Winter 2009

Ecology and Molecular Phylogenetics of *Hydnora* (Hydnoraceae) in Southern Africa

Jay Francis Bolin
Old Dominion University

ECOLOGY AND MOLECULAR PHYLOGENETICS OF *HYDNORA* (HYDNORACEAE) IN SOUTHERN AFRICA

by

Jay Francis Bolin
B.S. December 2000, Virginia Tech
M.S. May 2004, Old Dominion University

A Dissertation Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

ECOLOGICAL SCIENCES

OLD DOMINION UNIVERSITY
December 2009

Approved by:

Lytton Musselman (Director)

Rebecca Bray (Member)

Erika Maass (Member)

Timothy Motley (Member)

ABSTRACT

ECOLOGY AND MOLECULAR PHYLOGENETICS OF *HYDNORA* (HYDNORACEAE) IN SOUTHERN AFRICA

Jay Francis Bolin
Old Dominion University, 2009
Director: Dr. Lytton J. Musselman

The Hydnoraceae are a clade of root holoparasitic angiosperms that contain two small genera, *Hydnora* and *Prosopanche.* This study, focused on *Hydnora*, presents novel data regarding the pollination biology, germination ecology, parasite-host nutritional relationships, and the molecular systematics of this group. Experimental addition of the primary pollinator, *Dermestes maculatus* to *Hydnora africana* chamber flowers demonstrated beetle imprisonment during the carpellate stage. Changes in the inner surfaces of the androecial chamber allowed beetle escape after pollen release. Most beetles escaped, dusted with viable pollen, three days after pollen release. To investigate germination ecology, aqueous root extracts of host and non-host *Euphorbia* spp. were applied to seeds of *Hydnora triceps* which germinated only in response to root extracts of its exclusive host, *Euphorbia dregeana,* and not for co-occurring non-host *Euphorbia* spp. This pattern of host specific germination suggests that germination response to host-root cues may be responsible for host partitioning. There are large gaps in our understanding of holoparasitic plant-host nutrient relationships and the mechanisms of solute uptake. Transdermal water loss, parasite-host mineral relationships, and heterotrophy were evaluated for *Hydnora.* Transdermal water loss in *Hydnora* ranged from 0.14±.02 to 0.38±.04 mg cm^{-2} hr^{-1}, comparable to transpiration rates recorded for xerophytes. Concentrations of P and K were higher in *Hydnora* relative to their CAM

(Crassulacean acid metabolism) hosts; other mineral concentrations were significantly lower in the parasite or were not different. Stable isotope fractionation in host tissues dictated significant differences between parasite and host $\delta^{13}C$ signatures. A phylogeny of the Hydnoraceae was generated using plastid (*rpoB*) and nuclear ITS (internal transcribed spacer) DNA sequences. The analyses supported the monophyly of *Hydnora* and *Prosopanche*, their relationship as sister genera, and validated subgeneric sections of *Hydnora*. Optimization of the character of host preference suggests the Fabaceae as the ancestral state of *Prosopanche* and *Hydnora*. A well resolved Hydnora clade parasitizing Fabaceae was resolved as sister to a clade parasitizing exclusively *Euphorbia*, indicating a single host shift. In order to examine the specific limits of *H. africana* phylogenetic and morphological data were compared. In the section *Euhydnora*, floral morphometric data was congruent with phylogenetic data, revealing three cryptic taxa within *Hydnora africana sensu lato*, *Hydnora africana, Hydnora longicollis,* and a new *Hydnora* species.

This thesis is dedicated to my loving wife Carmony and the Bolin-You-Hartwig family.

ACKNOWLEDGMENTS

I am indebted to my major advisor Lytton J. Musselman, whose relentless enthusiasm for botany and natural history and his huge investments of time and resources in me, have profoundly and positively steered my development as a scientist. Tim Motley has provided invaluable technical assistance and guidance as a committee member. My thanks to Rebecca Bray are from the heart, for her editorial and botanical skill and countless pounds of caponata. Erika Maass and her entire family in Windhoek, selflessly provided logistical support and fellowship during my fieldwork in Namibia and during my Fulbright appointment. Kushan Tennakoon provided inspiration and expertise to pursue ecophysiological studies of *Hydnora* and served on my committee during his Fulbright appointment to Old Dominion University. Frank Day and Joseph Rule served on my initial guidance committee; their assistance is gratefully acknowledged. The true list of individuals who assisted my work and deserve acknowledgment at the Biology Departments of Old Dominion University and the University of Namibia, and numerous other institutions and campgrounds across Africa, cannot possibly fit in the remaining space here. Let me state simply that this dissertation would not have been possible to complete without the direct assistance of dozens and dozens of colleagues, family members, fellow graduate students, friends, willing botanists, and just plain friendly people from around the world.

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CHAPTER 1

INTRODUCTION

Hydnoraceae is a compelling family of root holoparasitic angiosperms composed of two small genera, *Hydnora* and *Prosopanche*. The Hydnoraceae and the stem parasites *Cassytha* (Lauraceae), represent the earliest diverging lineages of haustorial plant parasites (Barkman et al. 2007). Unlike *Cassytha*, Hydnoraceae has completely recused the photosynthetic lifestyle (De la Harpe et al. 1981). Once commonly allied with Rafflesiaceae (i.e. Cronquist 1981; Takhtajan 1997), the Hydnoraceae have been placed with Aristolochiaceae (Nickrent et al. 2002). However, the precise phylogenetic position of the Hydnoraceae within the Piperales is not known nor is its nearest photosynthetic relative. This is due in large part to persistent unresolved relationships within the Piperales and the exclusion of Hydnoraceae in several recent phylogenetic analyses (Stevens 2008). Interestingly, the Hydnoraceae embryological and seed characters are atypical for Piperales (González and Rudall 2003).

Lacking leaves, scales, and roots, *Hydnora* was first misidentified as the fungus *Hydnum* by Thunberg (1775) in the karoo of South Africa. Numerous additional species of *Hydnora* and *Prosopanche* were described through the 19th and 20th centuries; the most recent monographs of the genus describe 9-12 *Hydnora* and 5-6 *Prosopanche* species (Harms 1935; Vaccaneo 1934). *Hydnora* is distributed from South Africa to East Africa, Madagascar, and the Arabian Peninsula (fig. 1.1). In East Africa, the Arabian Peninsula, and Madagascar (Musselman and Visser 1989), *Hydnora* spp. parasitize

The model journal for this dissertation is the *International Journal of Plant Sciences.*

primarily Fabaceae (Bosser 1994; Jumelle and Perrier de la Bâthie 1912; Miller and Morris 1988; Musselman and Visser 1987). The center of *Hydnora* diversity is the Northern Cape Province of South Africa and the adjoining Karas Region of Namibia (fig. 1.2), where in addition to Fabaceae hosts, *Hydnora* is a specialist parasite of *Euphorbia*. The distribution and diversity of *Prosopanche* is centered in Argentina extending into Paraguay and potentially parts of Brazil, Chili, and Uruguay (Cocucci 1965; Cocucci and Cocucci 1996). *Prosopanche costaricensis* L.D. Gómez is known only from Costa Rica,

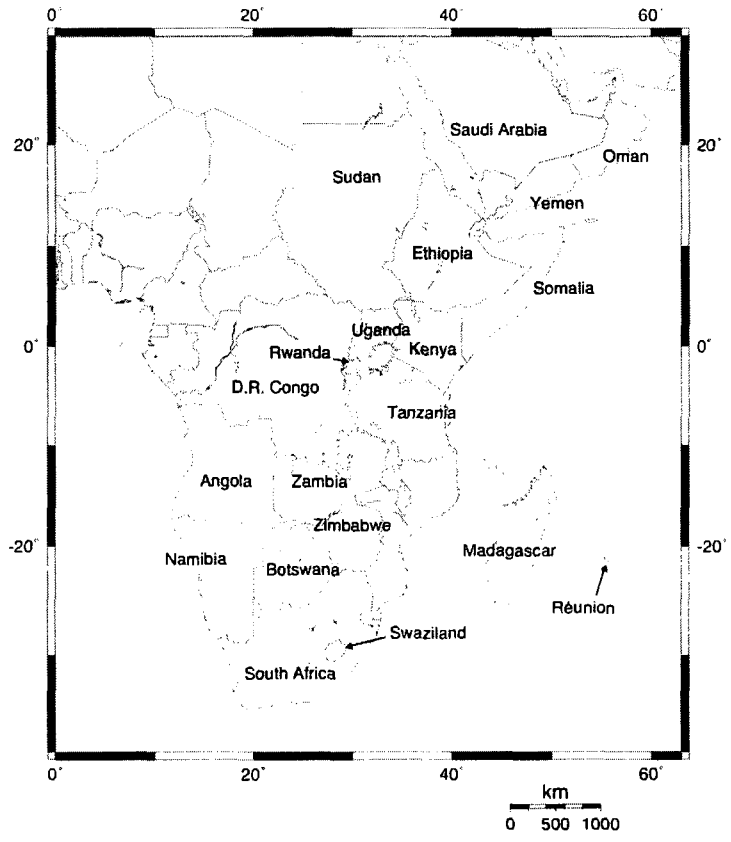

Fig. 1.1 Map of Arabia, East Africa, Southern Africa, and Madagascar. Labeled countries and territories indicate the confirmed *Hydnora* occurrence. The

Fig. 1.1 (continued) population on Réunion may have been introduced and is considered extirpated (Bosser 1994).

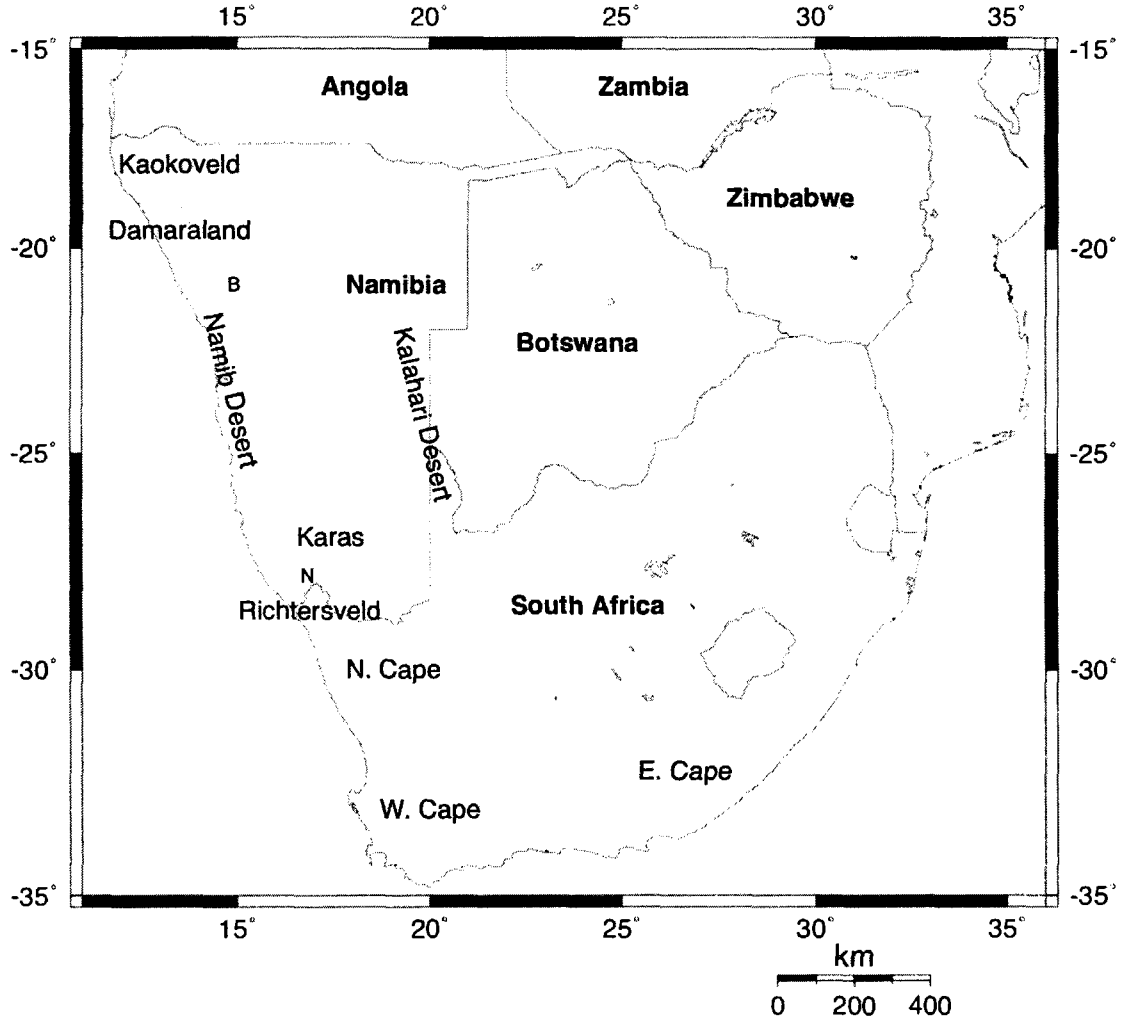

Fig. 1.2 Map of Southern Africa. Bolded labels indicate countries where *Hydnora* is confirmed. Other labels indicate regional names including the Brandberg Massif (B) and the Farm Namuskluft (N). The center of *Hydnora* diversity is the Karas Region and Northern Cape Province of Namibia and South Africa, respectively.

representing a significant disjunction for what was once thought to be a strictly South American group (Gómez and Gómez 1981). Synonymy is rife in the literature and attributable to under collection, morphological convergence, and poor preservation of herbarium material (Musselman and Visser 1987, 1989). Some taxonomic uncertainty remains unresolved because type specimens and other important collections were destroyed during World War II (Musselman and Visser 1987).

Increased awareness and interest in the genus is largely the work of Kuijt (1969), Visser (1981), and Musselman (1991). The modern body of Hydnoraceae literature is mainly descriptive in nature, and despite recent progress, much remains to be learned about this enigmatic genus. The fleshy chamber flowers of *Hydnora* and *Prosopanche* are usually apparent only when flowering. The first detailed descriptions of *Hydnora* pollination biology were by Marloth (1907) who first noted the insect trapping mechanism of *Hydnora africana* and detailed the osmophores and putrid floral odor. *Hydnora* spp. use resource mimicry to lure insects with foul odors that mimic insect brood sites, rotting flesh or dung. Since Marloth's observations only anecdotal reports of floral visitors have been made for *H. abyssinica* (Musselman 1984), *H. africana* (Visser and Musselman 1986), and *H. triceps* (Visser 1989). Odors of a single *H. africana* flower were captured and analyzed by Burger et al. (1988); dimethyl disulfide and dimethyl trisulfide were identifed. They made no specific inferences about the compounds isolated. Interestingly, recent work by (Stensmyr et al. 2002) showed that some of the compounds isolated from the *H. africana* odor profile illicit antennae responses and attract blowflies. Host specific nitidulid beetles, drawn to the fruity floral odor of *Prosopanche americana*,

have been suggested as pollinators (reviewed in Cocucci and Cocucci 1996), however in-depth studies are lacking.

The vegetative organ of Hydnoraceae spreads horizontally through the soil and is ornamented with lateral appendages or bumps that can differentiate into buds, haustoria, or bifurcations of the main body. Due to its unusual appearance, the vegetative organ of Hydnoraceae has been difficult to classify. Schimper (1880) considered the vegetative body of *Prosopanche* rhizome-like, a line of thinking followed by subsequent workers (Cocucci 1965; Cocucci and Cocucci 1996). Whereas for *Hydnora*, Kuijt (1969) employed the terms "pilot roots" for the main vegetative branches and "haustorial roots" for the lateral appendages. Recently, Tennakoon et al. (2007) presented anatomical evidence for *H. triceps* that supported the classification of *Hydnora* vegetative bodies as rhizomes with chimeric root cap-like meristems.

Dastur (1921) studied the seed development of *H. africana* and described a tetrasporic embryo sac and an undifferentiated embryo. Nothing is known about the seedling development or seed germination of *Hydnora* or *Prosopanche* (Kuijt, 1969). Overall, other than for agronomically important root holoparasites such as *Orobanche* (i.e. Abu-Shakra et al. 1970; Sunderland 1960), little is known about the germination requirements of root holoparasites. Unfortunately, for Hydnoraceae and many other groups of holoparasites their host specific germination requirements are implied based on little or no data (i.e., Joel et al. 1994; Press et al. 1990).

Ethnobotanical surveys have showed numerous uses of the fruits of Hydnoraceae. The fruits of Hydnoraceae are large turbinate berries with thousands of small seeds embedded in a fleshy pulp relished by traditional cultures, either eaten raw when ripe or

cooked when immature (Cocucci 1965; Harms 1935; Musselman 1984; Musselman and Visser 1987; Nyafuono et al. 2000; Vaccaneo 1934). Fruits are also eaten by other mammals, that may act as seed dispersers. The South African Afrikaans names for *Hydnora africana*, *jackkalskos* and *bobbejaankos*, meaning jackal-food and baboon-food, respectively, are illustrative of the importance of *Hydnora* as veld food. Medicinally plants in the Hydnoraceae have several reported uses. Patagonians were reported to use *Prosopanche* pollen in dressings for wounds (Cocucci 1965). The dried and powdered *Hydnora* rhizomes are treatment for intestinal ailments in Sudan (Musselman 1984) and South Africa (Dold et al. 2003). Moreover, pastes made of powdered *Hydnora* rhizomes are a topical treatment for acne and skin problems (Dold and Cocks 2005). The medicinal use of *Hydnora* should not be considered an archaic tribal practice; *Hydnora* rhizomes are still actively traded in herbal markets of South Africa's Eastern Cape Province (Dold and Cocks 2002) and Kampala, Uganda (Nyafuono et al. 2000). Recently, Saadabi and Ayoub (2009) demonstrated the antibacterial and antifungal activity of *Hydnora* rhizome extracts using *in vitro* assays. Powdered rhizome extracts with high tannic properties have also been applied in tanning and for staining and preservation of fishing nets (Miller and Morris 1988; Welwitsch 1869). A large quantity of *Hydnora* rhizomes was captured from the Germans during the First World War and identified at Kew (Musselman 1984); the precise use of these rhizomes is unknown but they were probably intended for tanning.

Although a potentially intriguing model of host-parasite relationships, only a handful of studies have investigated host-parasite ecophysiological aspects of *Hydnora,* and no studies exist for *Prosopanche*. Using spectrophotometry, De la Harpe et al. (1981)

showed that *Hydnora* has no trace of chlorophyll. *Hydnora* host-parasite comparisons are limited to one-off measurements of carbon (De la Harpe et al. 1981; Ziegler 1996) and deuterium (Zeigler 1994) stable isotope ratios for *Hydnora africana*.

SPECIFIC AIMS

1) The insect trapping mechanism and pollination biology of *Hydnora* was experimentally evaluated using a beetle addition experiment and pollen viability assay coupled with observations of floral visitors and flower phenology.

2) The germination ecology of *Hydnora* was experimentally evaluated by exposing *Hydnora* seeds to *Euphorbia* host root and *Euphorbia* non-host root extracts.

3) The carbon, nitrogen, and mineral relationships between *Hydnora* and hosts were evaluated by estimating transdermal water loss for *Hydnora*, measuring parasite and host macro- and micro-nutrient profile data, and $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures.

4) Host preference, character evolution, and the systematics of the Hydnoraceae were explored by generating a molecular phylogeny using plastid and nuclear DNA sequence data.

CHAPTER 2

POLLINATION BIOLOGY OF *HYDNORA AFRICANA* IN NAMIBIA: BROOD-SITE MIMICRY WITH INSECT IMPRISONMENT

INTRODUCTION

The genus *Hydnora* (Hydnoraceae) is part of a remarkable basal angiosperm lineage composed entirely of root holoparasites with extremely reduced vegetative morphology (Kuijt 1969; Tennakoon et al. 2007). *Hydnora* ranges from South Africa across sub-Saharan Africa to the Arabian Peninsula and Madagascar (Beentje and Luke 2001; Jumelle and Perrier de la Bâthie 1912; Musselman and Visser 1989). The apparent center of diversity is southern Africa where at least three species are currently recognized (Maass and Musselman 2004; Musselman and Visser 1989; Schreiber 1968). The Hydnoraceae also includes *Prosopanche*, a new world genus with distinct floral morphology (Cocucci and Cocucci 1996). Molecular data show that the Hydnoraceae is allied with the Aristolochiaceae in the Piperales (Nickrent et al. 2002).

All members of the family have chamber flowers and use odor to attract pollinators. Insect imprisonment, defined as a mechanism that temporarily detains insects, was reported for *Prosopanche* (Cocucci and Cocucci 1996) and *Hydnora* (Marloth 1907). However, it was suggested that insects trapped within the *Hydnora* chamber flower do not escape (Visser 1981). Chamber flowers with insect imprisonment are known from other basal angiosperm families, i.e., Araceae (Gibernau et al. 2004; Lack and Diaz 1991)

and Aristolochiaceae (Proctor et al. 1996). Many of the Araceae-Aristolochiaceae insect trapping mechanisms use erect trichomes to detain insects and subsequently slough

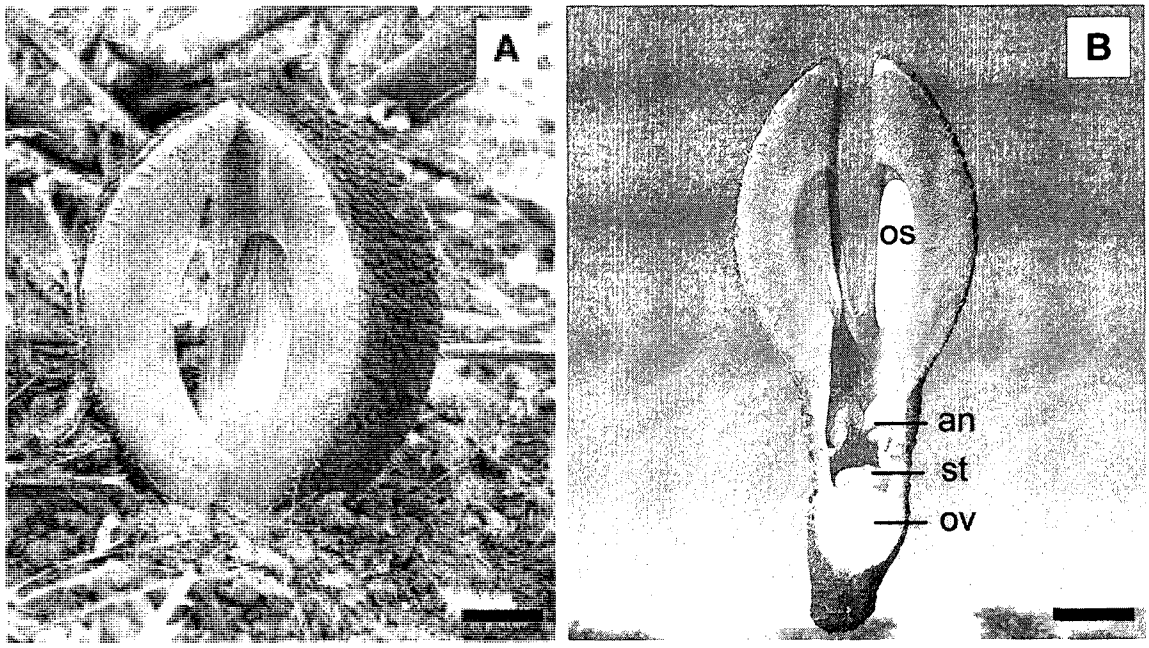

Fig. 2.1 Flower of *Hydnora africana.* Only a portion of the flower of *H. africana* emerges from the soil surface. *A*, The flower at the base of its host *Euphorbia gregaria* (background) (scale bar = 1.5 cm). *B,* The structure of the trimerous flower. The osmophore is recessed on the interior surface of each tepal (os). The fused antheral ring (an) is trilobed and forms the base of the androecial chamber. The trilobed stigma (st) forms a cushion at the base of the gynoecial chamber above the ovary (ov) (scale bar = 2.5 cm).

off trichomes to allow insect escape (Proctor et al. 1996). Floral visitors to chamber flowers with insect trapping mechanisms are mainly Coleoptera (i.e. Beath 1996; Sivadasan and Sabu 1989) or Diptera (i.e. Hall and Brown 1993; Sakai 2002).

