Adaptive evolution of cytochrome *c* oxidase: Infrastructure for a carnivorous plant radiation

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Much recent attention in the study of adaptation of organismal form has centered on developmental regulation. As such, the highly conserved respiratory machinery of eukaryotic cells might seem an unlikely target for selection supporting novel morphologies. We demonstrate that a dramatic molecular evolutionary rate increase in subunit I of cytochrome *c* oxidase (COX) from an active-trapping lineage of carnivorous plants is caused by positive Darwinian selection. Bladderworts (*Utricularia*) trap plankton when water-immersed, negatively pressured suction bladders are triggered. The resetting of traps involves active ion transport, requiring considerable energy expenditure. As judged from the quaternary structure of bovine COX, the most profound adaptive substitutions are two contiguous cysteines absent in ≈99.9% of databased COX I sequences from Eukaryota, Archaea, and Bacteria. This motif lies directly at the docking point of COX I helix 3 and cytochrome *c*, and modeling of bovine COX I suggests the possibility of an unprecedented helix-terminating disulfide bridge that could alter COX/cytochrome *c* dissociation kinetics. Thus, the key adaptation in *Utricularia* likely lies in molecular energetic changes that buttressed the mechanisms responsible for the bladderworts' radical morphological evolution. Along with evidence for COX evolution underlying expansion of the anthropoid neocortex, our findings underscore that important morphological and physiological innovations must often be accompanied by specific adaptations in proteins with basic cellular functions.

molecular adaptation | positive selection | cellular energetics | protein structure | developmental regulation

Considerable interest in molecular evolutionary biology has focused on the genetic basis for development, including similarity versus dissimilarity of gene interaction networks at the macroevolutionary level, and cis-regulation by transcription factors at the microevolutionary level (1, 2). Ontogeny undoubtedly holds clues to the evolution of new morphologies (3), and quite obviously, understanding its control at the molecular level must be important. Nonetheless, the adaptive buttressing of developmental-morphological innovation provided by structural genes has received relatively scant attention (but see refs. 4 and 5), even though novel phenotypes based on regulatory changes must survive to perpetuate.

Another recent research trend in molecular evolution has been the genetic detection of past positive selection. Much interest has focused on positive selection because it provides the footprints of adaptation at the molecular level (6). However, there are few clear examples in which specific amino acid substitutions caused by positive selection have been correlated with altered protein structure (7), and few where phenotypic change has been observed as an organism (8), or a radiation of organisms, adapts to new environmental/physiological conditions based on the selected trait as a key innovation (9).

In a recent examination of molecular substitution rates among the carnivorous plant sister-groups *Genlisea*/*Utricularia* and *Pinguicula* (all of the flowering plant family Lentibulariaceae), the first lineage was found to evolve significantly faster than the latter across all three genomic compartments, plastid, mitochondrial, and nuclear (10). The bladderworts (*Utricularia*) and corkscrew plants (*Genlisea*) are a highly specialized lineage of carnivorous plants that mainly consist of modified leaves that enable the capture of microscopic aquatic animals (11, 12). Leaf form has evolved into active, spherical suction bladders in *Utricularia* and nonactive, corkscrew-shaped traps in *Genlisea* (11). These aquatic prey-trapping modes, coupled with an overall relaxation of the higher-plant body plan, have permitted bladderworts and corkscrew plants to exploit a vast array of previously unoccupied and nutrient-deficient ecological niches (11, 12). The closest relatives of both genera, the carnivorous butterworts, *Pinguicula* (11, 12), have a more typical angiosperm morphology, trapping prey on sticky leaf surfaces (11). As such, this plant family presents an array of morphological forms that require developmental as well as adaptive explanations.

