.8 m above ground, so as to represent suspended soils (*20*); the three sizes of islands constructed correspond to the three size categories used in the observational study. These were left in the field for colonization by decomposer organisms until assessment of the humus for community- and ecosystem-level properties 195 days later (*20*).

Most of the main groups of decomposer organisms responded to both treetop island size and tree species identity, for both the observa-

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