Although *Hydnora* flowers lack typical entrapment mechanisms found in other species they do have a unique structure that may facilitate imprisonment and subsequent release. The chamber flower of *Hydnora* has two main components, an androecial chamber and a subtending gynoecial chamber (fig. 2.1A & B). The two chambers are joined by a ring of connate anthers with a central orifice that allows passage of pollen and floral visitors between the chambers. The connate anthers of *Prosopanche* lack the large central orifice and were described as the "antheral body" (Cocucci and Cocucci 1996). We use the term antheral ring for this homologous structure in *Hydnora* to emphasize the unique passage formed by the orifice within the stamens.

The striking chamber flower of *Hydnora africana* Thunb. and its strong fetid odor have attracted botanists interested in its pollination biology (Burger et al. 1988; Marloth 1907; Musselman 1984; Musselman and Visser 1989; Visser and Musselman 1986). *Hydnora africana sensu lato* is known from parts of Angola, Namibia, and South Africa. It follows the distribution of its hosts, various species of *Euphorbia*. Due to synonymy associated with *H. africana* and observed morphological and molecular variability (Bolin, unpublished data), we emphasize that we are presenting pollination biology data for only Namibian *H. africana* populations parasitizing *Euphorbia gregaria* Marloth. This host plant is restricted to southern Namibia and extreme northwestern South Africa (Curtis and Mannheimer 2005).

We investigated the ecology of *H. africana* in Namibia and address: (1) seasonal flowering and fruiting patterns, (2) individual flower phenology, (3) identification of floral visitors, and (4) an experimental evaluation of the insect imprisonment mechanism.

MATERIALS AND METHODS

Study Sites

Field observations on *H. africana* were conducted in south-central Namibia at two sites, approximately 80 km apart, the Farm Kanas (FK) located west of the town of Seeheim and the Gondwana Cañon Park (GCP) east of the Fish River Canyon. In the study areas, vegetation is dominated by the large shrub *E. gregaria,* the only host of *H. africana* at these sites. The vegetation type of both sites is classified as Dwarf Shrub Savannah and has limited and sporadic rainfall, approximately 50 – 150 mm per year (Mendelsohn et al. 2002). Air temperatures measured during the surveys of floral visitors at both sites were similar and ranged from highs of 30-38 °C during the day to 12-17 °C during the night. Observations of the seasonal flowering and fruiting phenology of *H. africana* at GCP were initiated Sept. 2001 and included seven visits through early 2008. At GCP, surveys of floral visitors and experimental manipulations were conducted between Oct. 6 – 14, 2005. FK was visited three times between Feb. 2004 and Nov. 2005 and surveys of floral visitors were conducted Oct. 31 - Nov. 6, 2005.

To observe *H. africana* flowering phenology and to assess floral visitors, a total of 37 flowers (KF = 18 and GCP =19 flowers) were followed from first opening of the flower. Flowers were visited three times daily (approximately 0700-0900, 1200-1400, 1700-1900 hrs) and all floral visitors were quantified for three days following pollen

release. These observations were supplemented with periodic nighttime observations for floral visitors, including one full night of bihourly observations. A Moritex endoscope was used to observe the insects within the gynoecial chamber (Moritex USA Inc., San Jose, CA USA). Insects were identified with the assistance of: John Irish, National Botanical Research Institute, Windhoek, Namibia, Dr. Jerry Cook, Sam Houston State University, Houston, TX: and the Namibian National Museum, Windhoek, Namibia. Floral morphometrics including flower length, width, portion of flower above ground level, tepal width, length and number, stamen width, stigma width, and interior orifice diameter formed by the antheral ring were taken for 48 flowers (KF = 23 and GCP = 25).

Beetle Addition Experiment

To assess the insect trapping mechanism of *H. africana* a manipulative experiment was conducted using flowers from individual plants at GCP (n = 9). At bud break, five *Dermestes maculatus* (Dermestidae) beetles marked with white corrective fluid were added to each floral chamber. The flowers were followed until three days after pollen release. Each evening the flowers were assessed for escape of marked *D. maculatus* with the aid of the endoscope, and when necessary, manual removal (with forceps) to facilitate the beetle count. At the termination of the experiment, remaining and dead *D. maculatus* were also quantified.

Pollen Viability

An estimate of pollen viability from seven plants (FRC) was assessed using a tetrazolium assay (Norton 1966) for dehydrogenase activity at 24 h intervals for three

days beginning with pollen release (Oct 7 – 10, 2005). Pollen was stained with a 1% tetrazolium salt solution and viewed at 100 X magnification with a field microscope (Ernst Leitz Co., Wetzlar, Germany). Pollen stained pink was scored as viable and translucent pollen was scored nonviable. Three random fields were scored per replicate by two researchers independently and subsamples (random fields) were averaged. A negative control for the staining procedure was included by devitalizing the pollen with ethanol (Dafni et al. 2005).

Statistical analyses of the beetle addition and pollen viability experiments were conducted using repeated measures analysis of variance (ANOVA) implemented in SPSS for Windows 14.0 (SPSS Inc., Chicago, IL USA). When assumptions of sphericity were violated, the conservative Huynh-Feldt correction was applied to produce a valid F-ratio (Huynh and Feldt 1976).

RESULTS

Phenology and Morphology

The flowering period of *H. africana* parasitizing *E. gregaria* in south-central Namibia peaks from Nov. - Feb. with low levels of sporadic flowering observed throughout the year. Fruit maturation is lengthy and may be longer than one year. Due to the hypogeous nature of *H. africana,* it was difficult to evaluate the flowering per plant, however careful excavation revealed common rhizomes. Adjacent spent flowers and buds (maximum buds and flowers per individual observed = six) of the season, associated with each open flower were usually present, indicating multiple flowers per individual per season. We observed that only 6.2 % (n = 32) and 19.4 % (n = 36) of individuals

observed at KF and GCP, respectively had more than one flower open simultaneously. A single individual at GCP was observed with three fresh open flowers.

Floral metric data from KF and GCP were pooled because they were consistently similar. The flowers averaged 11.7 ± 0.3 cm above the ground surface. Overall flower

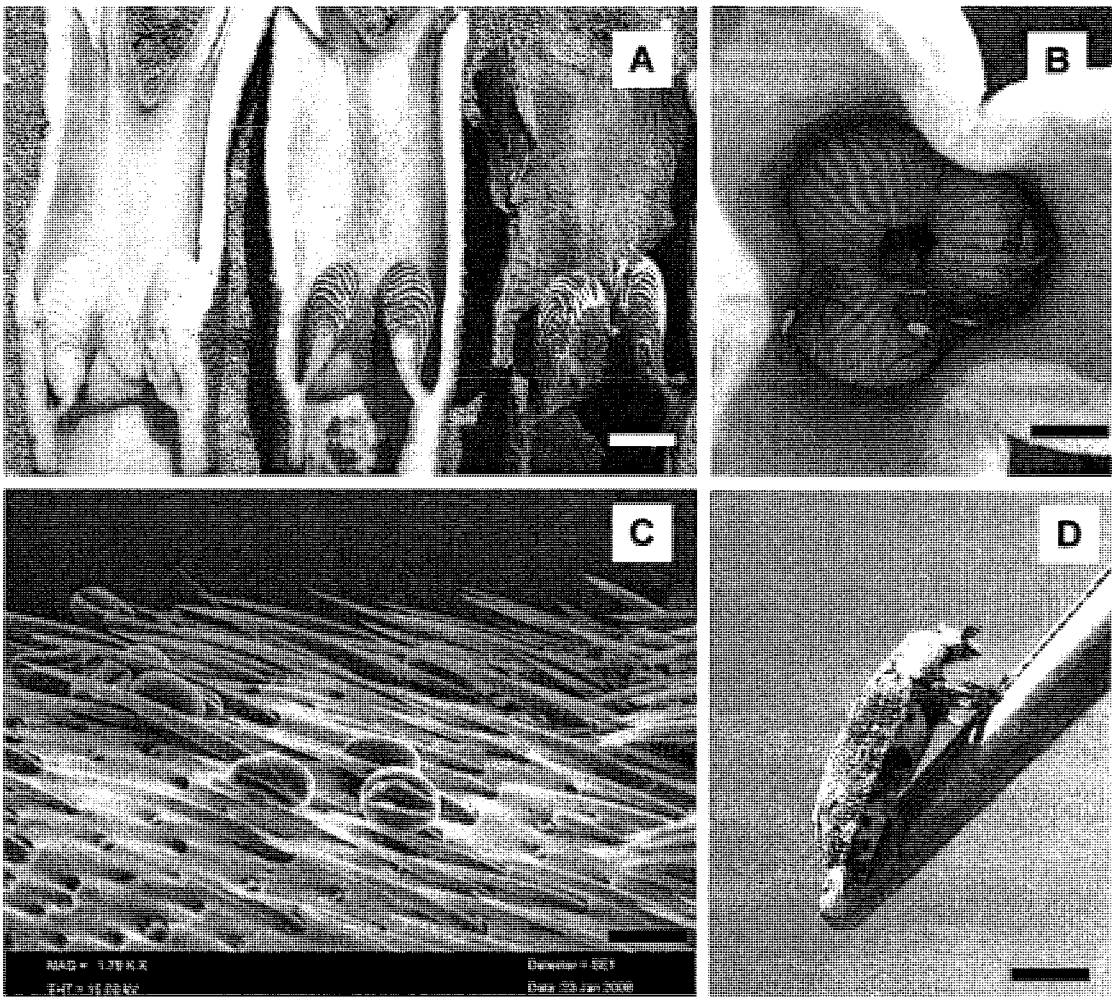

Fig. 2.2 Trapping mechanism and floral visitors. *A.* Three androecium chambers arrayed to show the "catch and release" mechanism of *Hydnora africana.* Left, flower is carpellate and the inner surface of the androecial chamber is smooth and orange-pink

Fig. 2.2 (continued) (day 1 - 3). At this stage, *D. maculatus* and other insects cannot escape. Center, flower is at pollen release, the inner surface of the androecial chamber begins to darken and becomes stippled. At this stage beetles begin to escape. Right, flower is three days past pollen release and the chamber wall is dry and textured (scale bar = 1 cm). *B.* View down into the androecial chamber, antheral ring is in the center, just prior to pollen release. Five *D. maculatus* marked with correction fluid are visible. (scale bar = 1 cm). *C.* SEM image of *H. africana* pollen on the dense hairs located on the elytra of *D. maculatus* (scale bar = 20 μm). *D.* The primary floral visitor, *D. maculatus* is the likely pollinator of *H. africana*, here dusted with a heavy pollen load after pollen release (scale bar = 2.5 mm).

length (measured from the base of the ovary) and width were 16.7 ± 0.4 and 7.1 ± 0.2 cm, respectively. Tepal width was 3.9 ± 0.1 cm and tepal length was 10.6 ± 0.3 cm. Nearly all bore three tepals with the exception of a few aberrant individuals with two or four tepals. At pollen release, the diameter of the orifice formed by the antheral ring was 4.0 ± 1.1 mm. Stamen width was 2.3 ± 0.1 cm and stigma width was 1.6 ± 0.1 cm.

The *H. africana* flower bud emerges from the soil surface, usually adjacent to or among the branches of the host (fig. 2.1A). Obstacles such as stones or other debris are lifted by bud emergence from the soil. A pungent odor resembling carrion, detectable at distances up to 10 m, is released when the flower opens, originating in the elongate osmophores recessed in each tepal (fig. 2.1B). It was not possible to quantify the intensity of the odor over the flowering period, but it lessens after pollen release.

When the flower first opens the osmophore is initially white and over several hours turns grey. Likewise the inner surfaces of the tepals are initially orange-pink and turn a deeper orange-red after opening. Stigmatic surfaces are moist and viscous at bud break and remain so throughout flowering. While putatively pistillate and receptive, numerous Coleopterans visit the flower. Insects alight on the tepals and crawl across the intermediate surfaces of the perianth lobes over elongated structures resembling trichomes (figs. 2.1A & 1B). Upon reaching the recessed osmophore they typically drop into the floral chamber. The smooth inner surface and steep vertical incline of the androecial chamber prior to pollen release (fig. 2.2A) prevents various species of insects from escaping (fig. 2.2B). No nectaries were observed.

After a mean of three days (range 2 – 5 days) of strong odor production, presumed stigmatic receptivity, and detention of insects, the trilobed anthers dehisce sequentially over a period of several hours. In rare instances pollen release between the first and last lobe of the stigmatic ring may take more than a day. Even without insect visitation, pollen drops passively from the interior facing portion of the anthers directly on to the stigma (fig. 2.2A). After pollen release, the surface of the androecial chamber begins to change with stippling and darkening of the tissues. One day after pollen release, changes in floral tissues create a textured surface on the inner wall of the androecial chamber facilitating insect release (fig. 2.2A).

Floral Visitors

GCP and KF floral visitor data were pooled because insect visitation rates were not significantly different between study sites ($t = 0.93$, $df = 35$, $P = 0.36$) and species

compositions were similar. A total of 128 floral visitors representing 18 species were observed within *H. africana* flowers (table 2.1). A total of 11 species, mainly beetles, were trapped within the carpellate-stage chamber flowers. An additional seven species were observed freely moving in and out of the flowers (table 2.1). Occurrence of detained insects within each flower was low, 2.8 ± 0.7 insects. *Dermestes maculatus* accounted for 76.9% of all trapped insects and was observed at a density of 2.2 ± 0.6 per flower. Floral visitors were observed that could readily move in and out of the chamber flowers. These insects only accounted for 20 individual observations, and those cannot be directly compared to the density of trapped insects due to inherent sampling differences. Half of the total insects not imprisoned in the chamber flower were ants. No insects were observed visiting the flowers during the night, other than those trapped. All trapped floral visitors in female stage flowers were observed with high pollen loads, entirely dusted in copious amounts of pollen (figs. 2.2C & 2.2D).

Beetle Addition Experiment and Pollen Viability

Marked *D. maculatus* did not escape prior to pollen release (fig. 3.1). *Dermestes maculatus* escape began on the day of pollen shed and continued in the days following. Three days after pollen release 55.5 ± 16.7 % of the marked beetles had escaped, 26.6 ± 16.7 % were still within the chamber, and 15.6 ± 6.8 % were dead. Repeated measures ANOVA indicated that this movement of beetles was statistically significant ($F = 7.21$, $df = 5$, $P = 001$). After pollen shed, the marked *D. maculatus* were heavily dusted in pollen. Pollen viability was high at pollen release, 90.0 ± 3.3 % and declined significantly ($F = 26.94$, $df = 3$, $P = 0.001$) to 20.9 ± 14.4 % after three days (fig. 3.1). Pollen devitalized

with ethanol was consistently translucent after staining, indicating that the negative control was effective.

DISCUSSION

The floral biology of *H. africana* follows many of the major patterns highlighted by Thien et al. (2000) in their review of basal angiosperm pollination biology, including brood-site mimicry and insect imprisonment. Flowers of *H. africana* are putatively protogynous, a strong floral odor is produced, and a chamber flower and insect trapping mechanism are present. These features are shared with many *Aristolochia* spp. (Burgess et al. 2004; Proctor et al. 1996). Such similarities between *Aristolochia* and *Hydnora* may represent ancestral traits rather than convergent evolution in light of the systematic placement of the Hydnoraceae with the Aristolochiaceae (Nickrent et al. 2002). These traits are also congruent with the patterns associated with beetle pollination (saprocantharophily) (Bernhardt 2000), although *Aristolochia* are mainly fly pollinated (sapromyophily) (Proctor et al. 1996).

After bud break the flower of *H. africana* immediately begins production of a foul odor reminiscent of carrion. This odor is produced in the osmophore, a spongy white area that is recessed within the inner surface of each tepal and soon turns grey (fig. 1.1B). These osmophores were identified by Marloth (1907), who termed them "white bodies" and bravely reported that these putrid smelling bodies are "like a spongy pudding, not only in appearance but also in taste". Harms (1935) used the term "bait bodies" (Koderkörpen) to describe the osmophores. Subsequently, the term "bait bodies" has been erroneously applied to hair-like outgrowths on the outer margins of the tepals

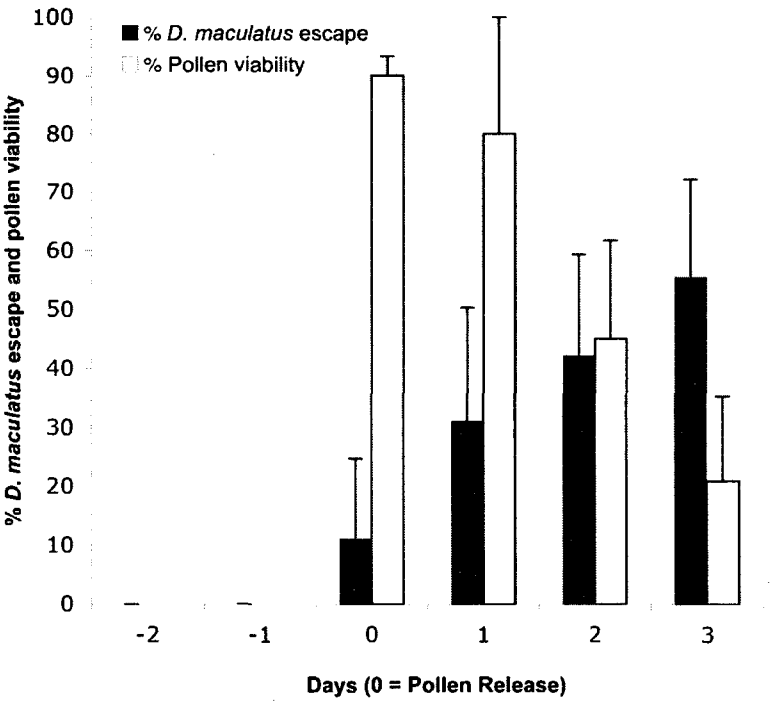

Fig. 2.3 Marked beetle (*Dermestes maculatus*) escape from *Hydnora africana* chamber flowers (n = 9) and pollen viability (n = 7). Error bars indicate standard error.

(Musselman and Visser 1989) that have no role in odor production. Burger et al. (1988) investigated the chemical composition of the floral odor produced by a single *H. africana* flower and reported a suite of compounds, including dimethyl disulfide and dimethyl trisulfide. These two compounds are also found in the floral odor of the dead horse-arum, *Helicodiceros muscivorus* Engl. (Araceae), which attracted blowflies (Stensmyr et al. 2002). Floral odor is a key component of the brood-site mimicry in *H. africana.*

We observed *D. maculatus* floral visitors investigating the osmophores, lured to the flowers by the putrid odor. Often they dropped into the floral chamber due to slick inner surfaces of the tepal (fig. 2.2B). The floral visitors that were temporarily imprisoned in the floral chamber were all Coleoptera with the exception of one ant lion larva (Myrmeleontidae) (table 2.1). Numerous Tenebrionidae species and one Scarabidae were found imprisoned but at very low densities. *Dermestes maculatus* accounted for 76% of all imprisoned insects and occurred at the highest density 2.2 ± 0.6 per flower (fig. 2.2D).

The adults and larvae of *D. maculatus* feed on animal connective tissues and dried carrion (Begum et al. 1983); hence their common name hide beetle. Previous observations of *H. africana* in South Africa also reported the presence of *D. maculatus* within the chambers of *H. africana* (Marloth 1907; Visser and Musselman 1986). All of the imprisoned insects became coated with sticky pollen after pollen release (figs. 2.2C & 2D). Similarly, Coleoptera are reported as floral visitors of *Hydnora abyssinica* Braun representing Hybosoridae, Trogidae, Scarabaeidae, and three species of Tenebrionidae (Musselman 1984). Interestingly, we did not observe Diptera visiting *H. africana* in the study area (GCP and FK). In contrast, in the Richtersveld of South Africa, and Namuskluft, Namibia, both Coleoptera and Diptera, particularly flesh flies and blowflies, were observed commonly visiting *H. africana* and *Hydnora triceps* Drège & Meyer (Bolin *et al.* unpublished data). Perhaps environmental or host specific factors are influencing *H. africana* floral odors or conceivably, differences in odor may reveal a cryptic species.