Ontogenetically, the trapping leaves of *Genlisea* and *Utricularia* are homologous (13), but structurally and functionally they differ greatly. *Genlisea* traps are open systems with a basal digestive bladder (13); microscopic prey organisms crawl into entranceways along the helically shaped trap arms (Fig. 1) and are prevented exit by forward facing hairs (11). In contrast, *Utricularia* suction bladders are sealed with trap doors that open and shut within 30 ms (11). Upon triggering, prey are sucked in by considerable internal negative pressure, achieved by actively pumping water to the exterior environment (11). The active pumping of water occurs by means of a two-step ATP-driven ion-pumping process, initiated by respiration-dependent transport of chloride ions against an electrochemical gradient (14). The second step involves flow of water from intercellular space to external glands (14). The rate of ion movement has been found to be approximately four times greater than in animals or other plant transport systems (14). In addition, supply of ATP is not a limiting factor during trap resetting, which is thought to be a continuous process (11), with respiratory rates at least 10% greater in immature bladders than leaves (15). Respiratory enhancement in resting structures is directly indicative of preparation for activity. Although it remains untested, it is highly likely that the respiratory rate at full trapping function is even higher because bladderwort traps have two-phase activity, first by setting the trap by means of water pumping, and second in response to triggering.

Here, we demonstrate part of the adaptive scaffolding underlying the suction-bladder traps of *Utricularia*. We discovered that the increased respiratory capacity needed for active ion pumping is correlated with positive selection in the key respiratory

Abbreviation: COX, cytochrome *c* oxidase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY128568, AY600087–AY600139, AY601869, and AY601870).

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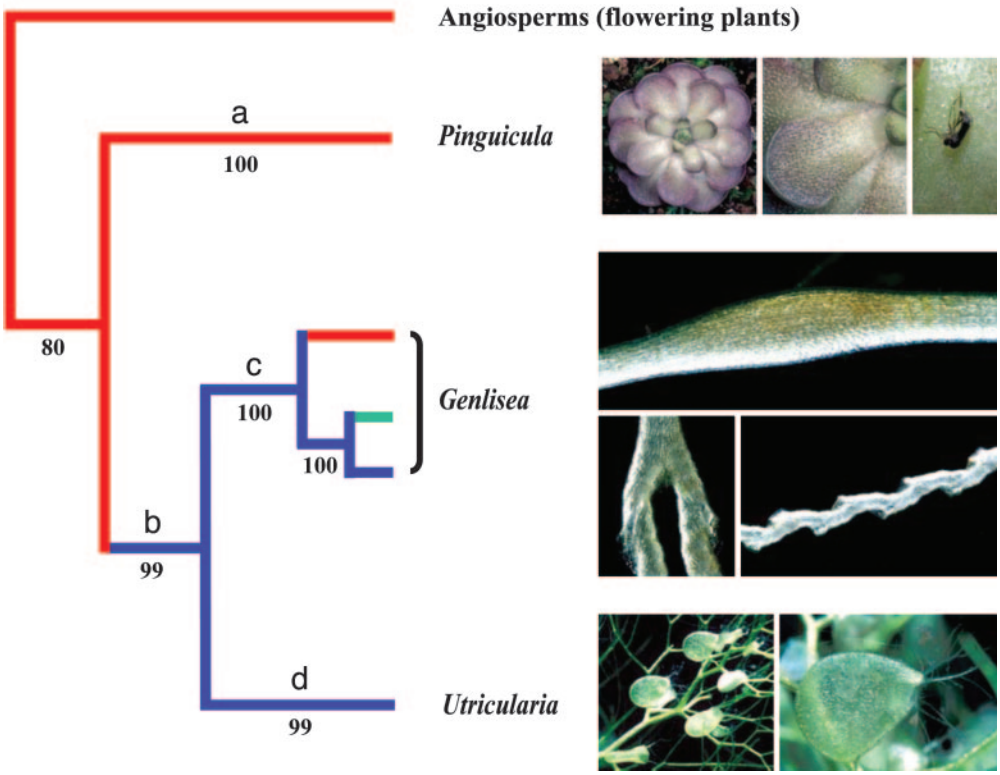