Table 2.1

HYDNORA AFRICANA FLORAL VISITORS

Insects Trapped				Insects Not Trapped			
	Floral Visitor	*N*	%		Floral Visitor	*N*	%
Coleoptera:				Blattodea:			
Dermestidae	*Dermestes maculatus*	83	76.9	Polyphagidae	*Tivia termes*	4	20.0
Histeridae	*Saprinus bicolor*	1	0.9	Orthoptera:			
Scarabaeidae	*Gymnopleurus humanus*	1	0.9	Bradyporidae	*Acanthoproctus cervinus*	1	5.0
Tenebrionidae	*Cyphostethe sphaenaroides*	1	0.9	Coleoptera:			
Tenebrionidae	*Eurychora* sp.	3	2.8	Tenebrionidae	*Stenocara dentata*	1	5.0
Tenebrionidae	*Metriopus* sp.	2	1.9	Staphylinidae	*Philonthus* sp.	4	20.0
Tenebrionidae	*Rhammatodes* sp.	7	6.5	Hymenoptera:			
Tenebrionidae	*Stips dohrni*	6	5.6	Formicidae	*Camponotus fulvopilosus*	6	30.0
Tenebrionidae	*Zophosis* sp. 1	2	1.9	Formicidae	*Pheidole* sp. 1	3	15.0
Tenebrionidae	*Zophosis* sp. 2	1	0.9	Formicidae	*Pheidole* sp. 2	1	5.0
Neuroptera:							
Myrmeleontidae	Myrmeleontidae sp.	1	0.9				
	Total	108	100		Total	20	100

In addition to imprisoned insects, seven additional species were occasionally observed freely moving in and out of the floral chambers (Table 2.1). The visitation rate of the transient insects was low, but not comparable to the density of imprisoned insects. In more than 500 individual observations on the 37 study flowers, only 20 individual transient floral visitors were observed. These transient floral visitors consisted of three species of ant (Formicidae), a desert cockroach (Polyphagidae), an armored cricket (Orthoptera), and two beetle species (Coleoptera). The transient beetles had two different means of escape, *Stenocara dentata* (Tenebrionidae) was large enough to reach the lip of

the androecial chamber, while the *Philonthus* sp. (Stapylinidae) flew or crawled out. Interestingly, *D. maculatus*, also a strong flyer, did not attempt to fly out of the chamber, but repeatedly attempted to crawl out. This might be explained because *D. maculatus* lacked a minimum clear takeoff distance when imprisoned in the chamber.

Marloth (1907) first outlined insect imprisonment in *H. africana*. However, Visser (1981) expressed doubts that the trapped insects eventually escaped to affect pollination. The marked beetle addition clearly demonstrates that *D. maculatus* is imprisoned during the pistillate stage and only begins to escape after pollen shed (fig. 2.3). To our knowledge, our marked beetle trial was the first experiment to evaluate the efficacy of an insect imprisonment mechanism. Changes in the inner surface of the androecial chamber, related to the drying and senescence of the perianth, allowed *D. maculatus* to escape the chamber (Fig 2.2D). When escaping, *D. maculatus* were observed flying away once they reached the apex of the tepal. Over 55.5 % of the beetles escaped by the third day after pollen release, 26.6 ± 16.7 % were still within the chamber, and the remainder were dead (perhaps from handling for counts or from the marking paint). The mean and standard error for the beetles still within the chamber at the termination of the experiment is relatively high. This can be explained by a single flower (one experimental unit) where all of the beetles were trapped within the gynoecial chamber by the closure of the antheral ring. The passage at the center of the antheral ring is wide enough (4.0 ± 1.1 mm) at pollen release to allow insect movements between the androecial and gynoecial chambers (fig. 2.2B). Antheral ring diameter obviously precludes some floral visitors from acting as pollinators. Notably, all of the observed imprisoned insects could enter and exit the gynoecial chamber. Completely dried flowers

from past years and herbarium specimens often have closed antheral rings. The closure of the antheral ring is likely a passive movement as portions of the flower dry. But it is possible that it affords some protection to the ovary. However, sporadic entombment of insects in the gynoecial chamber may be purely incidental.

To ensure that the beetles leaving the chamber flower had access to viable pollen one to three days after pollen release we used a tetrazolium salt pollen viability assay. As expected, pollen viability declined over time. However, viable pollen was available to potential pollinators as many as three days after pollen release; albeit, at significantly reduced viability levels. Still, the combination of the marked beetle trial and the pollen viability assay demonstrate the efficiency of the *H. africana* insect imprisonment mechanism.

Floral thermogenesis has been reported from several basal angiosperm lineages i.e., Araceae, Magnoliaceae, and Nympheaceae (i.e. Azuma et al. 1999; Dieringer et al. 1999; Nagy et al. 1972; Prance and Arias 1975). Moreover, thermogenesis has been reported from two parasitic genera *Prosopanche* (Cocucci and Cocucci 1996) and *Rafflesia* (Patino et al. 2000). We investigated thermogenesis, with 4-channel H12 HOBO Data loggers equipped with Type-K 5 mm diameter thermistors (Onset Computer Corp. Borne, MA USA). Temperatures were recorded for four days within the androecial and gynoecial chambers of three plants *in situ* and compared to soil and air temperatures as controls. Our investigation of thermogenesis in *H. africana* was inconclusive (Data not reported). However, more sensitive thermocouples inserted directly into floral tissues may reveal elevated temperatures. Still, using similar methodology and equipment we

observed thermogenesis in pistillate stage *Hydnora esculenta* in Madagascar (Bolin unpublished data).

Basic information about the breeding system for all *Hydnora* spp. is sorely lacking. The long fruit development time hindered our first attempts at classical breeding system experiments, but further research is planned. *Hydnora* spp. have varying levels of host specificity and thus offer an good model for testing the relationship between increasing self compatibility and host specificity in parasitic plants (Bernhardt 1983; Molau 1995).

Because the primary floral visitor *D. maculatus*, oviposits exclusively in carrion, the pollination syndrome of *H. africana* can be classified as brood-site mimicry with imprisonment (Dafni 1984; Faegri and van der Pijl 1979; Proctor et al. 1996). Sakai (2002) described pollinator mutualisms for two *Aristolochia* spp., with chamber flowers and without trapping mechanisms, whose spent floral materials were larval development sites for Dipterans. No evidence of insect larvae development in *H. africana* flowers past pollen release was observed. Some chamber flowers with insect imprisonment offer rewards to increase visitation (Diaz and Kite 2006). However, in this low productivity study system (Mendelsohn et al. 2002), carrion feeders such as *D. maculatus* probably cannot afford to pass up any potential feeding and oviposition opportunity.

More pollination studies of *H. africana* across its range are required to determine the precise pollinators involved in this brood-site mimicry syndrome and the potential role of fly mediated pollination. Unlike our study sites (GCP and KF) that lacked Dipteran visitors, Dipteran floral visitors were observed at *H. africana* and *H. triceps* populations that we observed in the Richtersveld in South Africa and Namuskluft,

Namibia. However, it is unknown if flies make contact with the stigma. Flies would certainly circumvent the trapping mechanism, unless they are trapped in the gynoecial chamber following antheral ring closure. Clearly, more research is required to understand the observed floral visitor variation between our study sites and those elsewhere in Namibia and South Africa. Combined with further investigations of the breeding system, a fascinating model of insect imprisonment pollination will surely emerge.

CHAPTER 3

HOST SPECIFIC GERMINATION OF *HYDNORA TRICEPS*

INTRODUCTION

Root holoparasitic angiosperms require immediate haustorial attachment to their hosts following germination in order to survive. Agriculturally important weedy root holoparasites in the genus *Orobanche* and *Striga* require the presence of a host root or root extracts to stimulate germination (Abu-Shakra et al. 1970; Joel et al. 1994; Sunderland 1960). Largely based on those data, the working assumption for all root holoparasites from nine lineages (Apodanthaceae, Balanophoraceae, Cynomoriaceae, Cytinaceae, Hydnoraceae, Lennoaceae (included in Boraginaceae), Mitrastemonaceae, Orobanchaceae, and Rafflesiaceae) is that germination requires chemical stimulants from the host root (Boone et al. 1995; Press et al. 1990; Stewart and Press 1990). Remarkably, for root holoparasitic plants, aside from agronomically important weeds, basic germination data remain scanty and inconclusive: *Bdallophytun bambusarum* (Liebm.) Harms (Cytinaceae) (García-Franco and Rico-Gray 1997), *Dactylanthus taylorii* Hook.f. (Balanophoraceae) (Ecroyd 1996), *Epifagus virginiana* (L.) W.P.C.Barton (Orobanchaceae) (reviewed in Williams and Zuck 1986) and *Pholisma sonorae* (Torr. Ex A. Gray) Yatsk. (Lennoaceae) (Cothrun 1969) are the only examples.

Parasitic plant germination stimulants from host root exudates broadly classified as strigolactones were first characterized from cotton (Cook et al. 1966; Cook et al. 1972) and then isolated from host plants (Siame et al. 1993). Recently strigolactones have been

identified as signals for mutualistic arbuscular mycorrhizal fungi (Akiyama et al. 2005) and novel forms from a variety of plants have been described (i.e. Awad et al. 2006 ; Yoneyama et al. 2008).

We investigated the germination ecology of a narrow endemic of South Africa and Namibia, *Hydnora triceps* Drège & Meyer (Hydnoraceae). An unusual plant, the rhizome of *H. triceps* traverses the soil to parasitize its exclusive host, *Euphorbia dregeana* Meyer (Tennakoon et al. 2007). The chamber flower of *H. triceps* is entirely subterranean (fig. 3.1A). As the flower develops, it displaces surface soil and generates cracks in the soil surface, through which putrid odors produced by osmophores attract carrion flies and beetles (Maass and Musselman 2004; Visser 1989). The fruit of *H. triceps* is a large berry (5-15 cm) and contains thousands of tiny seeds. The embryo of *Hydnora africana* Thunb. was described as undifferentiated and spherical (Dastur 1921), and nothing is known about the germination or seedling morphology of *Hydnora.* Kuijt (1969) presaged that "a fascinating story awaits the botanist who is fortunate enough to have access to viable seeds."

We describe the germination biology of *H. triceps.* To address potential host specificity in the germination response, we compared the germination responses of *H. triceps* seeds from two distinct populations to root extracts of host and non-host *Euphorbia* spp. Moreover, we include brief observations on seedling morphology, and field observations of frugivory and seed dispersal.

MATERIALS AND METHODS

Fruit were collected from two sites in southern Africa, Farm Namuskluft in southwestern Namibia (S 27° 56.427", E 16° 48.141"; Oct. 2005; four fruits) and Farm

Gemsbokvlei in northwestern South Africa (S 29° 29.299", E 17° 07.078"; Sept. 2005, nine fruits). Fruits were 10 – 30 cm below the soil surface in the vicinity of host plants. Seeds were bulked separately from each respective population. Seeds were dry stored, in brown paper bags, at ambient laboratory temperatures in dark conditions until the initiation of the experiment. From each site, mature roots (1-2 cm diameter) from host (*E. dregeana*) and non-host *Euphorbia* species occurring sympatrically (*Euphorbia mauritanica* L. and *Euphorbia gummifera* Boiss.) were collected and stored at 4 °C and used within ten days of collection.

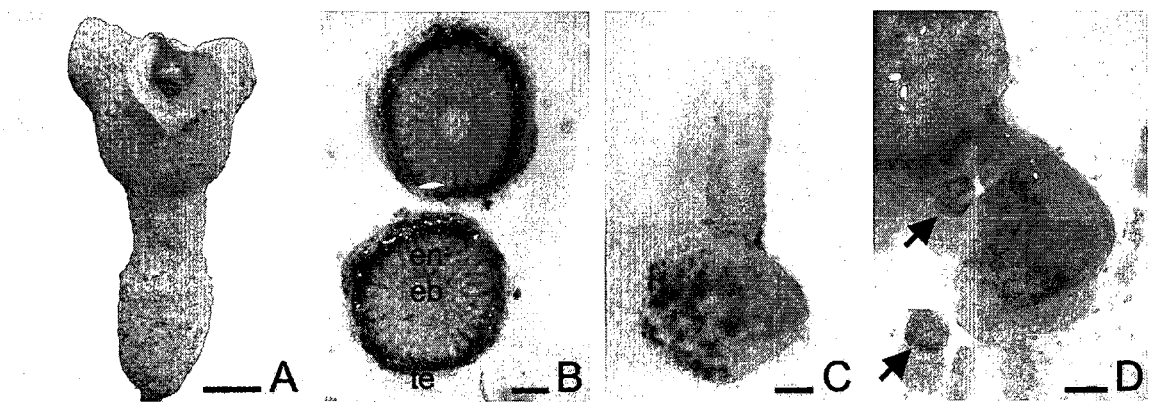

Fig. 3.1 *Hydnora triceps* flower, seed, and seedling. *A*, The hypogeous flower of *H. triceps*, connate tepals form three lateral vents that convey fetid floral odors and pollinators (Scale bar = 2 cm). *B*, Cross sectional view of *H. triceps* seed showing the spherical embryo (eb), endosperm (en), and testa (te) (Scale bar = 0.25 mm). *C*, Germinated seed showing radicle (Scale bar = 0.25 mm). *D*, Small mammal dropping containing intact *H. triceps* seeds, indicated by arrows (Scale bar = 1 mm).

Germination studies were conducted at the University of Namibia and initiated 16 Dec. 2005. Seed viability was estimated by cutting 100 seeds in half. Seeds were considered viable if a full and fleshy endosperm and embryo were evident. Estimation of viability was only conducted for the South African (SA) population due to low seed harvest from the Namibian (NAM) population. Germination protocols and apparatus were adapted from the International Institute of Tropical Agriculture *Striga* "cut root" germination methods (Berner et al. 1997). Each experimental unit was an individual 15 cm diameter Petri dish with two layers of filter paper. We placed a 1.5 cm diameter x 1.5 cm tall aluminum foil ring in the center of the dish. For each root extract treatment replicate we placed one g of fresh and shredded root tissues (from an individual host plant per replicate to avoid pseudoreplication) in the aluminum foil ring. Three mL of deionized water was added to the center of the aluminum ring, saturating the root tissues and spreading the root extracts to the seeds. We used three replications of 50 seeds and 20 seeds each for each treatment, for the SA and NAM populations, respectively. All seeds were first surface sterilized with a 10% bleach solution and triple rinsed. Filter papers of control groups were moistened only with deionized water, using the same germination apparatus.

Germination treatments for the SA provenance *H. triceps* seeds were (1) control, and root extracts from (2) *E. dregeana* (SA), (3) non-host *E. mauritanica* (SA), (4) non-host *E. gummifera* (NAM), and (5) *E. dregeana* (NAM). Due to limited number of seeds, treatments for the NAM provenance seeds were (6) control, (7) *E. dregeana* (NAM), (8) *E. dregeana* (SA). Germination was considered the emergence of the radicle. Seeds were

maintained at ambient laboratory temperatures (23-27 °C) in darkness and observations for germination were concluded after 30 days.

Observations of seed dispersers were made from 10-19 Sept. and 9-13 Dec., 2005 at the SA population. Twenty intact rodent droppings from within and around partially eaten fruits were collected and investigated for intact seeds. Seed viability was estimated destructively by inspection for intact endosperm and embryo.

Because no *H. triceps* seeds germinated in control and non-host root extracts treatments, statistical analyses were applied to arcsine transformed data to parse the effects of seed provenance (SA and NAM) and *E. dregeana* root exudate provenance (SA and NAM) using two way Analysis of Variance (ANOVA) in SPSS 16.0 (SPSS, Chicago, IL, USA).

RESULTS

The seeds of *H. triceps* were obviously water permeable as seed swelling indicated passive imbibition of water. Seed diameter ranged from 0.9 to 1.2 mm. An undifferentiated spherical embryo was observed surrounded by ruminate endosperm and a thin hard testa (fig. 3.1B). Radicles were observed up to 3 mm long (fig. 3.1C).

Germination rates of *H. triceps* were low and only occurred when seeds were exposed to the root extracts of its exclusive host *E. dregeana* (table 1). Excluding control and non-host treatments (all 0 % germination), two way ANOVA indicated no significant differences between the seed provenance (d.f. = 1, F = 2.8, $P = 0.13$), *E. dregeana* root exudate provenance (d.f. = 1, F = 0.03, $P = 0.86$), or their interaction (d.f. = 1, F = 1.7, $P = 0.23$). Seed viability was 96 % for the *H. triceps* from the SA population.

The round-eared elephant shrew (*Macroscelides proboscideus*) and the striped mouse (*Rhabdomys pumilio*) were observed feeding on the fleshy pulp of *H. triceps* fruits. Inspection of small mammal dropping ($n = 20$) found in and around partially eaten fruits showed that 25% contained viable *H. triceps* seeds (Range: 0-19, Mean: 1.9 ± 1.1 (s.e.) seeds/dropping) (fig. 3.1D). All seeds observed in the droppings had intact embryos and endosperm and showed no signs of testa damage.

Table 3.1

GERMINATION OF *HYDNORA TRICEPS* SEEDS

H. triceps seed provenance	Root extract	Root extract provenance	Percent germination
Gemsbokvlei (SA)	Control	n/a	0.0
Gemsbokvlei (SA)	*E. dregeana* †	Gemsbokvlei (SA)	14.0 ± 6.1
Gemsbokvlei (SA)	*E. mauritanica* ‡	Gemsbokvlei (SA)	0.0
Gemsbokvlei (SA)	*E. gummifera* ‡	Namuskluft (NAM)	0.0
Gemsbokvlei (SA)	*E. dregeana* †	Namuskluft (NAM)	12.0 ± 3.1
Namuskluft (NAM)	Control	n/a	0.0
Namuskluft (NAM)	*E. dregeana* †	Namuskluft (NAM)	20.0 ± 8.7
Namuskluft (NAM)	*E. dregeana* †	Gemsbokvlei (SA)	3.3 ± 1.7

Note.- Each treatment and control was applied independently in triplicate. The mean percent germination ± standard error ($n = 3$) is presented. Two factor ANOVA of the *E. dregeana* treatments demonstrated no significant effects of *H. triceps* seed or *E. dregeana* root extract provenance.

† Host Root

‡ Nonhost Root Extracts

DISCUSSION

The major result of this study showed that *H. triceps* seeds germinate only in response to root extracts of its exclusive host *E. dregeana,* and not for co-occurring species *E. mauritanica* and *E. gummifera.* Though *H. africana* parasitizes *E. mauritanica* and *E. gummifera* and occurs sympatrically with *H. triceps,* the parasites are not known to share hosts, an apparent case of host partitioning. Our data suggests that host partitioning for *H. triceps* occurs at germination via host and non-host root recognition. Single host fidelity is not a common trait for plant parasites. In the case of extreme host specialization and assuming the formation of a seed bank, it makes intuitive sense for plant holoparasites to approach germination in a conservative manner, evolving mechanisms to insure successful germination and attachment, and to limit suicidal germination. Additional host partitioning mechanisms cannot be ruled out. Post-germination and attachment failure of the parasite might be attributed to host root anatomy and response that can limit parasite development (Rümer et al. 2007).

A reciprocal transplant experiment evaluating mistletoe germination and establishment on hosts with different provenances demonstrated population level adaptation (Rödl and Ward 2002). Conversely in similar mistletoe experiments, Norton et al. (2002) showed that within population host variability was a more important variable than host provenance. In our study there was no significant effect of *H. triceps* seed or *E. dregeana* root extract provenance, the latter suggesting an absence of parasite-host local adaptation. However, these results should be interpreted with caution because of the low overall germination percentage.

Our observation that the *H. triceps* seed has a spherical undifferentiated embryo agrees with the findings of Dastur (1921) for *H. africana.* Seeds of plants with

undifferentiated embryos are excluded from some current classifications of seed dormancy (i.e. Baskin and Baskin 2004; Nikolaeva 1977). Still, Baskin and Baskin (2004) highlight that seeds of holoparasitic plants with undifferentiated embryos have some component of morphological dormancy, since they have underdeveloped embryos. For holoparasitic *Orobanche* spp., Baskin and Baskin (1998) review several lines of evidence suggesting morphophysiological seed dormancy, including an undifferentiated embryo, afterripening in dry storage conditions, and promotion of germination with giberillic acid. For *H. triceps*, seeds have a component of morphological dormancy, due to its undifferentiated embryo. The degree to which it can be classified as physiologically dormant requires more study because the potential dormancy breaking cue in host root exudates requires identification and nothing is known of its afterripening requirements, if any.

The fruits of *Hydnora* spp. are reportedly consumed by a variety of mammals including jackals, baboons, humans, rhinos, elephants, porcupines, and small mammals in southern Africa (Musselman and Visser 1989). Our direct observations of endozoochory by striped mice and round eared elephant shrews indicate that small mammals may be important seed dispersers and unlike some other *Hydnora* frugivores, may occasionally bring *H. triceps* seeds in close proximity to host roots in their burrows. Additionally, small mammal middens were often observed at the bases of *Euphorbia* shrubs.

For the holoparasites, *Bdallophytum americanum* (García-Franco and Rico-Gray 1997), *Dactylanthus taylorii* (Ecroyd 1996), *Epifagus virginiana* (reviewed by Williams and Zuck 1986), *Pholisma sonorae* (Cothrun 1969) germination studies resulted in unsatisfying results: very low and sporadic germination. In fact, these studies could not

link holoparasite germination to a requirement for host root extracts or exudates. In contrast, clear patterns of host specific germination emerged for *H. triceps*, despite low germination percentages. Low germination percentages for non-agronomically important root holoparasites in the literature and in this study may be an artifact of imperfectly simulated soil microenvironments and unaccounted for physiological germination inhibiting mechanisms (physiological dormancy). Our *H. triceps* germination percentages may have been retarded by inadvertent light exposure when checking for germinated seeds and a lack of an extended afterrippening period.

As Job Kuijt (1969) foretold, the germination ecology of this furtive genus is indeed captivating. Unfortunately, the limiting factor to further experimentation and comparative studies of other *Hydnora* spp. is a shortage of seeds. Mammals covet the fruits in the frugal arid-karoo of Namibia and South Africa. Thus, even when immature fruits are marked and buried for later excavation, inevitably most have been discovered and consumed prior to recovery.

CHAPTER 4

MINERAL NUTRITION AND HETEROTROPHY IN THE WATER CONSERVATIVE HOLOPARASITE *HYDNORA* THUNB. (HYDNORACEAE)

INTRODUCTION

Parasitic plants derive all or part of their mineral and carbon requirements from their host plants. All parasitic plants share a specialized organ known as the haustorium, through which they mediate solute uptake from the host by a variety of mechanisms. Transpiration (mass flow/passive transport), osmotica, and active transport may all play important roles in solute and water uptake (Hibberd and Jeschke 2001; Shen et al. 2006). The relative importance of these modes of transport may depend on haustorial anatomy (especially the host-parasite interface), the rate of parasite transpiration, and the mode of parasitism (from hemiparasitism to holoparasitism). It is generally accepted that most hemiparasites, and particularly the well studied mistletoes, drive solute uptake primarily via greater transpiration rates than their respective hosts (Ehleringer et al. 1985). In contrast, holoparasites without the presence of extensive light gathering surfaces generally have drastically lower rates of transpiration relative to their hosts (Seel et al. 1992) but are still strong sinks for host derived solutes and water.

Thus how do holoparasites, without the benefit of high transpiration, drive water and solute transport from the host? Hibberd and Jeschke (2001) state in their review of solute flux that the precise answer is still unclear, however progress has been made. Several studies have shown selective transport and processing of solute at or near the

haustoria in hemiparasites using radiotracers (Govier et al. 1967) or analysis of xylem sap (Pate et al. 1994; Tennakoon and Pate 1996; Tennakoon et al. 1997). Notably, in the hemiparasite *Rhinanthus minor*, haustorial anatomy dictated by host resistance strongly influenced transport of solute (Cameron and Seel 2007). Perhaps the simplest model of transport is an osmotic gradient from host to parasite. However, this is not easily measured within vascular elements of a parasite-host association. Integrated models of water and solute fluxes combined with sap analysis and other direct measurements have shown a trend of strong dependence on phloem borne nutrients in the holoparasites, *Cuscuta* (Jeschke et al. 1994) and *Orobanche* (Hibberd et al. 1999).