Fig. 1. The hypothesized evolution and development of ATP-dependent active pumping in Lentibulariaceae. a, divergence of the flypaper trapping strategy (*Pinguicula*); b, ancestral *Utricularia*/*Genlisea* active-pumping suction pitcher prototype; c, corkscrew trapping strategy (*Genlisea*) with the loss of active pumping; d, *Utricularia* maintains ATP driven active pumping, with the development of the trap door innovation, which seals a negative internal pressure required for suction-bladder trapping. Colored branches indicate the ancestral Leu-113-Ser-114 motif (red), with gain of functional changes Cys-113-Cys-114 (blue) in the *Utricularia*/*Genlisea* ancestor (with *Genlisea* represented here by *Genlisea hispidula*; Table 1) followed by partial loss to Cys-113-Ser-114 (green) in *Genlisea aurea* or complete reversal to Leu-113-Ser-114 (red) in *Genlisea violacea* (Table 1). The above hypothesis assumes coevolution of the Cys-113-Cys-114 motif, as suggested by our hypothesis of a vicinal disulfide bridge in COX I helix 3. Numbers below branches represent parsimony jackknife support values (63–100 = strong support), based on analysis of two noncoding plastid DNA regions (10, 12). Images show *Pinguicula* (*Top*), with flypaper trapping leaves; *Genlisea* (*Middle*) with digestive bulb (uppermost), forked region where corkscrew traps begin, and an individual corkscrew trap; *Utricularia* (*Bottom*) with suction-trapping bladders and branched appendages at their trap doors.

enzyme cytochrome *c* oxidase (COX), the main catalytic subunits of which are highly conserved among prokaryotes and eukaryotes (16). Comparison with the structure of bovine COX (17) implicates a 2-aa motif, absent from ≈99.9% of all other cellular organisms studied, that may alter the dissociation kinetics of cytochrome *c* docking to the COX holoenzyme (18).

Materials and Methods

PCR, Sequencing, and Alignment. The 3′ and 5′ exons of the intron-containing COX I gene were sequenced for 21 *Utricularia*, three *Genlisea*, and five *Pinguicula* taxa representing all major clades of Lentibulariaceae (Table 1, which is published as supporting information on the PNAS web site). *Pinguicula* sequences did not vary at any nonsynonymous site across the five sequenced taxa, so one taxon was selected for use as outgroup for positive selection analysis (Table 1). In addition, COX I was sequenced for seven other carnivorous plant taxa from five families (Droseraceae, Cephalotaceae, Byblidaceae, Nepenthaceae, and Sarraceniaceae) by using methods described in ref. 10 (refer to Table 1 for GenBank accession numbers). Alignment of all COX I sequences was unambiguous.

RNA-edited sites (cytosine → thymine) in the COX I gene (19) were determined through sequencing of cDNA for five species of *Utricularia* and *Genlisea* and one species of *Pinguicula*. mRNA was isolated by using MICROFASTTRACK 2.0 (Invitrogen), and the SuperScript System for RT-PCR was used to synthesize cDNA. Refer to Table 1 for GenBank accession numbers.

Direct TBLASTN search (20) of the National Center for Biotechnology Information (NCBI) sequence database for plant COX subunit VIIa and VIIc sequences using the bovine proteins as queries did not produce any structurally reasonable hits, regardless of search parameters. A stepwise strategy was therefore adopted: from the original searches with mammalian sequences, the most distant sequences were selected for new searches, each time taking care that the results of these searches overlapped. The first plant sequences of subunit VIIa were found by using the silkworm sequence from EST database. The results were checked for the length of the ORF, for the signal sequence and the actual structural protein, and for the putative transmembrane segment (21). The sequences were also found to be the only matches to COX VIIa in HMMer search (22). Despite the low overall similarity scores, the best conserved segments in the known oxidase subunits have good matches in the plant sequences (see Fig. 3, which is published as supporting information on the PNAS web site). No subunit VIIc sequences could be identified with confidence.