Another starting point for understanding the role of osmotica in solute uptake is general mineral relationships between the parasite and host. Unfortunately mineral profile comparisons have often raised more questions about transport than they have answered. This may be due to the coarse nature of these comparisons, differences in sampling strategies, omissions of important elements, and the fact that only a small portion of parasitic plants have been analyzed in this manner (Pate 1995). In his review Pate (1995) indicates that plant parasite mineral nutrition data are biased towards aerial parasites and herbaceous parasitic plants of agronomic importance (*Cuscuta, Orobanche,* and *Striga*), a situation that has changed little. The mineral relationships of mistletoes and their hosts are the most extensively studied and were reviewed in depth by Lamont (1983) these aerial parasites have elevated tissues concentrations in of most elements evaluated relative to hosts. Intuitively, modeling solute uptake in water conservative holoparasites should be conceptually simpler than systems where mass flow due to high rates of transpiration may be confounding. A gap remains in the plant parasite literature for

mineral relationships of holoparasitic root parasites (but see Brotherson et al. 2005; Singh et al. 1971).

Naturally abundant stable isotope methods are increasingly useful tools that have made important contributions to our understanding of parasite heterotrophy, nutrition, and water relations (Bannister and Strong 2001; Cernusak et al. 2004; Dawson et al. 2002; Ehleringer et al. 1985; Farquhar et al. 1989b; Pate 2001; Press et al. 1987; Schultz et al. 1991; Tennakoon and Pate 1996). An implicit assumption in the estimation of heterotrophy in parasitic plants is that without parasite autotrophic C contributions, identical or similar $\delta^{13}C$ values should be observed in the host and parasite (Marshall and Ehleringer 1990; Press et al. 1987). However, estimation of plant parasite heterotrophy using $\delta^{13}C$ values can be unreliable where carbon isotope discrimination during photosynthesis is similar between parasite and host (Bannister and Strong 2001). Moreover, selective uptake may decouple holoparasite and host $\delta^{13}C$ signatures. Fractionation of plant $\delta^{13}C$ occurs at multiple scales from molecules to tissue types and whole plants (reviewed in Badeck et al. 2005; Hobbie and Werner 2004). For example, carbohydrates can be enriched in $\delta^{13}C$ relative to amino acids (e.g. Winkler et al. 1978). Thus the assumed tight linkage of holoparasite and host $\delta^{13}C$ values requires further study (but see Cernusak et al. 2004).

This present study was undertaken to investigate the parasite-host mineral and stable isotope relationships ($\delta^{13}C$ and $\delta^{15}N$) in the genus *Hydnora* (Hydnoraceae), a group of apparently water conservative root holoparasites. Five *Hydnora* spp. are currently recognized in Madagascar and Africa: *Hydnora abyssinica, H. africana, H. esculenta, H. triceps, H. sinandevu* (Beentje and Luke 2001; Maass and Musselman

2004; Musselman and Visser 1989). Two taxa, *H. africana* and *H. triceps* exclusively parasitize *Euphorbia* spp. having CAM metabolism, while *H. abyssinica, H. esculenta,* and *H. sinandevu* have a variety of Fabaceae hosts (Beentje and Luke 2001; Musselman and Visser 1989). Among these holoparasites, *H. triceps* is entirely subterranean with underground (hypogeous) flowering; other *Hydnora* taxa emerge briefly only to flower (Bolin et al. 2009). The vegetative body of *H. triceps* is a rhizome entirely covered with a suberized periderm with a chimeric growth tip that shows both root and shoot characters. The highly reduced rhizome bears no stomata, leaves, or leaf scales (Tennakoon et al. 2007). No chlorophyll was detected in tissues of *H. africana* using spectrophotometric methods (De la Harpe et al. 1981). Furthermore, *H. triceps* showed aggressive haustoria with direct parasite xylem-host xylem contacts and parasite parenchyma-host phloem contacts (Tennakoon et al. 2007). Their subterranean habit, lack of transpirative surfaces, stomata, and apparent holoparasitism make *Hydnora* a good model organism for investigating parasite-host relationships.

In this study we provide the first account of mineral relationships of holoparasites on CAM hosts. Moreover, this paper estimates the rate of transdermal water loss in *Hydnora* and provides the first comprehensive $\delta^{13}C$ and $\delta^{15}N$ analysis of *Hydnora* parasitizing CAM and C_3 hosts in southern Africa and Madagascar (but see De la Harpe et al. 1981; Ziegler 1996). Stable isotope values of *Hydnora* spp. are compared to mistletoes collected on a variety of hosts at same study locations to provide a novel example of complete and partial heterotrophy.

MATERIALS AND METHODS

Study Sites and Sampling

Tissue samples for mineral and stable isotope analyses were collected from paired host-parasite associations in Madagascar, Namibia and northwestern South Africa (table 4.1). Collections sites were clustered at seven main sites in Madagascar and southern Africa: Berenty Preserve (BP), Madagascar, Parc Andohahela (PA), Madagascar, Etosha National Park, Okakuejo, Nambia (OK), Brandberg, Nambia (BR), Farm Kanas, Seeheim, Nambia (K), Gondwana Canon Park, Nambia (GC); Farm Namuskluft, Rosh Pinah, Namibia (NA), Farm Gemsbokvlei, Northern Cape Province, South Africa (G), and Farm Kanikwa, Northern Cape Province, South Africa (KF). Sites were chosen to represent the diversity of *Hydnora*-host associations. In southern Africa, mean annual precipitation was highest at the Okakuejo, Namibia site (400-450 mm). Other southern African study areas were arid with less than 150 mm of precipitation annually (Mendelsohn et al. 2002). At the Madagascar study sites, mean annual precipitation was higher, with 546 mm recorded at the Berenty Preserve (Jolly et al. 2002) and 700-900 mm estimated in the Malio area of Parc Andohahela, a transitional area between dry spiny-forest and rainforest. In total, thirteen *Hydnora*-host and twelve mistletoe-host associations were sampled. Site locations, taxa sampled and photosynthetic metabolism of each host are given in table 4.1. Field collections were made from September to December 2005 for southern African sites and December 2007 in Madagascar. The subterranean rhizomes of *Hydnora* spp. were located by excavating around flowers or remnants of perianth parts from previous seasons. For the *Hydnora*-host associations, samples were collected from the parasite rhizome and from host plant, root and shoot.

Tissue was sampled from new growth of the *Hydnora* vegetative body. Root tissue of the host was sampled from a 4-5 cm section distal to the haustorium attachment point and shoot tissue was collected from either newly emerged stems of the year or fully expanded leaves, for stem succulent *Euphorbia* (CAM) and Fabaceae (C_3) hosts respectively. The *Euphorbia* hosts sampled were all stem succulent plants with small deciduous leaves, usually not present when sampling. For mistletoe samples (*Plicosepalus undulatus, Tapinanthus oleifolius,* and *Viscum capense*) fully expanded leaves of the host and parasite were collected. In this paper, we treat *H. africana* as a single species according to the most recent treatment of the genus for Namibia (Schreiber 1968). However, our field and laboratory studies indicate that *H. africana* may comprise several closely related taxa (Bolin, unpubl. res.).

Transdermal water loss in Hydnora

To confirm water conservatism, we estimated transdermal water loss for *H. africana* and *H. triceps* rhizomes *in situ*. We compared portions of the rhizome with the least developed periderm, less than one mm thick (within 10 cm of the apical meristem), to rhizome portions that had visibly thicker periderm, greater than one mm thick (10-20 cm from the apical meristem). We used 18 sections of rhizome (length 45.4 – 116.3 mm; diameter 6.62 – 16.63 mm) for each species and level (thin periderm vs. thick periderm); in total 72 sections were used. The surface area (cm^2) of the rhizome sections was estimated by modeling the sections as cylinders (excluding the cut surfaces). This model is a simplification and an underestimate of the rhizome surface area. Since we did not

Table 4.1

LOCATIONS OF SAMPLING AREAS

Site	Location	Parasite	Host	Parasite:Host Metabolism
Berenty Preserve MAD (**BP**)	S24° 59.861' E46° 17.762'	*Hydnora esculenta* Jumelle & Perrier	*Pithecellobium dulce* (Roxb.) Benth.	Holo:C_3
Parc National Andohahela, MAD (**PA**)	S24° 56.186' E46° 38.546'	*Hydnora esculenta*	*Albizia tulearensis* R.Vig	Holo-C_3
Okakuejo, Etosha National Park, NAM (**OK**)	S19°10.855' E15°55.021'	*Hydnora abyssinica* A.Braun	*Acacia luderitzii* Engl.	Holo-C_3
Brandberg, NAM (**BR**)	S21°09.808' E14°50.712'	*Hydnora africana* Thunb.	*Euphorbia damarana* Leach.	Holo-CAM
Kanas Farm, NAM (**K**)	S26°43.666' E17°32.451'	*Hydnora africana*	*Euphorbia gregaria* Marl	Holo-CAM
		Plicosepalus undulatus E. Mey.	*Acacia erioloba* E.Mey ex. Harv.	Hemi-C_3
		Plicosepalus undulatus	*Acacia hebeclada* DC.	Hemi-C_3
		Plicosepalus undulatus	*Acacia karroo* Hayne	Hemi-C_3
Gondwana Cañon Park, NAM (**GC**)	S27°32.731' E17°52.820'	*Hydnora africana*	*Euphorbia gregaria*	Holo-CAM
		Tapinanthus oleifolius (Wendel.) Danser	*Acacia melifera* (Vahl) Benth.	Hemi-C_3
		Tapinanthus oleifolius	*Euphorbia gregaria*	Hemi:CAM
		Tapinanthus oleifolius	*Rhigozum trichotomum* Burch.	Hemi-C_3
		Tapinanthus oleifolius	*Parkinsonia aculeata* L.	Hemi-C_3
Namuskluft Farm, NAM (**NA**)	S27°56.383' E16°48.196'	*Hydnora africana*	*Euphorbia chersina* N.E. Br.	Holo-CAM
		Hydnora africana	*Euphorbia gummifera* Boiss.	Holo-CAM
		Hydnora africana	*Euphorbia mauritanica*	Holo-CAM
		Hydnora triceps Drege	*Euphorbia triceps* Drege et Meyer	Holo-CAM
		Tapinanthus oleifolius	*Aloe dichotoma* Masson	Hemi-CAM
		Tapinanthus oleifolius	*Rhus populifolia* Sond.	Hemi-C_3
		Viscum capense L.f.	*Euphorbia triceps*	Hemi-CAM
		Viscum capense	*Euphorbia gummifera*	Hemi-CAM
		Viscum capense	*Euphorbia gummifera*	Hemi-CAM
Gemsbokvlei Farm, SA (**G**)	S29°18.304' E17°04.142'	*Hydnora africana*	*Euphorbia mauritanica*	Holo-CAM
		Hydnora triceps	*Euphorbia triceps*	Holo-CAM
Kanikwa Farm, SA (**KF**)	S29°18.716' E17°01.716'	*Hydnora africana*	*Euphorbia mauritanica*	Holo-CAM

Note.- Madagascar (MAD), Namibia (NAM), Republic of South Africa (SA) listed north to south, parasite-host species pairs sampled at each site, photosynthetic metabolism of host, and mode of parasitism (Holo = holoparasite; Hemi = hemiparasite).

account for surface area of lateral appendages and the round to pentagonal shape of the rhizome sections, the model provides a conservative overestimate of transdermal water loss rate. Very thick portions of the rhizome (> 20 mm diameter) were clearly angular rather than terete and therefore excluded from this experiment. Transdermal water loss was estimated by evaluating water lost over 12 hours *in situ*. Before dawn, fresh *Hydnora* rhizome sections were carefully excavated and weighed. The cut ends of each rhizome section were sealed with plastic wrap and bound tightly with a rubber band. The rhizome sections were buried in sandy soil, 10 cm below the surface. After 12 hours the sections were excavated and reweighed. A data logger HOBO U12 (Onset Computer Corp., Pocasset, MA, USA) equipped with soil-air thermocouples was used to monitor soil and ambient air temperatures during the experiment.

Stable Isotope and Mineral Nutrition

In the field, samples were stored in paper bags, and then they were oven dried until a constant mass was attained at 75 °C in the laboratory. Dried samples were first manually cut into small pieces then reduced to fine powder with a ball grinder.

Mineral analyses of the parasite vegetative body and corresponding host root were investigated for three associations: *H. africana- E. gregaria, H. africana-E. mauritanica*, and *H. triceps-E. dregeana*. Microwave nitric acid tissue digestions (Huang et al. 2004) were conducted for metals with a MDS-2100 (CEM Corporation, Matthews, NC, USA). The concentrations of Ca, K, Fe, Mg, Mn, Na, P, and Zn were assayed using a Model 5300 (PerkinElmer Life and Analytical Sciences, Inc. Wellesley, MA, USA) optical emission spectrometer (Jones 1975). Total C, N, and S were determined with a NA 1500

Elemental Analyzer (Carlo Erba, Milan, Italy). Soluble Cl was determined using an ion chromatography system Model ICS-1000 (Dionex Corporation, Sunnyvale, CA, USA). Fe, Mn, and Zn were not analyzed for *H. africana-E. mauritanica* due to insufficient sample material.

Replicate tissue samples were combusted in an ANCA-SL elemental analyzer and the resulting gases were analyzed for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios on an Isotope Ratio Mass Spectrometer (PDZ Europa Scientific 20/20). $\delta^{13}C$ values are presented as the relative difference between isotope ratios of the sample and the standard Pee Dee Belemnite. $\delta^{15}N$ values are presented relative to the standard, atmospheric air. Precision of measurement based on triplicate assays of a single sample of dry matter was ± 0.11‰ calibrated against a set of variable weight standard reference asparagine.

Hydnora-host mineral profiles were statistically assessed using three true replicates per association, independent values from three *Hydnora*-host pairs. Paired t-tests were used to test for significant differences in the host and parasite mineral concentrations. For $\delta^{13}C$ and $\delta^{15}N$ values, standard errors are presented for all analyses with more than one true replicate per association. One sample t-tests (test value = 0) were conducted on the differences of parasite shoot (rhizome) and host tissues. All statistical analyses were performed with SPSS version 16.0 (SPSS, Chicago, IL, USA).

RESULTS

Transdermal water loss in Hydnora

Estimates of transdermal water loss from rhizome lengths with the least developed (near the growth tip of the rhizome) and more developed periderm showed low

transdermal water loss under field conditions over 12 hours. For *H. africana,* transdermal water loss was significantly lower ($P<0.001$, $n = 19$) for rhizome lengths of developed periderm (0.14±.02 mg cm^{-2} hr^{-1}) compared to least developed periderm lengths (0.22±.02 mg cm^{-2} hr^{-1}). For *H. triceps,* transdermal water loss was significantly lower ($P<.01$, $n = 19$) for rhizome lengths of developed periderm (0.19±.02 mg cm^{-2} hr^{-1}) compared to least developed periderm sections (0.38±.04 mg cm^{-2} hr^{-1}). Transdermal water loss was greater for *H. triceps* relative to *H. africana.* Temperatures logged during the 12 hour study period ranged from 23.3-46.9 °C and 23.7-38.3 °C for air and soil temperatures, respectively.

Mineral Nutrition

The concentrations of elements analyzed for *Hydnora* (rhizome tissue) and *Euphorbia* hosts (root tissue) are presented in table 4.2. Comparisons of the mineral profiles and the parasite to host ratios (P:H) show that relative concentrations of P and K were all greater in the parasite relative to the host. Levels of P and K in the parasite were significantly enriched in the *H. triceps-E. dregeana* association ($P<0.05$, $n = 3$), and parasite P was significantly enriched in the *H. africana-E. mauritanica* association ($P<0.01$, $n = 3$). Other P and K values in *Hydnora*-host associations were elevated in the parasites but not significantly. Other mineral nutrients Ca, Cl, Fe, Mg, Mn, N, Na, S, and Zn, were significantly lower in the parasite relative to the host in most cases, with the exceptions of nonsignificant differences for Mn and Zn in the *H. triceps-E. dregeana* association; Ca, Cl, Mn, and Zn in the *H. africana-E. gregaria* association; and N and S in the *H. africana-E. mauritanica* association. Total C values were significantly elevated

in the parasite relative to the host only in the *H. africana-E. mauritanica* association; other associations were not significantly different (table 4.2).

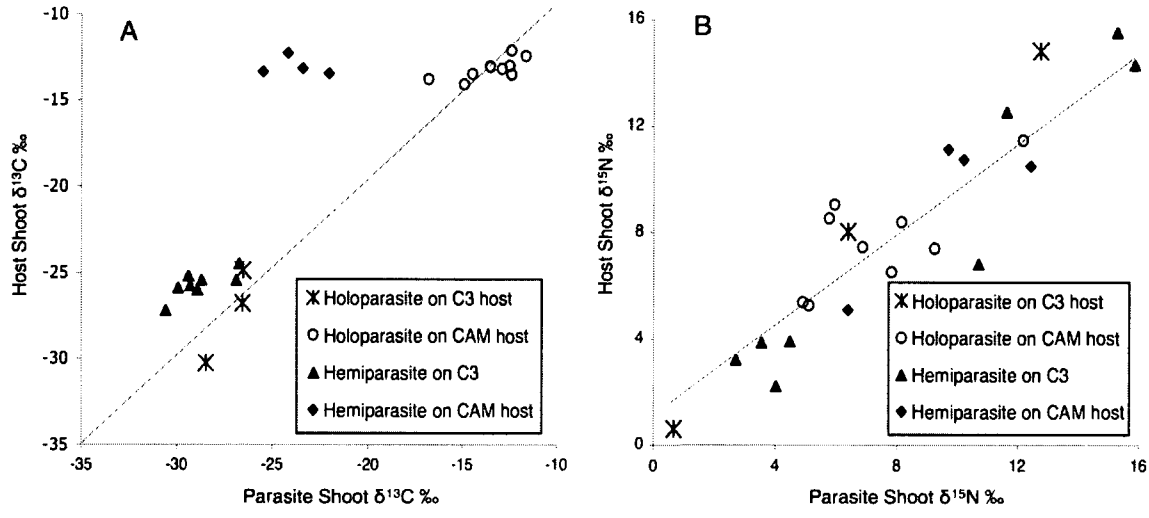

Fig. 4.1 *A*, Parasite-host $\delta^{13}C$ and $\delta^{15}N$ relationships using mean values for each parasite-host association. $\delta^{13}C$ relationships of host and parasite shoot tissues. Points above the 1:1 line indicate parasites with more negative $\delta^{13}C$ values relative to their hosts. *B*, $\delta^{15}N$ relationships of host and parasite shoot tissues mean values. Correlation of mean data per association is positive and statistically significant. Regression equation, $y=0.8754x + 0.7413$, $r^2=0.86$, $P<0.0001$.

Table 4.2

MINERAL COMPOSITION OF *EUPHORBIA* SPP. ROOT (HOST) AND *HYDNORA* SPP. RHIZOME (PLANT PARASITE) TISSUES

	Macronutrients								Micronutrients			
	Total C (%)	Total N (%)	Total P (mg/g)	Total K (mg/g)	Total Ca (mg/g)	Total Mg (mg/g)	Total S (mg/g)	Soluble Cl (mg/g)	Total Fe (mg/g)	Total Mn (mg/g)	Total Na (mg/g)	Total Zn (mg/g)
Euphorbia dregeana	46.91±0.77	0.78±0.12 *	0.98±0.19 *	6.40±0.99 *	8.63±1.04 *	3.01±0.39 *	2.32±0.36 *	12.01±1.13 *	0.42±0.01 *	0.041±0.001	11.13±0.74 *	0.010±0.001
Hydnora triceps	47.76±0.93	0.41±0.02 *	1.86±0.20 *	15.80±2.68 *	2.75±0.92 *	1.75±0.12 *	1.05±0.09 *	5.91±1.10 *	0.25±0.02 *	0.018±0.009	6.73±0.73 *	0.013±0.001
P:H	1.02	0.53	1.89	2.47	0.32	0.58	0.45	0.49	0.6	0.44	0.6	1.3
Euphorbia gregaria	46.98±0.66	0.63±0.03 **	0.72±0.28	11.63±2.24	9.56±3.56	3.37±0.25 **	1.74±0.18 **	7.35±1.75	0.67±0.03 *	0.024±.001	7.22±0.37 **	0.012±0.003*
Hydnora africana	51.75±3.09	0.26±0.01 **	0.87±0.03	14.82±1.57	1.28±0.07	0.34±0.03 **	0.50±0.03 **	3.24±0.17	0.33±0.04 *	0.015±.004	2.26±0.71 **	0.007±0.001*
P:H	1.1	0.41	1.2	1.27	0.13	0.1	0.28	0.44	0.49	0.63	0.31	0.58
Euphorbia mauritanica	46.16±0.41 **	0.36±0.04	1.21±0.10 **	25.23±2.43	3.43±0.36 **	1.43±0.15 **	2.02±0.22	8.25±1.53 *	N.D.	N.D.	6.60±0.36 **	N.D.
Hydnora africana	51.46±1.04 **	0.36±0.003	2.41±0.14 **	27.46±1.27	1.13±0.12 **	0.57±0.01 **	1.71±0.10	1.98±0.24 *	N.D.	N.D.	2.66±0.33 **	N.D.
P:H	1.11	0.99	1.98	1.09	0.33	0.4	0.85	0.24			0.4	

Note.-Each value is a mean (±SE) of three true replicates. A parasite to host total concentration ratio is also presented for each element (P:H). Paired t-tests were conducted to compare mean values of host and parasite. Significant differences are indicated with asterisks (* $P<0.05$; ** $P<0.01$).