After discovery of putative COX VIIa in plants, PCR primers were developed for isolating further sequences. The putative VIIa gene was sequenced from *Utricularia subulata* cDNA (GenBank accession number AY601870) by using nested PCR with external primers F-VIIa (5′-CATTTAGGCCACGAGAGAA) plus R-VIIa 5′-TTGCCGATTCCATTCGACA in the first round, and internal primers F-VIIa-2 (5′-AGGTATTCCAGAGCATTCATAA) plus R-VIIa-2 (5′-

GAACCAGCCATAGCCAAAGGAA) in the second round. These primers amplified a 183-bp region of the 207-bp gene (in reference to the *Arabidopsis thaliana* expressed protein At4g21105) under the following PCR conditions: 29 cycles of annealing time 30 s at 65°C, and extension time 1 min at 72°C. Gene purification and sequencing was done as described in ref. 10.

Positive Selection Analysis. To detect positive selection, we used the methodology described in refs. 23 and 24 as implemented in the computer program PAML (25). In brief, we fit two models to the data using maximum likelihood, assuming a fixed gene tree. Both models assume that the d_N/d_S ratio varies among sites, but one model allows only for negative selection (M7), whereas the other model allows for both negative and positive selection (M8). To test model M7 a likelihood ratio test is used that employs the difference in maximum log likelihood (log likelihood ratio) as a test statistic. If the log likelihood ratio between the two models exceeds a certain critical value (6.00 at the 5% significance level), the simpler model allowing no positive selection can be rejected with statistical significance and we conclude that the sequences have been subject to positive selection. If model M7 is rejected, the sites that most likely are undergoing positive selection are identified by using an empirical Bayes approach (ref. 25 and Z. Yang, W. S. W. Wong, and R.N., unpublished work). Because this method relies on knowledge regarding the gene tree, we used different methods for estimating the tree and repeated the statistical analysis on all estimated trees. Trees were generated with the following data and methods (see ref. 26): (*i*) with the plastid DNA intergenic regions (10, 12), parsimony, neighbor joining with HKY85 distances, maximum likelihood with the Jukes–Cantor model, maximum likelihood with the general time reversible model with γ distributed rates, and (*ii*) with the COX I data itself, parsimony (four random most-parsimonious trees) in addition to neighbor joining with HKY85 distances. Tree-search parameters and results are available upon request. For positive selection analysis of the COX I data, 14 putative (19) and cDNA-determined RNA edited sites were removed from the data matrix to eliminate erroneous inclusion of nontranscribed synonymous and nonsynonymous changes. Residues are numbered according to the bovine enzyme (17).

Protein Modeling. CHARMM C30B1 (27) was used for protein structural modeling. PROCHECK 3.3 (28) was used to determine conformations by Kabsch–Sander definitions. The protein model was visualized by using VMD 1.8.2 (29).

Results and Discussion

Evidence for Positive Selection. The most common method for detecting positive selection in nucleotide sequences is to demonstrate that the rate of synonymous substitution (d_S) is greater than that of nonsynonymous changes (d_N), such that $d_N/d_S > 1$ (6). By using this approach, positive selection has been detected in many systems, including viruses (30, 31), reproductive proteins (23, 32), after gene duplications (33), and more generally, among multiple proteins in a taxonomic comparison (34). We tested the hypothesis of no positive selection ($d_N/d_S \leq 1$ for all sites) in the mitochondrially encoded COX I gene of *Genlisea/Utricularia* by using models allowing site-specific variation (25). When applying the method, we assumed that a species tree estimated is the correct tree. For the parsimony tree using plastid DNA intergenic regions (10, 12), the maximum log likelihood value for model M7 (neutral evolution model) was −2823.27, and the maximum log likelihood value for model M8 (positive selection model) was −2817.93. Two times the log likelihood ratio is, therefore, 10.68, corresponding to rejection of model M7 with a *P* value of 0.005. Under model M8, the maximum likelihood estimate of the proportion of positively selected sites and the d_N/d_S ratio in these sites was 0.043 and 2.52, respectively. As such, the hypothesis of no positive selection could be rejected with strong statistical significance ($P < 0.005$). To test the robustness of this conclusion, we analyzed the same data, as well as COX I data itself, using various methods of phylogeny construction, which produced equivalent results. Using an empirical Bayes approach, we then identified the putative positively selected residues, accepting a cut-off of posterior probability (*pp*) > 0.95 (Table 2, which is published as supporting information on the PNAS web site).

Positively Selected COX I Residues. Along the COX I subunit, we identified twelve putatively positively selected amino acids that were confined to the *Genlisea/Utricularia* lineage (Table 2). Of particular interest was an otherwise conserved Leu-113-Ser-114 motif replaced by a radical Cys-113-Cys-114 change across all examined *Utricularia* species. *Pinguicula* species did not vary at either site relative to Asteridae outgroup taxa (refs. 10 and 12; see Pfam PF00115), whereas *Genlisea* was found to have the Cys-113-Cys-114 changes in only one of three species examined. Two other *Genlisea* species have either Leu-113-Ser-114 or Cys-113-Ser-114, suggesting a full or partial loss of the Cys-113-Cys-114 motif after the *Genlisea-Utricularia* divergence (Fig. 1). As a further control, we obtained new COX I sequences to demonstrate that the Cys-113-Cys-114 motif does not occur in any other genus of carnivorous plants, excluding the possibility that the changes could be part of the general carnivorous plant habit (Table 3, which is published as supporting information on the PNAS web site). Indeed, the gnetalean gymnosperm *Welwitschia mirabilis*, a relict member of an ancient seed plant lineage that inhabits one of Earth's driest environments (35), was the only other organism found to have the Cys-113-Cys-114 motif in the entire Pfam COX 1 database (PF00115) >14,500 sequences (see, e.g., Table 3).

Altered Protein Structure and Function. Mitochondrial membrane-bound COX catalyses the respiratory reduction of oxygen to water and couples this reaction to translocation of protons (36), generating a transmembrane proton electrochemical gradient that is used for the synthesis of ATP. As such, it is a reasonable hypothesis that positively selected amino acid changes in COX I could be involved in altering cellular energetic capacity. In known COX structures, the residues corresponding to Leu-113 and Ser-114 are situated in transmembrane helix 3 of subunit I at the interface to the intermembrane space. This area interacts with the nuclear-encoded COX VIIa and COX VIIc subunits in the bovine heart enzyme (PDB accession code 1v54; ref. 17), of which VIIa moreover occurs in tissue-specific forms (16). Extensive studies with yeast have shown that subunit VIIa (corresponding to VII in yeast) is essential for assembly of the COX holoenzyme, and that subunit VIIc (corresponding to VIII in yeast) is not necessary but modulates COX catalytic activity (37, 38). Leu-113 and Ala-114 of the bovine enzyme lie near the apex of a "tripod" formed by the C-terminal tips of helix 3 of COX I and those of VIIa and VIIc, not far from where the electron donor to COX, cytochrome *c*, is known to bind (Fig. 2). Surprisingly, when we least-squares fitted (29) the C_α coordinates of the transmembrane helical segments of subunits I and II of the *Paracoccus denitrificans* oxidase (1ar1.pdb)/cytochrome *c* docked complex (18) to the corresponding subunits of bovine COX, the C-terminal tail of subunit VIIa (residue 58) touched the surface loops of the electron donor (residues 45–47). As such, the dissociation kinetics of cytochrome *c* could be modulated in part by this tripod-like interaction region.