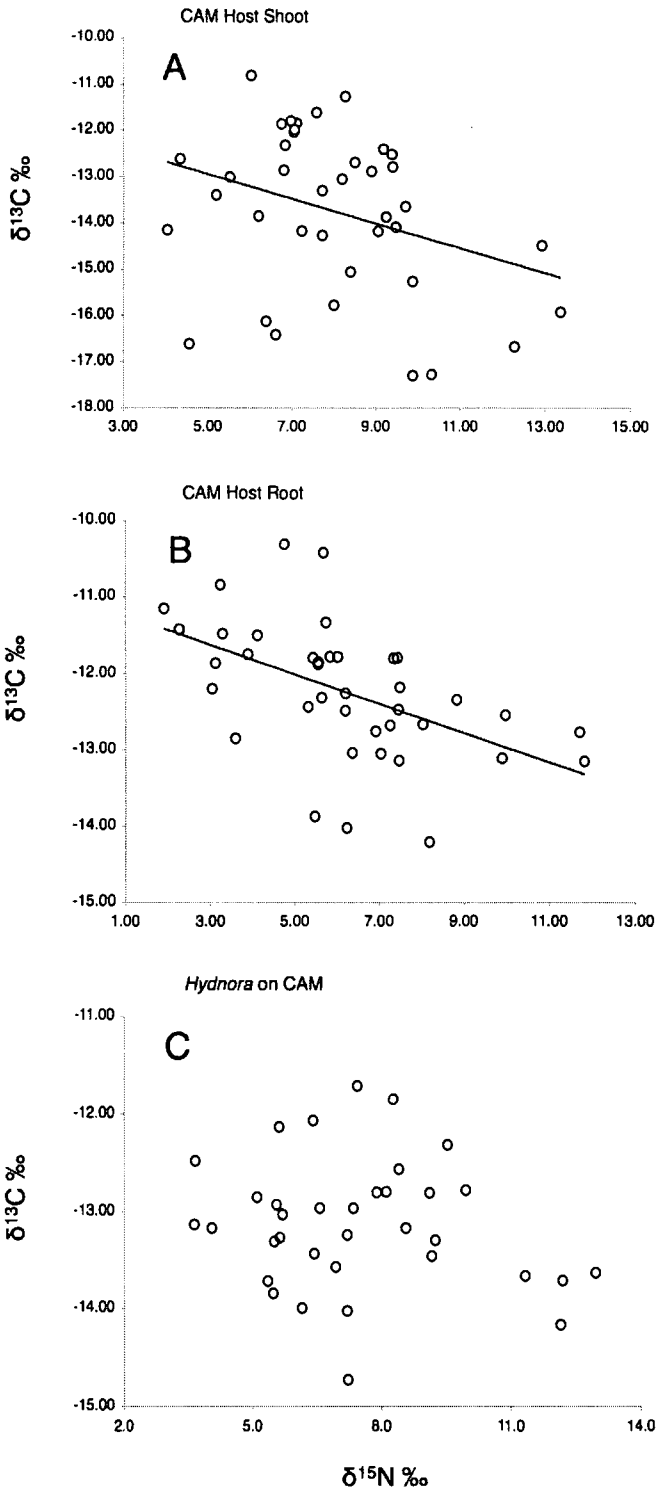

Fig. 4.2 *A & B*, Tissue $\delta^{13}C$ and $\delta^{15}N$ relationships for CAM host, shoot and root. Both correlations are statistically significant. Regression equations, shoot y =-0.2671*x* –

Fig. 4.2 (continued) 11.619, r^2= 0.11, P=0.043, root y=-0.1922x – 11.049, r^2=0.26, P=0.001, respectively. *C*, $\delta^{13}C$ and $\delta^{15}N$ relationships for *Hydnora* rhizome on CAM hosts. No significant correlation, y =-0.0493x – 12.764, P=0.316.

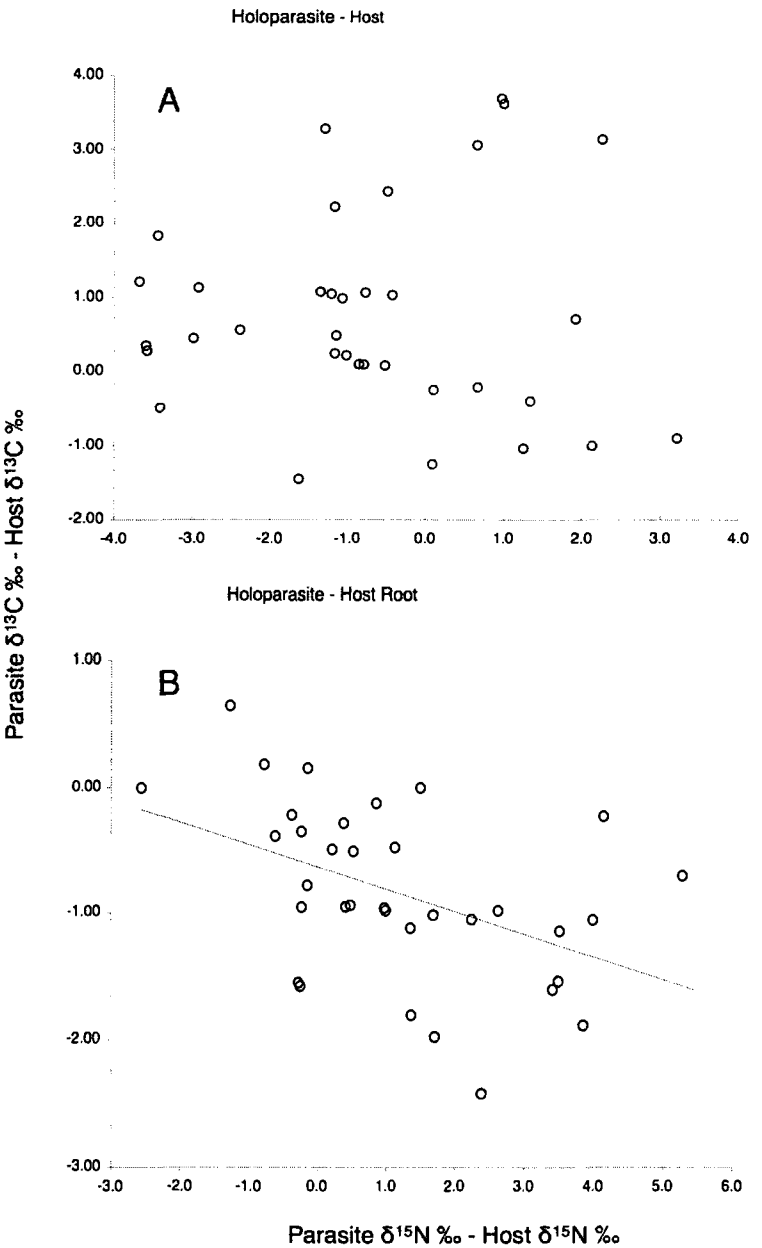

Fig. 4.3 Correlations of differences between *Hydnora* and CAM host $\delta^{13}C$ and $\delta^{15}N$. Each host tissue types were plotted separately, holoparasite shoot (rhizome) minus CAM host

Fig. 4.3 (continued) shoot values and holoparasite shoot minus CAM host root values. *A*, The correlation of $\delta^{13}C$ and $\delta^{15}N$ for holoparasite shoot-host shoot differences was not significant, P=0.994. *B*, The correlation of $\delta^{13}C$ and $\delta^{15}N$ for parasite shoot-host root differences was significant, regression equation $y=-0.184x - 0.6133$, r^2=0.22, P=0.005. Outlier holoparasite:host data from BR (*H. africana*:*E. damarana*) were excluded from these correlations (see results section for definition of outliers).

Holoparasite and Hemiparasite $\delta^{13}C$

The $\delta^{13}C$ stable isotopic signature of *Hydnora* holoparasites on CAM and C_3 hosts closely mirrored the values of their hosts (fig. 4.1A). The parasite-host differences were small but significant. The mean difference and direction of the relationship between parasite and host $\delta^{13}C$ values depended on the host tissue type. When compared to host stem tissues, the holoparasite $\delta^{13}C$ was significantly enriched by 0.55‰ ± 0.23 (P = 0.02, n = 46), summary data per association in table 4.3. In contrast, when compared to host root tissues, holoparasite $\delta^{13}C$ was significantly more negative by -0.97‰ ± 0.11 (P < 0.001, n = 46). Holoparasite $\delta^{13}C$ values when compared to the estimated whole host $\delta^{13}C$ value, approximated by averaging host shoot and root values, were not significantly different (-0.21 ± 0.15‰, P = 0.17, n = 46).

The $\delta^{13}C$ values of all hemiparasite shoot tissues were significantly more negative than host shoot tissues (-5.43‰ ± 0.70, P<0.001, n = 31), summary data per association in table 4.3. The $\delta^{13}C$ values for hemiparasites on CAM hosts only (fig. 4.1A) were -11.07‰ ± 0.49 (P<0.001, n = 9), relative to host values and were -3.12‰ ± 0.29 (P<0.001, n = 21) for hemiparasites on C_3 hosts, relative to host values.

Table 4.3

STABLE ISOTOPE RATIOS ($\delta^{13}C$ & $\delta^{15}N$) FOR PLANT PARASITES AND HOSTS

					$\delta^{13}C$ (‰)					$\delta^{15}N$ (‰)				
Hydnora (holoparasite)	Host	Site	Date	*n*	Parasite Shoot	Host Root	Host Shoot	Dif. S-S	Dif. S-R	Parasite Shoot	Host Root	Host Shoot	Dif. S-S	Dif. S-R
H. abyssinic a	*A. luderitzii*	OK	23/11/05	2	-26.51±0.79	-24.79±0.02	-24.90±0.84	-1.61	-1.72	14.87±1.19	10.64±0.79	12.75±0.54	2.12	4.23
H. africana	***E. chersina***	NA	14/10/05	4	-14.08±0.24	-13.22±0.37	-14.90±0.90	0.82	-0.86	6.87±0.25	7.37±0.38	7.46±0.44	-0.59	-0.50
H. africana	***E. damarana***	BR	27/12/05	4	-13.51±0.25	-11.72±0.13	-12.40±0.65	-1.11	-1.79	7.82±0.85	5.57±0.08	6.52±0.24	1.30	2.25
H. africana	***E. gregaria***	GC	7/10/05	4	-13.02±0.20	-12.05±0.14	-13.54±0.31	0.52	-0.97	8.17±0.41	4.72±0.72	8.40±0.54	-0.23	3.45
H. africana	***E. gregaria***	K	20/10/05	4	-13.19±0.06	-11.23±0.15	-12.91±0.48	-0.28	-1.96	5.11±0.5	2.89±0.5	5.26±0.7	-0.15	2.22
H. africana	***E. gummifera***	NA	15/12/05	4	-12.44±0.23	-11.08±0.41	-11.64±0.13	-0.80	-1.36	9.23±0.36	6.31±0.65	7.40±0.34	1.83	2.92
H. africana	***E. mauritanica***	G	27/9/05	4	-13.79±0.13	-12.89±0.14	-16.80±0.32	3.01	-0.90	12.16±0.38	10.85±0.61	11.46±0.95	0.70	1.31
H. africana	***E. mauritanica***	KF	31/1/05	3	-12.99±0.22	-15.39±0.99	-12.50±0.19	-0.49	2.40	4.9±0.45	3.99±0.68	5.38±0.53	-0.48	0.91
H. africana	***E. mauritanica***	NA	15/12/05	4	-12.10±0.16	-11.88±0.21	-12.40±0.15	-0.22	0.30	5.78±0.8	5.34±0.68	8.53±0.53	-2.75	0.44
H. esculenta	*Alb. tulearensis*	PA	10/12/07	2	-26.54±0.48	-24.86±0.22	-26.79±0.18	0.25	-1.68	8.02±0.33	7.02±2.33	6.39±1.5	1.63	1.00
H. esculenta	*P. dulce*	BP	7/12/07	3	-28.46±0.96	-28.04±0.75	-30.27±0.45	1.81	-0.42	0.61±0.96	-2.51±0.33	0.67±1.53	3.12	-0.06
H. triceps	***E. dregeana***	G	27/1/05	3	-13.49±0.19	-13.43±0.22	-14.46±0.31	0.97	-0.06	5.95±0.32	6.42±0.36	9.03±0.47	-3.08	-0.47
H. triceps	***E. dregeana***	NA	13/10/05	4	-13.06±0.11	-12.47±0.12	-13.53±0.36	0.47	-0.59	8.17±0.4	4.72±0.72	8.40±0.54	-0.23	3.45
Aerial parasites														
P. undulatus	*A. erioloba*	K	8/11/05	3	-26.73±1.05		-24.48±0.66	-2.25		15.29±1.01		15.53±0.84	-0.24	
P. undulatus	*A. habeclada*	K	8/11/05	3	-29.39±0.74		-25.20±0.79	-4.19		15.86±0.62		14.31±0.94	1.55	
P. undulatus	*A. karroo*	K	8/11/05	3	-30.56±0.14		-27.18±0.29	-3.38		4.02±0.87		2.24±1.07	1.78	
T. oleofolius	*A. melifera*	GC	7/10/05	3	-28.92±0.08		-26.00±0.37	-2.92		2.7±1.25		3.22±0.68	-0.52	
T. oleofolius	***Al. dichotoma***	NA	13/10/05	1	-22.04		-13.45	-8.59		6.39		5.09	1.30	
T. oleofolius	***E. gregaria***	GC	9/10/05	2	-25.51±0.72		-13.32±0.31	-12.19		12.41±1.09		10.5±0.24	1.91	
T. oleofolius	*R. trichotomum*	GC	13/10/05	3	-29.91±0.62		-25.86±0.97	-4.05		10.7±0.62		6.82±0.47	3.88	
T. oleofolius	*Rh. populifolia*	NA	16/10/05	1	-28.70		-26.77	-3.27		3.56		3.87	-0.31	
T. oleofolius	*T. usneiodes*	GC	13/10/05	3	-29.29±1.32		-25.76±0.53	-3.53		11.62±1.06		12.55±1.58	-0.93	
V. capense	***E. dregeana***	NA	15/10/05	3	-23.42±0.38		-13.14±0.31	-10.28		10.2±1.15		10.74±0.18	-0.54	
V. capense	***E. gummifera***	NA	10/15/05	3	-24.19±0.55		-12.26±0.50	-11.93		9.68±0.84		11.12±0.63	-1.44	
V. capense	*Rh. populifolia*	NA	10/16/05	3	-26.89±0.43		-25.42±0.23	-1.47		4.49±0.27		3.92±0.03	0.57	

Note.- Differences given for parasite shoot and host shoot (S-S) and root (S-R), relationships respectively. Complete species names and site locations given in Table 1. CAM hosts are bolded.

Holoparasite and Hemiparasite $\delta^{15}N$

There was a significant positive relationship between parasite and host shoot mean $\delta^{15}N$ tissue values across all functional groups and host types, regression equation: $y = 0.8754x + 0.7413$, $r^2 = 0.86$ ($P<0.001$) (fig. 4.1B), summary data per association in table 4.3. The mean difference and direction of the relationship between parasite and host $\delta^{15}N$ values depended on the host tissue type. Holoparasite $\delta^{15}N$ values were not significantly different relative to host shoot tissues, only enriched 0.59‰ ± 0.56 ($P=0.296$, $n = 46$). However, holoparasite tissues relative to host root tissues $\delta^{15}N$ values were significantly enriched by 2.40‰ ± 0.53 ($P<0.001$, $n = 46$). Hemiparasite $\delta^{15}N$ values for shoot tissues were not significantly enriched, and differed only by 0.56‰ ± 0.34 ($P = 0.117$, $n = 31$).

$\delta^{13}C$ and $\delta^{15}N$ holoparasite-host relationships

For the holoparasite CAM host tissues there was a significant negative relationship of $\delta^{13}C$ and $\delta^{15}N$ in both shoot ($y = -0.2671x - 11.619$, $r^2 = 0.11$, $P = 0.043$) and root tissues ($y = -0.1922x - 11.049$, $r^2 = 0.26$, $P = 0.001$). In CAM host tissues, more negative $\delta^{13}C$ values were related with higher $\delta^{15}N$ values (fig. 4.2A and B). C_3 host tissues were excluded from this analysis because their $\delta^{13}C$ values are approximately double the values for CAM hosts and thus obscure the overall CAM host $\delta^{13}C$ and $\delta^{15}N$ relationship. Moreover, holoparasites using C_3 hosts were not plotted separately due to low replication in this group ($n = 7$) (see table 4.3). $\delta^{13}C$ and $\delta^{15}N$ values were not correlated in holoparasite rhizome tissues ($P = 0.316$) (fig. 4.2C).

When the differences between holoparasite and host shoot values of $\delta^{13}C$ and $\delta^{15}N$ were plotted, there was no significant relationship ($P = 0.994$) (fig. 4.3A). However a significant relationship was apparent between differences of the holoparasite and host root values of $\delta^{13}C$ and $\delta^{15}N$ ($y = -0.184x - 0.6133$, $r^2 = 0.22$, $P = 0.005$) (fig. 4.3B). Outlier data (considered $\delta^{15}N$ values > 2 s.d. from the mean) from the BR population (*H. africana:E. damarana*) were excluded from these correlations. With BR values included, the correlations (not shown) of the $\delta^{13}C$ and $\delta^{15}N$ differences between holoparasite and host values were similar and significant for parasite shoot-host root ($y = -0.1192x - 0.6466$, $r^2 = 0.11$, $P = 0.043$) and parasite shoot-host shoot relationships ($y=-0.1047x - 0.6884$, $r^2=0.29$, $P<0.0001$), but these outlier values appeared to leverage the slope unduly.

DISCUSSION:

Transdermal water loss

Estimated transdermal water loss was low in *H. africana* and *H. triceps,* and confirmed the extremely water conservative nature of these plant parasites. As expected intuitively rhizome sections with thicker periderm (0.19±.02 mg cm^{-2} $hr^{-1}$and 0.14±.02 mg cm^{-2} hr^{-1}, *H. africana* and *H. triceps,* respectively) lost water at approximately half the rate as rhizome sections with thinner periderm (0.38±.04 mg cm^{-2} hr^{-1} and 0.22±.02 mg cm^{-2} hr^{-1}, *H. africana* and *H. triceps,* respectively). These low rates of water loss are not surprising in light of the absence of stomata on *Hydnora* rhizome surfaces (Tennakoon et al. 2007). The highest estimated rate of *Hydnora* transdermal water loss was more than nine times lower than the transcuticular leaf water loss from the water

conservative halophyte *Suaeda maritima* (Chenopodiaceae) (Hajibagheri et al. 1983) and comparable to the daily transpiration losses of CAM xerophytes *Seyrigia humbertii* (Cucurbitaceae) (0.15 mg cm^{-2} hr^{-1}) and *Xerosicyos danguyi* (Cucurbitaceae) (0.20 mg cm^{-2} hr^{-1}) (de Luca et al. 1977). Estimated transdermal water loss from *Hydnora* was orders of magnitude lower than transpirational rates estimated for mistletoes in central Australia (approx. 324 – 2460 mg cm^{-2} hr^{-1}) (Ehleringer et al. 1985).

The *Hydnora* spp. included in this study were from arid or semi-arid areas, with the exception of the *H. esculenta,* sampled in an unusual transitional region between dry spiny desert and rainforest. Host plants in these harsh environments have well understood adaptations to xeric environments (i.e. CAM photosynthesis, stem succulence, sunken stomata). Correspondingly, in these environments successful perennial plant parasites cannot freely transpire their host's water; the *modus operandi* of numerous hemiparasites to maintain a favorable water potential gradient (reviewed in Ehleringer and Marshall 1995). Notably, holoparasites generally have an absence or paucity of stomata (i.e. Kuijt and Dong 1990; Tennakoon et al. 2007). Still, these transdermal water loss estimates for *Hydnora* may underscore an extremely conservative water use strategy probably required in these arid environments.

Mineral Nutrition

Mineral nutrition analyses of mistletoes have revealed higher parasite tissue concentrations of numerous elements (including Ca, Fe, K, Mg, Mn, N, Na, and P) relative to the host (Ehleringer and Schultz 1985; Lamont 1983). This is attributed largely to passive accumulation of minerals in xylem water driven by high transpiration rates.

Conversely, in our water conservative *Hydnora* model we observed increased concentrations of parasite minerals in only P and K; other minerals analyzed (Ca, Cl, Fe, Mg, Mn, N, Na, S, and Zn) were at significantly lower levels in parasite relative to the host or were not significantly different (table 4.2). Our holoparasite mineral nutrition data largely conformed with nutritional profiles reported for two *Orobanche*-host associations that also reported elevated parasite concentrations of P and K (Brotherson et al. 2005; Singh et al. 1971). For an *Orobanche fasciculata – Artemisia pygmaea* association, Na was also elevated in the parasite (Brotherson et al. 2005). Na, like K is a potentially important element in maintenance of favorable osmotica.

The profound differences between mistletoe and root holoparasite mineral accumulation can be in part attributed to the lack of a strong transpiration stream in the latter and to differences in haustorial anatomy. Of course, holoparasitic plants have very different nutritional requirements without the need to maintain photosynthetic systems. Thus, holoparasites might be expected to have lower requirements for integral components of chlorophylls and chloroplasts such as Mg and Mn. It is tempting to interpret the differential concentration of P and K in this system as evidence for the maintenance of an osmotic gradient from the host to parasite; however this conclusion cannot be drawn, due to unaccounted for portions in the mineral budget such as annual losses of parasite flowers and fruits. Moreover, the measurement of the host root tissue mineral profile may not represent the host xylem and phloem fluid mineral profiles.

The haustorium of *H. triceps* has direct parasite xylem-host xylem contacts and parasite parenchyma-host phloem contacts with *E. dregeana* (Tennakoon et al. 2007). Thus we would expect the parasite to have access to both phloem mobile and immobile

elements. In *Hydnora* the apparent differential uptake of P and K, relative to other elements evaluated, coupled with the conservative transdermal water loss suggests that active processes rather than passive processes (i.e. bulk flow/diffusion) likely contribute to parasite solute uptake across the haustorium.

$\delta^{13}C$ & $\delta^{15}N$ Relationships

Host plants $\delta^{13}C$ values demonstrated carbon isotope fractionation based on photosynthetic metabolism (Farquhar et al. 1989a; O'Leary 1981). As expected C_3 and CAM host plants $\delta^{13}C$ shoot values ranged from -30.27 to -24.90 and -16.80 to -11.64, respectively. The parasite-host shoot tissue $\delta^{13}C$ plot (fig. 4.1A) of all parasite and host functional types, partitioned each group based on host metabolism and parasite photosynthetic ability. Holoparasitic *Hydnora* spp. mirrored the photosynthetic metabolism of their hosts (either C_3 or CAM). Hemiparasitic mistletoe $\delta^{13}C$ signatures on both C_3 and CAM hosts were significantly more negative than their hosts. This result demonstrates the partial heterotrophy of mistletoes and is in agreement with numerous studies of mistletoes in arid environments (Ehleringer et al. 1985; Marshall and Ehleringer 1990; Schultz et al. 1991).

An implicit assumption in the estimation of heterotrophic carbon gain in plant parasites, is that the differences between $\delta^{13}C$ signatures of the parasites and hosts can be attributed to autotrophic carbon gain of the parasite (Marshall and Ehleringer 1990; Press et al. 1987). However, these relationships can be confounded due to interactions of the water use efficiency and carbon metabolism of the parasite and host, that can each independently influence $\delta^{13}C$ signatures. Bannister and Strong (2001) demonstrated that

the Marshall and Ehleringer (1990) $\delta^{13}C$ methods of heterotrophy estimation can not be applied to mistletoes on hosts without severe water limitations, because of similar parasite-host $\delta^{13}C$ signatures, attributed to similar water use and photosynthesis of parasites and hosts.