In the bovine COX structure, there are no obvious associations between Leu-113-Ala-114 and their surroundings. The possibility that *Utricularia* Cys-113-Cys-114 could be in proximity

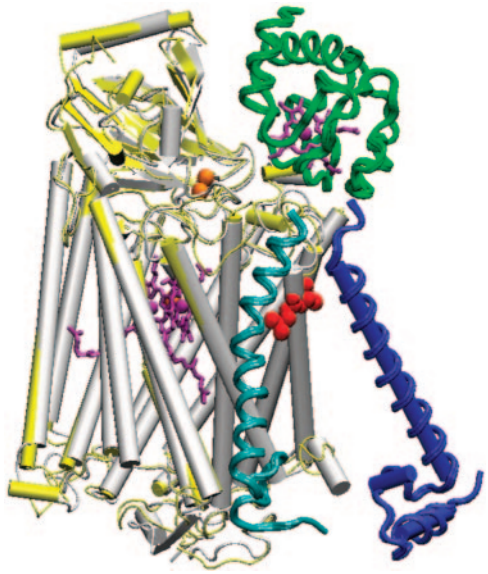

Fig. 2. Protein structural reconstruction of COX I and COX VIIa,c based on the bovine enzyme model (15). Mitochondrially encoded COX I is silver, and hemes and coppers show through in purple and orange, respectively. COX subunit VIIa is shown in dark blue; subunit VIIc is in light blue. We identified COX VIIa in plants, including *Utricularia*, but COX VIIc remains undiscovered. Residues 113 and 114 of helix 3 of COX subunit I are shown as red space-filling models. Cytochrome *c*, in green, is shown superimposed by using coordinates from the *P. denitrificans* oxidase (1ar1.pdb)/cytochrome *c* docked complex (16). COX I residues 113 and 114 and the C terminus of COX VIIa lie directly at the cytochrome *c* docking base. The model was visualized by using the application VMD (23).

to subunits VIIa and VIIc prompted us to examine whether intersubunit interactions by means of disulfide bridges could dampen or eliminate movements of the three helices with respect to each other. First, we had to clarify whether plant COX I interacts with proteins homologous to mammalian VIIa and VIIc, and in so doing, we identified the first plant homologues to subunit VIIa (Fig. 3). We could not, however, recognize a subunit VIIc with confidence beyond animals and fungi. Discovery of subunit VIIa was complicated by the small size of its polypeptide chain and the generally incomplete knowledge of the COX holoenzyme in plants. Nevertheless, we were able to recognize a number of putative homologues with appropriately conserved domain structure by using BLAST searches (20) of GenBank and the *A. thaliana* database (39). Although the overall sequence similarities to animal proteins are low, the proposed *Arabidopsis* homologue of COX VIIa is expressed (40) and predicted to be a transmembrane protein in domains matching bovine counterparts (41). Additionally, the segments corresponding to the short helix and turn on the matrix side of the inner mitochondrial membrane (Fig. 2) are especially well conserved (Fig. 3). The sequence we determined for *Utricularia* subunit VIIa does not differ importantly from that of other organisms, and in particular, no cysteines were found that could have formed disulfide bridges to subunit I (Fig. 3). Regarding subunit VIIc, it is entirely possible that neither *Utricularia* nor any other green plant possesses this protein, which is nonessential in yeast (37, 38).

An intriguing possibility for the contiguous cysteines in *Utricularia* COX I is that they could have coevolved if they formed a disulfide bridge between themselves. Such vicinal disulfides are rare but not unknown in proteins. The best studied example of a conserved vicinal disulphide is in the α subunit of the nicotinic acetylcholine receptor (42). In all known cases, the backbone of the cysteines forms a β-turn and a reversal of the peptide chain orientation. No cases of vicinal disulfides are known in α-helices. Except for the Cys-113-Cys-114 motif, the inferred *Utricularia* COX I proteins are very similar to mitochondrial oxidases in general, so a major change in the helix bundle seems unlikely. The double cysteines are, however, just one turn from the C-terminal end of COX I helix 3, and a vicinal disulfide at this position could cause unraveling of the helix end, which in turn could affect the docking base for cytochrome *c*.