We tested the underlying assumption that identical parasite-host $\delta^{13}C$ signatures equate complete heterotrophy in a system where the confounding factors of parasite photosynthesis and water use efficiency are minimized. Our $\delta^{13}C$ values for *Hydnora*-host relationships revealed small but significant differences from expectations, with the direction of the relationship depending on the host tissue sampled. *Hydnora* rhizome tissues were enriched in $\delta^{13}C$ (0.55 ± 0.23‰, $P = 0.02$, $n = 46$) relative to the host shoot tissues and had more negative $\delta^{13}C$ (-0.97 ± 0.10‰, $P < 0.001$, $n = 46$) values relative to the host root tissues. However, *Hydnora* rhizome tissues were not significantly different (-0.21 ± 0.15‰, P=0.17, $n = 46$) when compared to the estimated whole host $\delta^{13}C$ value, calculated by averaging host shoot and root values. Differences between host shoot and root values can be attributed to $\delta^{13}C$ partitioning within the plant tissue types and organs (reviewed in Hobbie and Werner 2004; O'Leary 1981); this inherent $\delta^{13}C$ variation within plants has been reported in numerous studies (i.e. Francey and Farquhar 1982; Tennakoon and Pate 1996; Waring and Silvester 1994).

The first comparison of *Hydnora*-host $\delta^{13}C$ values by De la Harpe *et al.* (1981) for a single *H. africana* on *E. mauritanica* association showed a 4.1‰ enrichment in the holoparasite relative to host stem tissue. Similarly, Ziegler (1996) reported $\delta^{13}C$ holoparasite enrichment of 0.6‰ for an *H. africana* on *E. damarana* association. Overall the literature suggests that holoparasites are enriched in $\delta^{13}C$ relative to host tissue. For

all holoparasites-hosts associations analyzed for $\delta^{13}C$ De la Harpe *et al.* (1981), Zeigler (1996), and Cernusak *et al.* (2004), report 1.4, 1.0, and 1.5‰ holoparasite $\delta^{13}C$ enrichment, respectively (data from De la Harpe *et al.* 1981 and Zeigler 1994 calculated by Cernusak *et al.* 2004). These data are consistent only with our results for *Hydnora*-host shoot $\delta^{13}C$ values, demonstrating 0.55 ± 0.23‰ enrichment in the parasite tissues. The endophytic mistletoe *Tristerix aphyllus*, an assumed holoparasite, breaks this general trend for holoparasites by showing $\delta^{13}C$ values -1.34‰ relative to host tissue (data calculated from Table 2 Kraus et al. 1995). However, the definition of holoparasite may be misapplied in this case because as seedlings *Tristerix* are green and apparently photosynthetic. Moreover, *Tristerix* contains small amounts of chlorophyll (Kraus et al. 1995). Badeck *et al.* 2005 report in a review of the $\delta^{13}C$ partitioning within the plant body that roots and stems were on average 0.96‰ and 1.91‰ enriched relative to leaves, respectively. Our *Euphorbia* host $\delta^{13}C$ data fit into that range; roots were 1.58‰ enriched relative to their photosynthetic stems.

Our $\delta^{13}C$ values for water conservative holoparasites and hosts support the underlying assumption of virtually identical $\delta^{13}C$ signatures in holoparasites and hosts. However, we caution that whole host $\delta^{13}C$ values should be determined or estimated since the type of host tissues selected for sampling influences the direction and magnitude of the difference between holoparasite and host $\delta^{13}C$ signatures.

Notably, Ziegler (1996) reported deuterium (δD) enrichment of 32.2‰ in *H. africana* relative to a *E. damarana* host and a similar pattern for several *Cuscuta*-CAM host associations. The explanation for deuterium concentration in holoparasites remains

ambiguous, but may be related to deuterium concentration in water-storage tissues or vacuoles of CAM plants (Ziegler 1996).

$\delta^{15}N$ values were significantly correlated between host and parasite across all parasite functional groups and host photosynthetic metabolisms (fig. 4.1B). This is attributable to the complete dependence of both holoparasites and hemiparasites on the host for N. All hemiparasites in this study were stem parasites, and thus have no other access to N. Likewise, *Hydnora* spp. have no access to soil N due to their lack of roots. For CAM hosts of *Hydnora*, the relationships between $\delta^{13}C$ and $\delta^{15}N$ values for both shoot and root portions were significant and negatively correlated (fig. 4.2A & B). CAM hosts with more negative $\delta^{13}C$ tended to have more positive $\delta^{15}N$ values. $\delta^{13}C$ and $\delta^{15}N$ relationships within *Hydnora* rhizome tissues were expected to mirror the same comparisons within their hosts, yet no significant correlation was evident (fig. 4.2C). However, when differences between holoparasite and host root $\delta^{13}C$ values were plotted against the differences between holoparasite and host root $\delta^{15}N$ values (fig. 4.3B) a significant negative correlation was evident, reflecting $\delta^{13}C$ and $\delta^{15}N$ relationships within the host tissues. This significant negative correlation is evidence of the tight coupling of the holoparasite and host. However, an unexpected result was the nonsignificant relationship when holoparasite differences from host shoot $\delta^{13}C$ and $\delta^{15}N$ values were plotted (fig 4.3A). A probable explanation is that phloem sugars and metabolites co-opted by the root holoparasite are the same as those destined for storage in the root, thus the $\delta^{13}C$ and $\delta^{15}N$ host root profile is apt to be more similar to the holoparasite than the host shoot profile.

Clearly more work is needed to understand the solute flux in plant holoparasites. However, this water conservative model may hold promise, by minimizing the dual confounding effects of parasite autotrophic carbon gain and transpiration. Well-defined culture protocols for *Hydnora* and host are obviously prerequisites for this to be a useful parasite-host model and have yet to be produced. Future studies that aim to estimate proportion of plant parasite heterotrophy should take care to estimate whole host $\delta^{13}C$. As our results demonstrate, improper host tissue selection will invariably bias the result.

CHAPTER 5

MOLECULAR PHYLOGENETIC RELATIONSHIPS OF THE HYDNORACEAE AND A REVISED TAXONOMY OF THE SECTION *EUHYDNORA*

INTRODUCTION

The holoparasitic Hydnoraceae contain only two genera, *Hydnora* and *Prosopanche* from the Old World and New World, respectively. *Hydnora* was first described as a fungus by Thunberg (1775), an error representative of the nomenclatural and phylogenetic uncertainty associated with Hydnoraceae since its discovery. Like other holoparasitic plants, extreme morphological reduction and convergence has made the phylogenetic position of the family uncertain. Traditional classifications associated Hydnoraceae with various angiosperm lineages including Aristolochiaceae (Ballion 1886; Meyer 1833), Rafflesiaceae (Brown 1844), Mitrastemonaceae in Raffesiales (Cocucci and Cocucci 1996), in Raffesiales (Cronquist 1981), in Hydnorales next to Rafflesiales (and related to Aristolochiaceae/Asorideae) (Takhtajan 1997). Nickrent et al. (2002) highlighted that many taxonomists appropriately linked the Hydnoraceae with Aristolochiaceae but erroneously associated the family closely with Rafflesiaceae. Presently data supports the Hydnoraceae in the Piperales with the Aristolochiaceae based on mitochondrial and nuclear ribosomal DNA evidence (Nickrent et al. 2002), validating the traditional taxonomic classifications of Meyer (1833) and Ballion (1886). However the precise placement of the Hydnoraceae in the Piperales, and its nearest photosynthetic

relative remains elusive, in large part due to the unresolved relationships within the Piperales.

The fleshy flowers of *Hydnora* and *Prosopanche* are difficult to preserve and herbarium material often lacks key diagnostic characters (Musselman and Visser 1987, 1989), which spawned synonymy. The most recent worldwide Hydnoraceae monographs named 5-6 *Prosopanche* spp. (Harms 1935) and 9-12 *Hydnora* spp. (Harms 1935; Vaccaneo 1934). Since the publication of those monographs, *Prosopanche costaricensis* L.D. Gómez (Gómez and Gómez 1981) was described from Costa Rica, representing a significant disjunction from the centers of distribution of *Prosopanche americana* (R.Br.) Baill. and *Prosopanche bonancinai* Speg. in Argentina. More recently, *Hydnora sinandevu* Beentje & Q.Luke (Beentje and Luke 2001) was described from the maritime districts of Kenya and Tanzania. The most recent reviews of Hydnoraceae taxonomy, combined with the two recently described species, depict a relatively small family with three species of *Prosopanche* (Cocucci and Cocucci 1996; Gómez and Gómez 1981) restricted to South and Central America and 5 species of *Hydnora* (Beentje and Luke 2001; Musselman and Visser 1987, 1989) from southern and eastern Africa, Madagascar, and the Arabian peninsula.

Relative to animal models, host-parasite relationships in parasitic plants have received scant attention (but see de Vega et al. 2008; Thorogood et al. 2008; Zuber and Widmer 2000). Of the ca. 3,000 parasitic plants only a handful show narrow host preference or a 1:1 relationship between parasite and host (Press and Graves 1995). A well-known example of narrow host preference in North America is the parasite *Epifagus virginiana* (L.) W. Bartram and its exclusive host *Fagus grandifolia* Ehrh. Cospeciation

of organisms has been demonstrated convincingly in several animal models, the seminal example being gophers and gopher lice (Demastes and Hafner 1993). In plant parasites the formation of host specific races, potentially a precursor to cospeciation, has been identified in agronomic (i.e. Bharathalakshmi et al. 1990; Botanga et al. 2002) and natural systems (de Vega et al. 2008; Thorogood et al. 2008). However, cospeciation of plant parasites and hosts has not been demonstrated.

Nuclear and mitochondrial DNA data have proved invaluable in placing "problematic" holoparasites in the tree of life (Barkman et al. 2004; Nickrent et al. 2004; Nickrent et al. 2005; Nickrent et al. 2002) and have illuminated at least eight independent origins of holoparasitism (Barkman et al. 2007). Plastid DNA data, relied upon heavily for our understanding of angiosperm relationships, are not always available or simple to obtain for holoparasites, due to reduced or modified parasite plastomes (dePamphilis and Palmer 1990; Funk et al. 2007; Krause 2008; McNeal et al. 2007). Adding to these complications, horizontal gene transfer between host and parasite is becoming increasingly evident in parasite lineages (Barkman et al. 2007; Davis et al. 2005; Davis and Wurdack 2004). *Hydnora* plastid data (*accD, matK, ndhJ, rpoB, rpoC1*) were sequenced as part of a larger bar coding study, though other plastid regions (*rbcL & ycf5*) failed to amplify (Lahaye et al. 2008). Nickrent et al. (2002) failed to amplify the plastid regions, *atpB* and *rbcL*, and as a result considered the plastome possibly absent or highly modified. Mitochondrial (*atp1, cox1,* and *matR*) and nuclear regions (nuclear ribosomal small and large subunits) were used effectively to infer the position of the Hydnoraceae in the Piperales (Barkman et al. 2007; Nickrent et al. 2002).

The taxonomy of the Hydnoraceae is challenging due to two primary factors: (1) a paucity of herbarium material and basic distributional information and (2) morphological reduction and convergence, evidenced by rampant synonomy in the group. The broad goals of this study were to generate the first phylogeny of the Hydnoraceae, to examine species and sectional boundaries, and to investigate patterns of character evolution and host preference. To address these questions, each Hydnoraceae species was either field collected or sampled from herbarium material. Marked differences in floral morphology, host preference, and floral visitors were observed within *Hydnora africana sensu lato* (subgenus *Euhydnora*) in southern Africa, and noted in our study of the *H. africana sensu lato* chamber flower and trapping mechanism (Bolin et al. 2009). Thus, for *Euhydnora* floral measurements were taken and additional specimens sampled for molecular work across its distribution to evaluate potential cryptic species.

MATERIALS AND METHODS

Taxon Sampling

All currently recognized species of the Hydnoraceae (Beentje and Luke 2001; Gómez and Gómez 1981; Musselman 1991) were sampled (table 5.1). Preliminary work showed that *Hydnora* rhizome tissues, including the rhizome meristems, amplified poorly (data not shown). When possible, perianth tissues from field-collected samples were desiccated in silica gel and used for DNA extractions. Samples of *H. africana sensu lato* were sampled from across its range from southwestern Angola to the Eastern Cape of South Africa. Black pepper, *Piper nigrum* L. was chosen as the outgroup due to its

phylogenetic position in the Piperales; outgroup sequences were obtained from GenBank (ITS: DQ868738; *rpoB*: EF590478).

Table 5.1

SPECIMEN LIST

Name of Taxon	Country	Host	Location	Herb. ID Voucher #	Sequence Data
H. abyssinica	NA	*Acacia luderitzii*	Oshikoto Etosha NP.	WIND JB05_02	ITS *rpoB*
H. abyssinica	YE	*Acacia* sp.	--	N/A	ITS *rpoB*
H. africana = *H. longicollis*	AN	*Euphorbia arenicola*	Namibe	ODU TL_2006	ITS
H. africana = *H. longicollis*	NA	*Euphorbia damarana*	Erongo Uis	WIND JB09_2	ITS
H. africana	NA	*Euphorbia chersina*	Karas Namuskluft	WIND JB05_3	ITS
H. africana	NA	*Euphorbia mauritanica* var. *fotens*	Karas Namuskluft	ODU JB05_5	ITS *rpoB*
H. africana = *H. africana sp. nov.*	NA	*Euphorbia gregaria*	Karas GCP	WIND JB05_1	ITS
H. africana = *H. africana sp. nov.*	NA	*Euphorbia gummifera*	Karas Namuskluft	WIND JB05_4	ITS *rpoB*
H. africana	SA	*Euphorbia caput-medusae*	E.Cape Karoo Bot. Gar.*	N/A	ITS *rpoB*
H. africana	SA	*Euphorbia mauritanica* var. *mauritanica*	N.Cape Gemsbokvlei	WIND JB09330	ITS *rpoB*
H. esculenta	MA	*Pithocellobium dulce*	Berenty Reserve	ODU JB09331	ITS *rpoB*
H. sinandevu	TA	?	Katavi NP	EA & K	ITS *rpoB*
H. triceps	NA	*Euphorbia dregeana*	Karas Region Namuskluft	ODU JB09332	ITS
H. triceps	SA	*Euphorbia dregeana*	N.Cape Gemsbokvlei	ODU JB09333	ITS *rpoB*
P. americana	AR	*Prosopis* sp.	Telteca, Mendoza	BCRU RVR52	ITS
P. bonacinae	AR	*Senecio* sp.	Malargüe Mendoza	ODU JB09334	ITS
P. costariciensis	CR	*Inga oerstediana*	San Jose	NY 1851	ITS *rpoB*

Note.- Country collected (AN = Angola; AR = Argentina; CR = Costa Rica; MA = Madagascar; NA = Namibia; SA = South Africa; TA = Tanzania), host plant, locality information, herbarium ID and accession number, and sequence data generated.

Table 5.1 (continued) * Plant collected in SA and cultivated in California by Sherwin Carlquist.

DNA Extraction, PCR Amplification, and Sequencing

Dried tissue samples were macerated using a Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA). DNA was extracted using the Qiagen DNeasy Plant Mini (Qiagen, Valencia, CA, USA) following the manufacturers' protocols. PCR reactions were prepared in 25 µl volumes with Promega GoTaq DNA Polymerase (Promega, Madison, WI, USA) and run on an ABI 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). The entire ITS region (ITS1, 5.8S rDNA, and ITS2) was amplified using combinations of internal (5.8S rDNA) and external primers (18S and 16S) from Baldwin (1992) and Nickrent et al. (1994). The plastid encoded *rpoB* gene was amplified using primers from the Plant DNA Bar Coding Phase 2 Protocols (http://www.kew.org/barcoding/protocols.html). Target PCR products assessed by size were excised from the agarose gels (1.5 %) and purified using the Qiagen gel purification columns. PCR products were prepared for sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Forward and reverse reads for each PCR product were generated with an ABI 3130 XL genetic analyzer.

Sequence Editing and Alignment

Sequences were assembled from forward and reverse sequence reads using Vector NTI Suite 7.1 (Invitrogen, Carlsbad, CA, USA). Sequences were initially aligned using ClustalX 1.83 (Thompson et al. 1997), then aligned visually using MacClade 4.06 (Maddison and Maddison 2003). Gaps were binary coded manually using the gap coding method of Simmons and Ochoterena (2000).

Cladistic Analyses

For all analyses, characters (nucleotide and gap data) were weighted equally. Maximum parsimony (MP) analyses were performed with Paup 4.0b10 (Swofford 2001) and Bayesian analysis (BA) was implemented using MrBayes 3.1(Ronquist and Huelsenbeck 2003). For MP analysis the following options were implemented: heuristic search, random addition of sequences (500 replicates), TBR branch swapping, MULTREES on, with maximum trees set to 1,000,000. Clade support values were assessed by bootstrap analysis with a heuristic search of 100 replicates. For BA, first Modeltest 3.7 (Posada and Crandall 1998) was used to identify the best fit model of nucleotide evolution for each data partition. The models of sequence evolution applied were HKY (ITS partition) and K2P models (*rpoB* partition). The scored gap partition was modeled as simple binary. BA was run using two simultaneous and independent analyses for a total of 1,500,000 generations. Trees were sampled every 100 generations until the standard deviation of the split frequencies fell below 0.01. The first 25% of the samples (3,250) were discarded as burnin. The *rpoB* analysis was run for 1,000,000 generations, and the ITS and combined analysis were run for 1,500,000 generations.

Morphology

For taxa within the *H. africana* complex (section *Euhydnora*), floral measurements (31 to 54 individuals per taxon) were taken on field collected specimens (2005-2009) and material from the major herbaria of Namibia (WIND) and South Africa (BOL, GRA, NBG, PRE). The following measurements were taken: total flower length (including pedicel) and width, tepal length, tepal lobe length, tepal width, ovary width, stamen width, and stigma width (fig 5.1).

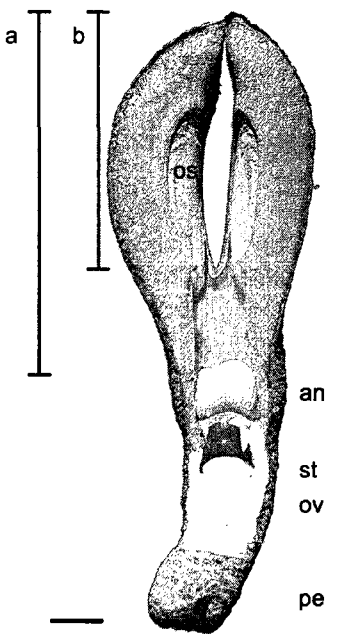

Fig. 5.1 The general floral plan of section *Euhydnora* including *H. africana sensu stricto*, *H. longicollis*, and *H. sp. nov.* Osmophores recessed into tepals (os), subtended by two floral chambers. Connate stamens form an antheral ring (an) with a central orifice on the floor of the androecial chamber. The cushion-like stigma (st) forms the floor of the

Fig. 5.1 (continued) gynoecial chamber. The unilocular ovary (ov) can be distinguished by a slight bulge above the fleshy pedicel (pe). The bars, indicate the tepal length (a) and tepal lobe length (b) measurements. Scale bar = 2 cm.

RESULTS

Internal Transcribed Spacer

The aligned region of ITS1, 5.8S, and ITS2 was composed of 659 characters plus 29 coded indels; 342 characters were parsimony informative (including coded indels). MP searches yielded one shortest tree of 838 steps with a consistency index (CI) of 0.77 and retention index (RI) of 0.84. The topology of the tree was well resolved and supported (fig. 5.2). The two genera, *Hydnora* (BS = 100) and *Prosopanche* (BS = 100) were recovered as well-supported sister clades. Non-*Euphorbia* parasitizing *Hydnora* were the earliest diverging lineages and formed a well-supported clade. Moreover, MP resolved a well-supported clade of *H. triceps* and *H. africana sensu lato*, hereafter known as the *Euphorbia* parasitizing clade. Baysian analysis (BA) yielded a phylogeny congruent with the MP tree (fig. 5.2).

rpoB

The aligned region of *rpoB* was 291bp and 103 characters were parsimony informative. MP searches yielded two shortest trees of 182 steps, with a CI of 0.69 and RI of 0.24. The strict consensus tree shows *Hydnora* and *Prosopanche* as well-supported

sister groups (fig. 5.3). The *Hydnora* clade is moderately supported (BS = 72) but largely unresolved; only one clade was recovered grouping two Fabaceae parasitizing taxa, *H. abyssinica* and *H. esculenta.*

Baysian analysis of *rpoB* data yielded a more resolved tree (fig 5.4). As in the MP analysis, *Hydnora* and *Prosopanche* were resolved as well-supported sister groups. The *Hydnora* species formed a well-supported clade, however like the MP analysis several taxa remained unresolved. In the *Hydnora* clade interior branch support values were weak except for a moderately well supported clade of Fabaceae parasitizing *Hydnora* spp. resolved near the terminus of the tree.

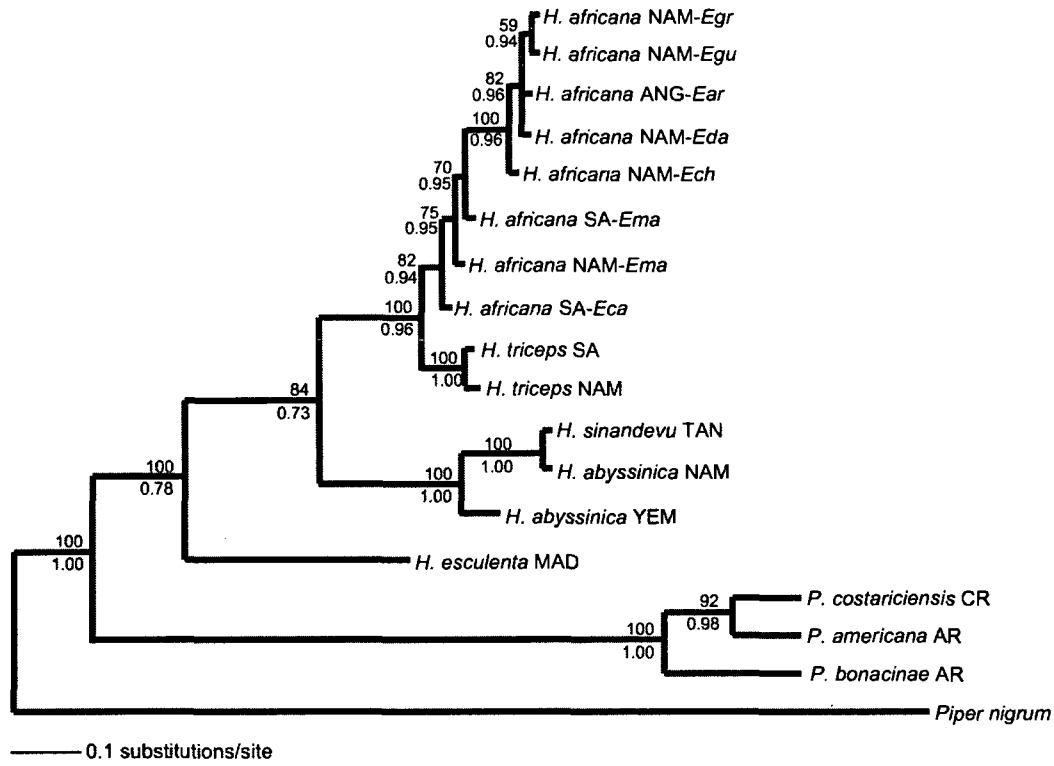

Fig. 5.2 Topology of the single most parsimonious tree derived from ITS and coded gap data only. Numbers above branches indicate MP bootstrap support and numbers below branches indicate BA posterior probabilities. Abbreviations indicating country of origin follow taxon name. For the *H. africana* complex truncated and italicized host names follow the country of origin (See table 5.1).

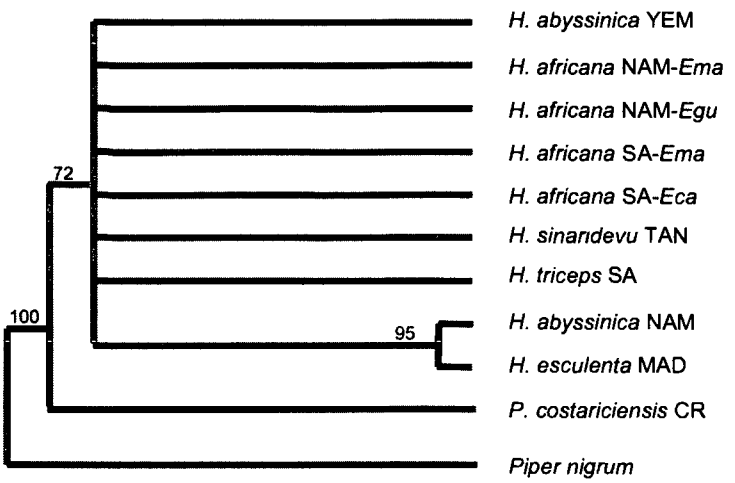

Fig. 5.3 Topology of the strict consensus of two MP trees derived from *rpoB* data. Numbers above branches indicate MP bootstrap support. Abbreviations indicating country of origin follow taxon name. For the *H. africana* complex truncated and italicized host names follow the country of origin (See table 5.1).

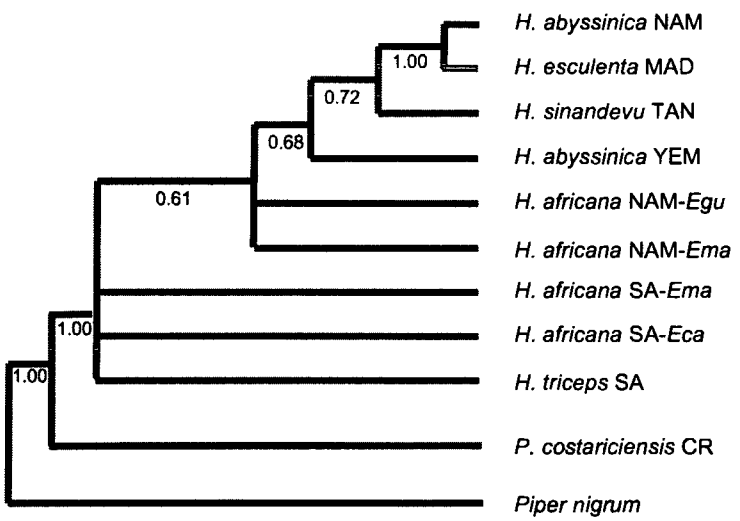

Fig. 5.4 Topology of BA cladogram derived from *rpoB* data. Numbers below branches indicate BA posterior probabilities. Abbreviations indicating country of origin follow taxon name. For the *H. africana* complex truncated and italicized host names follow the country of origin (See table 5.1).

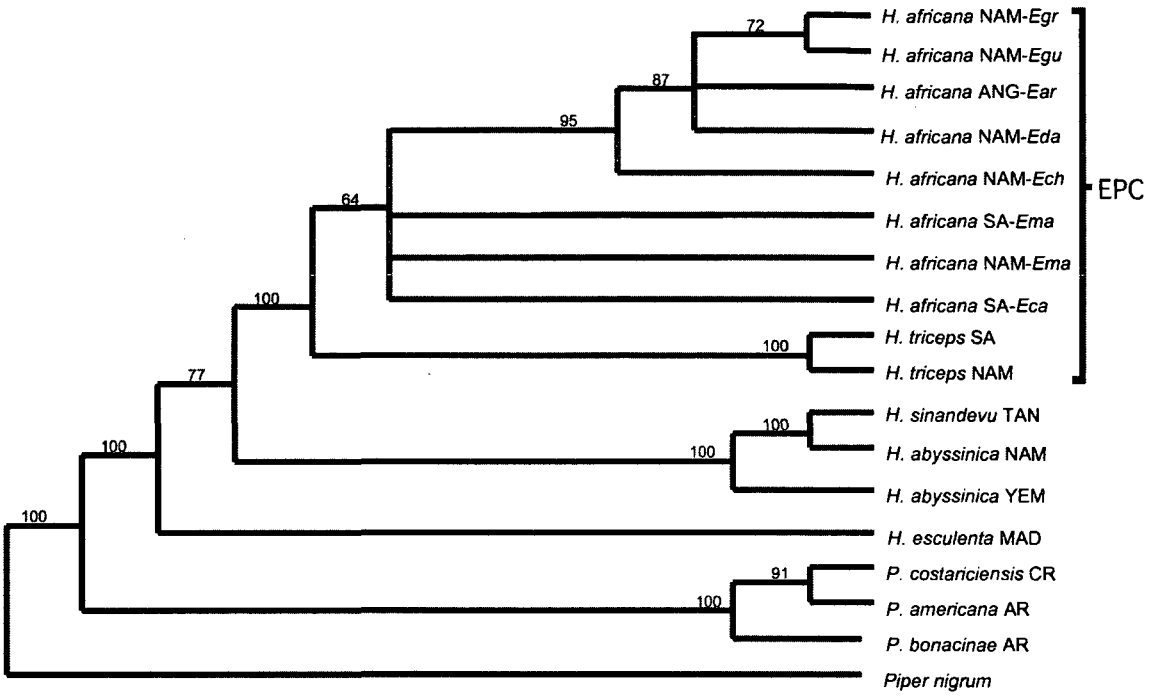

Fig. 5.5 Strict consensus of eight most parsimonious MP trees derived from the combined ITS and *rpoB* dataset. Numbers above branches indicate MP bootstrap support. ECP indicates the *Euphorbia* parasitizing clade. Abbreviations indicating country of origin follow taxon name. For the *H. africana* complex truncated and italicized host names follow the country of origin (See table 5.1).

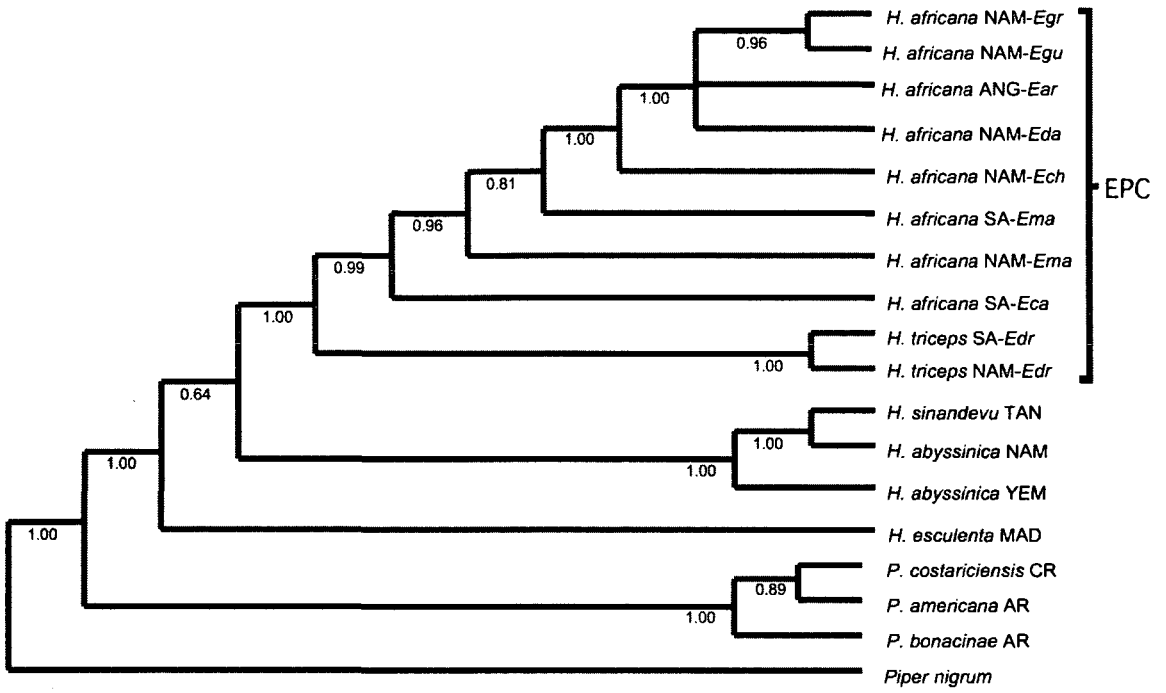

Fig. 5.6 Topology of the BA cladogram derived from the combined ITS and *rpoB* dataset. Numbers below branches indicate BA posterior probabilities. ECP indicates the *Euphorbia* parasitizing clade. Abbreviations indicating country of origin follow taxon name. For the *H. africana* complex truncated and italicized host names follow the country of origin (See table 5.1).

Combined Data

The aligned length of the combined data matrix, *rpoB* and ITS regions, was 979 characters, with 393 parsimony informative characters (including 29 coded gaps). MP searches yielded six most parsimonious trees, of 1,055 steps with a CI of 0.77 and RI of 0.69. The topology of the MP strict consensus tree was similar to the ITS tree with one exception, a polytomy of three *H. africana* taxa (fig. 5.6).

The BA of the combined data set yielded a tree with topology the same as the MP and BA ITS (plus indels) tree, and differed from the combined MP tree by resolving the polytomy of three *H. africana sensu lato* taxa (fig. 5.6).

Euhydnora Morphology

Floral metric data were collected for taxa within the *H. africana* complex (fig. 5.7). Using field and herbarium sample floral measurements, I could distinguish three morphologically distinct taxa within the *H. africana sensu lato* complex. The taxa could be discriminated based on variation in overall flower size and most reliably with tepal metrics (tepal length, tepal lobe length, and tepal width). Stamen, stigma, and ovary metrics were not diagnostic. The recognition of *H. africana sensu stricto*, *H. longicollis*, an unused but valid name, and an apparent new species *H. sp. nov.* was supported by congruence with the molecular phylogeny (fig. 5.8), distinct host preference, and geographic separation. The new species will be described in a separate publication.

DISCUSSION

Molecular Phylogeny

The molecular analyses of ITS, *rpoB*, and the combined dataset supported the monophyly of *Hydnora* and *Prosopanche* as well-supported sister clades. The *rpoB* MP and BA trees were poorly resolved relative to the ITS trees and may reflect conservative evolution in the *rpoB* gene relative to ITS. The *rpoB* MP analysis yielded a mostly unresolved *Hydnora* clade, in contrast to the BA that had more internal structure, although weakly supported (figs. 5.3 & 5.4). The topology of the *rpoB* BA tree has a weakly supported

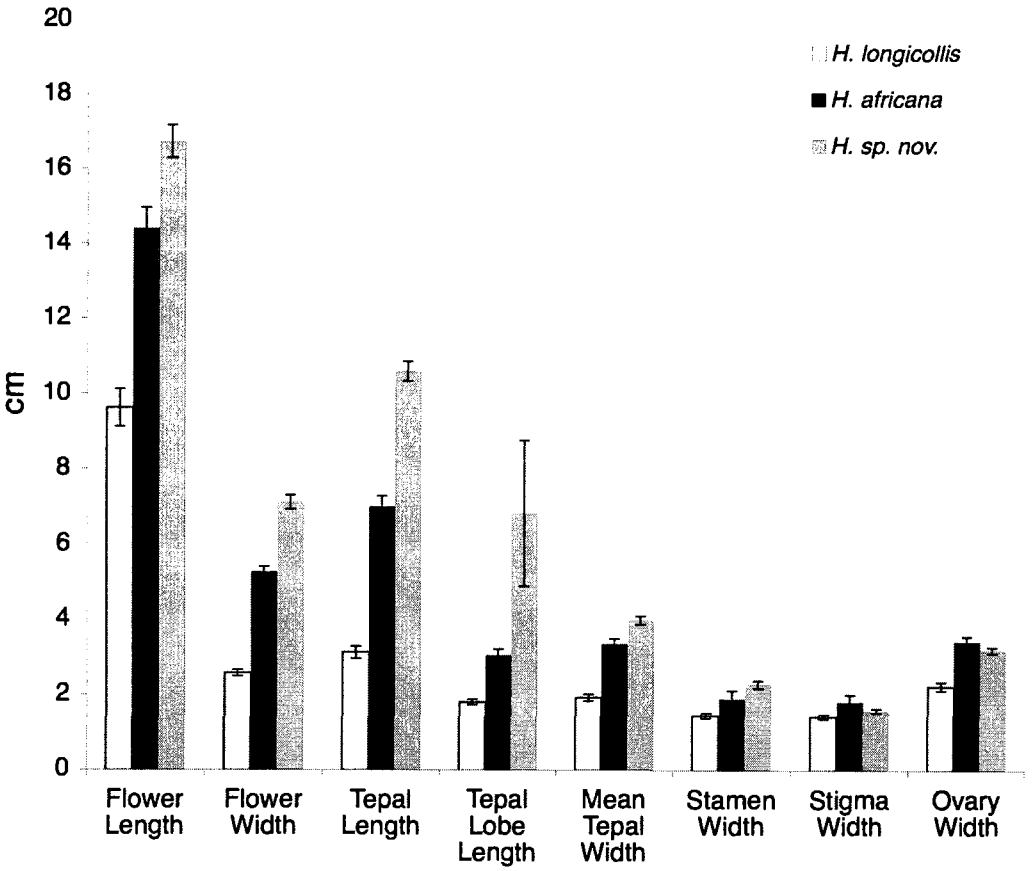

Fig. 5.7 Floral morphometrics for the *H. africana sensu lato* complex including *H. africana* (n = 31), *H. longicollis* (n = 27), and *H. sp. nov* (n = 54). Error bars indicate ± 1 standard error.

backbone with a posterior probability value of 0.61; if collapsed, both BA and MP *rpoB* topologies are congruent. Incongruence of the BA *rpoB* tree with the ITS trees can be attributed to weak branch support from an insufficiently informative *rpoB* dataset and undersampling of taxa relative to the ITS trees. For the ITS analysis the MP and BA trees were congruent (fig. 5.2). The ITS and *rpoB* combined data matrix retrieved trees similar

to the ITS tree demonstrating that the ITS data contained the majority of the phylogenetic signal. Two nodes were unresolved in the combined matrix MP tree relative to the combined matrix BA tree, yielding a polytomy of *H. africana sensu stricto* taxa (fig. 5.5 & 5.6).

Analyses of *rpoB* data showed open reading frames, suggesting a functional plastid encoded RNA polymerase (Allison 2000; Hudson et al. 1988). While some have suggested the possible absence of a Hydnoraceae plastome (Nickrent et al. 1997; Nickrent et al. 2002), our *rpoB* data and the findings of Lahaye et al. (2008) point to the alternative hypothesis that a vestigial plastome remains. Since our *rpoB* gene tree is largely congruent with the ITS data, contamination or other experimental error can be ruled out. In non-photosynthetic plants, plastids perform a variety of important functions (i.e. leucoplasts) and the presence of ubiquitous truncated "cryptic" plastomes is the prevailing view for plant parasites (Krause 2008).

Character evolution and host preference

The two well supported clades defined the genera *Hydnora* and *Prosopanche*. Symplesiomorphies for *Hydnora* are the antheral ring and prominent osmophores (fig. 5.8). The Malagasy taxon, *H. esculenta,* was the earliest diverging lineage of *Hydnora* and shared with *Prosopanche* the plesiomorphic feature of angular rhizomes (fig. 5.8). The next lineage was a well supported clade of *Commiphora*-Fabaceae parasitizing *Hydnora* (*H. abyssinica* YEM, *H. abyssinica* NAM, and *H. sinandevu* TAN) with, osmophores positioned on the tepal apex, white to tan (when fresh) interior tepal and floral chamber surfaces, mainly 4-merous flowers, and derived terete rhizomes. The

paraphyly of *H. abyssinica* suggests a potential cryptic *Hydnora* species on the Arabian Peninsula or revaluation of *H. sinandevu* as a good species. Little herbarium material exists to document reports of *Hydnora* from Oman, Yemen, and Saudi Arabia (but see Miller and Morris 1988; Musselman and Visser 1989).

Interestingly the ancestral state of host preference in the Hydnoraceae appears to favor Fabaceae. The name *Prosopanche* meaning "*Prosopis* strangler" is apt because *P. americana* and *P. costaricensis* parasitize only Fabaceae, while *P. bonancinai* has a more catholic host range including many families (Cocucci and Cocucci 1996; Gómez and Gómez 1981). Moreover, *H. abyssinica* and *H. esculenta* parasitize only Fabaceae (Bosser 1994; Jumelle and Perrier de la Bâthie 1912; Musselman and Visser 1987). The recently described *H. sinandevu* is reported to parasitize *Commiphora* spp. (Beentje and Luke 2001). Still, it is possible, and perhaps likely that this species parasitizes *Acacia* because it is known from *Acacia-Commiphora* savanna in Tanzania and Kenya and the determination of root holoparasite host preference is notoriously difficult to ascertain.

A single *Hydnora* host shift is suggested from mainly Fabaceae hosts to exclusively *Euphorbia* hosts (fig. 5.8). The *Euphorbia* parasitizing clade (EPC) of *Hydnora* include the sections *Euhydnora* and *Tricephalohydnum* and share three apomorphic features: (1) osmophores recessed within tepals, (2) pink (darkening to orange and red) internal tepal and floral chamber surfaces, and (3) mainly trimerous flowers. Also, the EPC apparently maintains the plesiomorphic state of angular rhizomes. The earliest diverging lineage of the EPC, is the hypogeous flowering *H. triceps*. Nested within a series of *H. africana* taxa near the terminus of the tree are two well-supported clades that circumscribe two morphologically distinct taxa: *H. longicollis* a valid but

synonymized name (Musselman 1991) recognized here, and a putative unpublished *Hydnora* species (here called sp. novon) from southwestern Namibia and extreme northwestern South Africa.

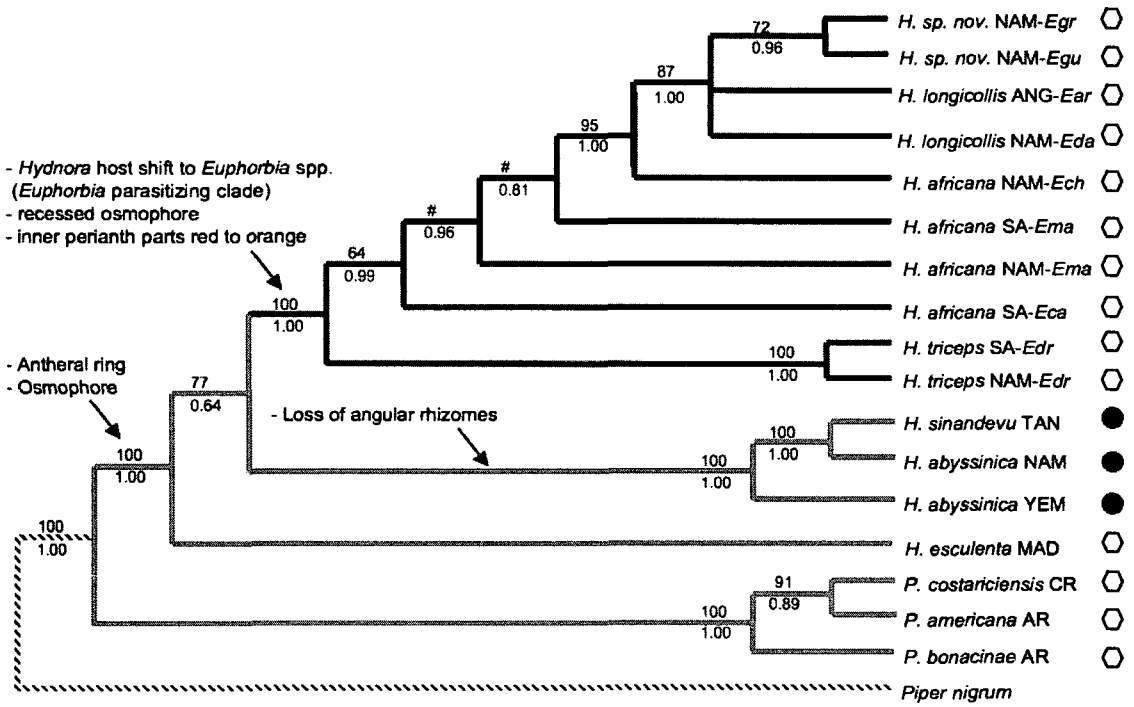

Fig. 5.8 Parasite host preference and morphology mapped onto the BA cladogram derived from the combined ITS and *rpoB* dataset. Numbers above branches indicate MP bootstrap support. Numbers below branches indicate BA posterior probabilities. The # symbols on the BA tree topology indicate collapsed branches in the MP topology. Synapomorpies indicated by arrows. Excluding the outgroup, *Piper nigrum,* the gray branches indicate parasites with non-*Euphorbia* hosts (mainly Fabaceae) and black branches indicate strictly *Euphorbia* hosts. Circles and hexagons to the right of the taxa names indicate terete and angular rhizomes, respectively. Abbreviations indicating

Fig. 5.8 (continued) country of origin follow taxon name. For the *H. africana* complex truncated and italicized host names follow the country of origin (See table 5.1).