To test the feasibility of a disulfide bridge between Cys-113 and Cys-114, we tried forcing a covalent bond between the S_γ atoms. To our surprise, the bridge formed without any major distortion of the helix, or the environment, all of which were permitted to move. The modeled structure differs from the bovine structure mainly for (φ, ψ) of Cys-114, which move from (-63,-45) to (-90,-28), but are still defined by the Kabsch-Sander algorithm (refs. 28, 43) to be α-helical. In summary, the two conserved cysteines can at least theoretically form a vicinal disulfide bridge, which would also be the first such bridge proposed within a helix.

In earlier theoretical studies, such a conformation has not been observed, but the calculations have been restricted to small model compounds, in which the major stabilizing effect of the helix environment has been absent. We hypothesize that, if such a vicinal bridge indeed exists in bladderworts, it may be linked to altered cytochrome *c* docking kinetics. Mass spectrometry could be used to test this hypothesis, because this technique has proven successful in identifying a His-Tyr bond in four distantly related cytochrome *c* oxidases (44).

COX Evolution in Bladderworts and Corkscrew Plants. In mammals, heart and skeletal muscle require significantly greater respiratory capacity than other tissues. In molecular evolutionary research on primate COX VIIa, accelerated protein evolution has been found for the heart/skeletal muscle isoform as opposed to less tissue-specific forms (45, 46). In bladderworts, both the uniquely present and positively selected residues of COX I helix 3 and the C terminus of COX VIIa are likely in close structural proximity to the cytochrome *c* docking base. This structural disposition of the Cys-113-Cys-114 motif would seem highly unlikely to have arisen by chance alone, and therefore very likely to be correlated to the demands of cellular respiration. Future structural analyses of bladderwort and model-plant COX holoenzymes should confirm the molecular interactions underlying the bladderwort key physiological innovation.

Our hypothesis for trap form evolution in bladderworts and corkscrew plants is that it was reinforced by altered cellular energetics. In simple open traps, essential prey-derived nutrients would be expected to quickly flow back out into the environment. This result may have been a functional difficulty for the prototypical *Genlisea*/*Utricularia* trap, which we hypothesize was an open bladder system that overcame this problem with active water pumping (Fig. 1). In *Genlisea*, long, tube-like traps that may reduce the rate of nutrient diffusion from the digestive bulb to the outside environment (11, 13) have probably evolved secondarily. Apart from the unlikely possibility of parallel Cys-113-Cys-114 evolution within the Lentibulariaceae, further evidence for the derived status of the passive "lobster-pot" trapping strategy relates to the *Utricularia* suction bladder requiring a sealed trap door to create a negative pressure: a hermetic trap without active water removal would not function for prey capture, necessitating the pumping feature to have evolved first (Fig. 1). As active pumping of water became less essential for trap function during *Genlisea* evolution, a relaxation on COX selective changes could then have allowed them to be lost (Fig. 1). To summarize our model, we propose that an accelerated rate of respiration was adaptively reinforced by specific amino acid changes in *Genlisea*/*Utricularia* COX I (e.g., residues Cys-113-

Cys-114), and we attribute the probable loss of these changes in *Genlisea* to the loss of active ion pumping (Fig. 1). Likewise, *Welwitschia* plants have a very high transpiration rate with little water storage capacity and therefore must satisfactorily intake limited ground or atmospheric moisture (35), a process that may require active water transport.

In conclusion, we present a first-order hypothesis for the enhancement of respiration in the bladderworts. The hypothesis points to the rate-limiting enzyme of the respiratory cycle, COX, and specifically, to its rate-limiting step, dissociation of the ternary complex between the oxidase and the electron donor, cytochrome *c*. The positively selected Cys-113-Cys-114 motif in bladderworts is found exactly in that region of the complex. Furthermore, COX as a target of selection is not unique to the evolution of bladderwort suction trapping. COX molecular evolution has been hypothesized to underlie expansion of the neocortex in anthropoid primates (46). Greater attention should be paid in the future to discovering other molecular adaptations in structural genes that serve as scaffolding for morphological and physiological novelty, including those acting as key innovations (9) for organismal radiations.

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