Classification

Subgeneric classifications were introduced by Decaisne (1873) to accommodate the burgeoning ranks of African species of *Hydnora* described in the 19th century. These classifications were based on limited material and poor or incomplete specimens. The first subgenera erected were based on floral merosity, *Dorhyna* Decaisne to accommodate 4-merous flowers and *Euhydnora* Decaisne for 3-merous flowers. Subsequently, Vaccaneo (1934) added rhizome characters to the existing subgeneric classifications, *Dorhyna*: 4-merous flowers with terete rhizomes and *Euhydnora*: 3-merous flower with angular rhizomes. Harms (1935) provided an overview of the genus following the system of Decaisne and contributed two subgenera (table 5.2). Harms circumscribed *Euhydnora* to contain *H. africana* and *H. longicollis*, and with caveats *H. angloensis* Decaisne, known only from a fruit, and *H. aethiopica* Decaisne, which he considered a dubious species. In *Dorhyna*, Harms placed *H. abyssinica* A. Braun, *H. bogosensis* Beccari, *H. cornii* Vaccaneo, *H. gigantea* Chiovenda, *H. hanningtonii* Rendle, *H. johannis* Beccari, H. *ruspolii* Chiovenda, and *H. solmsiana* Dinter. However, Harms cautioned that the differences among these putative taxa within *Dorhyna* were slight and that they were likely variations of a single species. This line of thinking was later implemented by Musselman and Visser (1987), in a review of *Dorhyna*. Harms introduced two new subgenera, *Tricephalohydnum* Harms to accommodate the unusual hypogeous species *H.*

triceps Drége & Meyer and *Neohydnora* Harms for the enigmatic Malagasy endemic, *H. esculenta* Jumell and Perrier. The utility of these *Hydnora* subgeneric classifications become limited in this species poor genus, however they do not conflict with the phylogeny. In that light, we propose that the recently described species, *H. sinandevu* should be placed in section *Dorhyna* because it forms a clade with *H. abyssinica*. Accordingly, *H. sp. nov.* forms a clade with a monophyletic *Euhydnora*.

Table 5.2

THE SECTIONS OF *HYDNORA* FOLLOWING HARMS (1935)

Euhydnora Decaisne	*Dorhyna* Decaisne
H. africana Thun.	*H. abyssinica* A. Braun
H. longicollis Welw.	*H. bogosensis** Beccari
H. angloensis† Decaisne	*H. cornii** Vaccaneo
H. aethiopica† Decaisne	*H. gigantea** Chiovenda
H. sp. novon. (this paper)	*H. hanningtonii** Rendle
	*H. johannis** Beccari
	*H. ruspolii** Chiovenda
	H. sinandevu Beentje & Luke
	*H. solmsiana** Dinter
Neohydnora Harms	*Tricephalohydnum* Harms
H. esculenta Jumelle & Perrier	*H. triceps* Drége & Meyer

NOTE.- * Considered synonyms following Harms (1935) and Musselman and Visser (1989).

† Harms considered *H. angloensis* and *H. aethiopica* doubtful species.

Taxonomic notes on section Euhydnora

Mainly subterranean, root holoparasitic perennial herb, without leaves and scales. Emerges from soil to flower. Mature rhizome angular, spreading laterally, and occasionally bifurcate or trifurcate. Rhizomes ornamented with lateral appendages or tubercles arrayed in rows. Lateral appendages can develop into floral buds, rhizome branches, or haustoria. Rhizome tips terete. Floral merosity usually 3, rarely 2 or 4, with free tepals. The bisexual chamber flowers of *Euhydnora* have pink internal tepal parts later darkening to orange and red. Osmophores recessed within each tepal are white darkening to gray and brown. Fruits are turbinate and contain numerous black seeds embedded in a white fleshy pulp.

Euhydnora is restricted to Angola, Namibia, and South Africa (fig. 5.9) and includes, *H. africana*, *H. longicollis*, and *H. sp. nov.*, the latter two taxa, formerly cryptic species in Namibia. The combined (ITS and *rpoB*) analysis suggests that *H. africana* is a variable species, that might warrant subdivision into geographic or host races. The most derived clade is composed of *H. longicollis* and *H. sp. nov.*. Each *Euhydnora* taxon is apparently specific to a narrow range of *Euphorbia* hosts. Due to their obligate relationships to their hosts, *Euhydnora* distributions mimic their host's mainly allopatric distributions (i.e. *E. damarana* & *E. gregaria*), with few areas of distributional overlap. The formerly cryptic complex of *H. africana sensu lato* (*H. africana*, *H. longicollis*, and *H. sp. nov.*) can be distinguished by morphology, overall flower size, tepal length, width, and tepal lobe length.

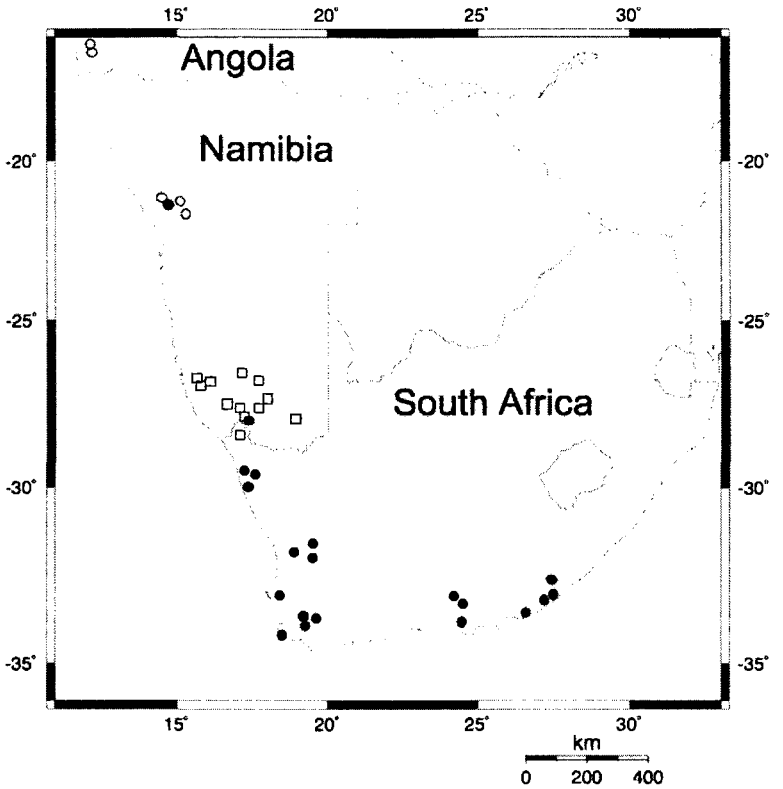

Fig. 5.9 Distribution of *H. africana* (filled circles), *H. longicollis* (open circles), and *H. sp. nov.* (open squares).

Hydnora africana Thunberg Kongl. Vetensk. Acad. Handle. 36:69. 1775.

TYPE: *H. africana* Thunb. karoo areas, Bokkeveld Mountains, South Africa, Western Cape, no date, *Thunberg 1542* (holotype: UPS).

Morphology: Tubular perianth, 8.2-19.5 cm long and 4.2-6.4 cm wide. Tepal length, 4.3-9.9 cm. Tepal lobe length measured from apex to point of connation with adjacent tepal, 2.5-4.2 cm. Tepal lobe width measured at midpoint, 2.0-6.5 cm. Perianth with two floral chambers; an androecial chamber subtended by gynoecial chamber. Chambers joined by

an antheral ring with a central orifice, formed by connate anthers. Antheral ring width, 1.0-2.1 cm. Pollen bisulcate. Perianth tissues fleshy, internal surfaces pink, then darkening to orange and red over several days. External perianth surfaces, scaly and brown. Internal perianth margins with numerous setae. Osmophores spongy, recessed on interior surface of tepal lobes. Flowers uniformly hermaphroditic. Sessile and cushion-like stigma forms floor of gynoecial chamber, stigma width, 1.9-2.4 cm. Ovary inferior and unilocular, with numerous ovules. Ovary width, 2.2-4.1 cm. Fleshy pedicel sometimes present, 0--5 cm. Fruit a partially subterranean turbinate berry, diameter 7-18 cm, with numerous spherical black-brown seeds, diameter 0.7-1.2 mm, embedded in a white pulp.

Distribution: *Hydnora africana* is the most widely distributed species in the section *Euhydnora* and occurs across a wide swath of southern Africa. This taxon is commonly associated with succulent karoo vegetation in the Eastern Cape, Western Cape, and Northern Cape Provinces of South Africa. In southwestern Namibia *H. africana* is found in the winter rainfall areas of the Karas Region. Outside of the winter rainfall areas of Namibia limited collections indicate that *H. africana* occurs on the Brandberg massif and thus may occur on other isolated inselburgs of the western escarpment of the Namib desert. These inselburgs have relict karoo vegetation and receive higher rainfall levels than the surrounding Namib Desert; some support robust colonies of its major host plant *E. mauritanica* (Burke 2002).

Hosts: Hydnora africana parasitizes a variety of shrubby and arborescent *Euphorbia* spp. Thunberg's original description indicated *Euphorbia mauritanica* L. as the host of *H. africana* from the Bokkeveld Mountains of the Western Cape of South Africa. *Euphorbia mauritanica* is frequently a dominant karoo component and has the widest range of the *H. africana* host species, from northwestern Namibia to the Western and Eastern Cape of South Africa. Thus it is no coincidence that *E. mauritanica* is the most commonly reported host of *H. africana.* Regionally, other *Euphorbia* hosts have been reported for *H. africana, Euphorbia caput-medusae* L. from the Western Cape (Adamson 1950) the arborescent *E. grandidens* Goebel and *Euphorbia triangularis* Desf. from the Eastern Cape, and *Euphorbia decussata* E.Mey. and *Euphorbia lignosa* Marloth from karoo habitats (Harms 1935). From the Namib Desert, *Euphorbia gariepena* Boiss. has been reported as a host (Craven and Marais 1992). However, there are no herbarium vouchers to document this association

Phenology: Flowering time for this wide ranging species depends on its location due to marked differences in rainfall patterns across southern Africa. In the Eastern Cape of South Africa flowering mainly occurs from Nov. to Jan. whereas in the Northern Cape flowering mainly occurs from Aug. to Oct. Limited information suggests that *H. africana* on inselbergs in Namibia flower from Feb. to April. However, like other *Hydnora* spp. occasional flowering can be observed year round in robust populations. Fruits are very slow to mature and are often observed concurrently with flowering suggesting maturation periods of 9-12 months.

Hydnora longicollis Welwitsch Trans. Linn. Soc. 27:66-67. 1869.

TYPE: *H. longicollis* Welwitsch, sandy areas, Mossamedes District, Southwestern Angola, date illegible, *Welwitsch 53* (holotype: K)

Nomenclature: Welwitsch's description of this taxon is somewhat unclear, the body of the description refers to a subspecific classification, *Hydnora africana* var. *longicollis* Welw. However the figure label in the same description refers to *Hydnora longicollis* Welw. Under the International Code of Botanical Nomenclature, *H. longicollis* is a valid name. Accordingly, the monographs of Vaccaneo (1934) and Harms (1935) recognized *H. longicollis*. Since the time of those monographs this taxon has been largely ignored due to its remote type locality in southwestern Angola and warfare and civil unrest in the border areas of Angola and Namibia in the latter half of the 20th century. *Hydnora longicollis* is disjunct from the distributions of other *Euhydnora* and has distinct morphology supported by DNA data from this study, thus should be recognized at a specific level.

Morphology: *Hydnora longicollis* has the smallest flower and fruit in *Euhydnora*. Tubular perianth, 5.1-14.5 cm long and 1.2-2.7 cm wide. Tepal length, 1.7-5.2 cm. Tepal lobe length measured from apex to point of connation with adjacent tepal, 1.1-2.4 cm. Tepal lobe width measured at midpoint, 1.2-2.7 cm. Perianth with two floral chambers; an androecial chamber subtended by gynoecial chamber. Chambers joined by an antheral ring with a central orifice, formed by connate anthers. Antheral ring width, 1.0-2.1 cm. Pollen bisulcate. Perianth tissues fleshy, internal surfaces pink, then darkening to orange

and red over several days. External perianth surfaces, scaly and brown. Internal perianth margins with numerous setae. Osmophores spongy, recessed on interior surface of tepal lobes. Flowers uniformly hermaphroditic. Sessile and cushion-like stigma forms floor of gynoecial chamber, stigma width, 1.0-1.8 cm. Ovary inferior and unilocular, with numerous ovules. Ovary width, 1.7-3.3 cm. Fleshy pedicel sometimes present, 0-3 cm. Fruit a partially subterranean turbinate berry, diameter 3-5 cm, with numerous spherical brown-black seeds, diameter 0.7-1.2 mm, embedded in a white pulp.

Distribution: *Hydnora longicollis* was described from the Mossamedes District of southwestern Angola, specifically in the vicinity of Giraul and Cabo Negro. Recent collections have confirmed the presence of *H. longicollis* in the type locality, south of Namibe, Angola. In northwestern Namibia *H. longicollis* is present in Damaraland. Collections have been made from areas south of the Omaruru River and as far north as Brandberg and the Mesum Crater. This taxon should follow the distribution of its common host in Namibia, *Euphorbia damarana,* north through the Kaokoveld. However much basic fieldwork is still required to delimit the distribution of *H. longicollis* in the relatively inaccessible areas of northwestern Namibia and adjoining southwestern Angola.

Hosts: Welwitsch listed the genus *Euphorbia*, in general, as the preferred host of *H. longicollis* in Angola. In Namibia, *E. damarana* is evidently the favored host. Two recent collections of *H. longicollis* south of Namibe, Angola confirm *Euphorbia* spp. as hosts, (1) herbarium voucher, TL-2006 (ODU) was made on a poorly known taxon, *Euphorbia*

virosa Willd. *arenicola* L.C.Leach and (2) herbarium voucher, WE Voigt 67-2009 (Harold Porter NBG Herbarium, Betty's Bay Cape Town SA); parasitized an undescribed arborescent *Euphorbia* sp. (Pers. Comm. Voight 2009). The original description of *H. longicollis* suggested *Zygophyllum orbiculatum* Wel. as a host (Welwitsch 1869). However, this host association is doubtful since no field collections or herbarium label information can confirm this association and all other hosts of the section *Euhydnora* parasitize *Euphorbia* spp. only. Clearly the host preference of *H. longicollis* requires more study. The inaccessible areas of the Kaokoveld in Namibia and adjoining areas of Angola are currently being catalogued floristically and may reveal new *Euphorbia* hosts.

Phenology: In Namibia, *H. longicollis* flowers mainly Feb. to April, however sporadic flowering has been observed year round. In Angola flowering has been observed in January. Fruits take many months to develop.

Hydnora sp. nov. This species description will be formally published separately from this dissertation.

Morphology: *Hydnora sp. nov.* has the largest flower in *Euhydnora*. Tubular perianth, 10.5-24.0 cm long and 4.8-11.2 cm wide. Tepal length, 6.4-14.8 cm. Tepal lobe length measured from apex to point of connation with next tepal, 6-9 cm. Tepal lobe width measured at midpoint, 2.0-5.7 cm. Perianth with two floral chambers; an androecial chamber subtended by gynoecial chamber. Chambers joined by an antheral ring with a central orifice, formed by connate anthers. Antheral ring width, 1.7-3.4 cm. Pollen

bisulcate. Perianth tissues fleshy, internal surfaces pink, then darkening to orange and red over several days. External perianth surfaces, scaly and brown. Internal perianth margins with numerous setae. Osmophores spongy, recessed on interior surface of tepal lobes. Flowers uniformly hermaphroditic. Sessile and cushion-like stigma forms floor of gynoecial chamber, stigma width, 1.0-3.0 cm. Ovary inferior and unilocular, with numerous ovules. Ovary width, 2.2-4.5 cm. Fleshy pedicel sometimes present, 0-6.5 cm. Fruit a partially subterranean turbinate berry, diameter 3-5 cm, with numerous spherical black-brown seeds, diameter 0.7-1.2 mm, embedded in a white pulp.

Distribution: *Hydnora sp. nov.* is centered in the winter rainfall and summer-winter transitional rainfall areas of southern Namibia and adjoining areas of the Richtersveld in the Northern Cape of South Africa. The distributions of *Hydnora sp. nov.* and *H. longicollis* in Namibia are separated by approximately 300 km and each follow the distributions of their hosts and the approximate boundaries of two floristic regions: the East Gariep District and the Central Namib Desert, respectively. The same pattern of vicariance is shared by numerous species pairs (Jürgens 1997). To explain this pattern of vicariance, Jürgens (1997) posits the intrusions of the Namib and Kalahari dunefields as the driving factors.

Hosts: *Hydnora sp. nov.* parasitizes *Euphorbia gregaria* and *Euphorbia gummifera*. Both host species commonly form the dominant shrub component of their preferred habitat, arid karoo.

Phenology: *Hydnora sp. nov.* flowers primarily from Oct. to Jan., however flowering has been observed sporadically year round. Fruits are slow to mature. Ripe fruits from the previous flowering season can sometimes be located among open flowers.

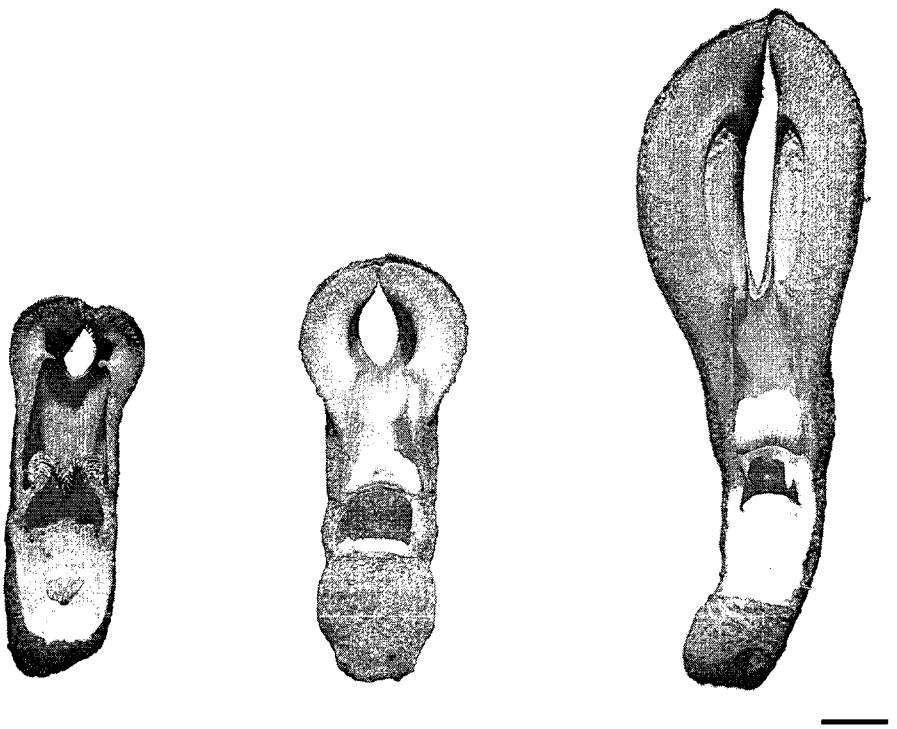

Fig. 5.10 Flowers of *H. longicollis* (left), *H. africana* (center), and *H. sp. nov.* (right). Each flower has one tepal removed, *H. longicollis* is after anthesis, *H. africana* and *H. sp. nov.* are before pollen shed. Scale bar = 2 cm.

KEY TO SECTION *EUHYDNORA*

1a. Tepal lobes less than 2.2 cm, generally less than 1/5 of the flower length protruding above ground.....*H. longicollis*

1b. Tepal lobes greater than 2.2 cm, generally more than 1/5 of the flower length protruding above ground.....2

2a. Tepal lobes between 2.2 cm and 4.5 cm.....*H. africana*

2b. Tepal lobes greater than 4.5 cm.....*H. sp. nov.*

CHAPTER 6

SUMMARY

This study examined the biology of *Hydnora* in southern Africa in a broad-based fashion due to the paucity of information about the group. Herein novel data was presented regarding the pollination biology, germination ecology, parasite-host nutritional relationships, character evolution, and systematics of this fascinating group of holoparasitic plants.

The pollination biology of *Hydnora* is remarkable. The trimerous flowers of *H. africana sensu lato* have androecial and gynoecial chambers and attract floral visitors with putrid odors emitted from prominent osmophores. Floral phenology and insect visitation was recorded for *H. africana* at two sites in southern Namibia, and the insect trapping mechanism was evaluated with beetle addition and pollen viability assays. Flowers were putatively protogynous for three days. Eighteen species of floral visitors were observed, including 10 Coleopteran species imprisoned by the smooth inner surface of the androecial chamber. The hide beetle *Dermestes maculatus* (Tenebrionidae) accounted for 76.9 % of the imprisoned insects with a density of 2.2 ± 0.6 per flower. The *D. maculatus* addition experiment ($n = 9$) clearly demonstrated imprisonment during the carpellate stage. Changes in the inner surfaces of the androecial chamber, stippling and texturing, allowed *D. maculatus* escape after pollen release. Over 55.5 % of the beetles escaped, dusted with viable pollen, three days after pollen release. The beetle addition and pollen assay demonstrate the efficiency of the *H. africana* imprisonment

mechanism. Differences in floral odor and insect visitation between sympatric *Hydnora* spp. are intriguing. Future pollination studies to describe floral odor profiles for closely related and sympatric *Hydnora* taxa, and correlation of those data to insect visitation should be rewarding. Differences in floral odors between closely related taxa may have contributed to sympatric speciation or the reinforcement of species boundaries.

The first germination data in the root holoparasitic Hydnoraceae was generated by applying aqueous root extracts of host and non-host *Euphorbia* spp. to seeds of *Hydnora triceps*, a narrow endemic of Namibia and South Africa. The seeds of *H. triceps* germinated only in response to root extracts of its exclusive host, *Euphorbia dregeana,* and not for co-occurring non-host *Euphorbia* spp. This pattern of host specific germination suggests that germination response to host-root cues may be responsible for host partitioning. Provenance of *H. triceps* seeds and *E. dregeana* root extracts did not significantly affect germination rates. The round-eared elephant shrew (*Macroscelides proboscideus*) and striped mouse (*Rhabdomys pumilio*) were observed feeding on the fleshy pulp of *H. triceps* fruits. Small mammal dropping collected from the same partially consumed fruits contained intact seeds (1.9 ± 1.1 seeds/dropping), providing indirect evidence of seed dispersal by small mammals. Similar germination trials for other *Hydnora* taxa were largely inconclusive due to negligible or zero germination (data not shown). Clearly, while this information regarding host specific germination of *Hydnora* is important, much work remains to determine the precise germination requirements. Frustratingly, seedling attachment to the host root has not been observed. Reproducible high germination percentages are requisite for further ecophysiological and developmental studies of *Hydnora.*

Parasite-host nutrient relationships and the mechanisms by which parasitic plants mediate solute uptake have been subject to intensive study. However, there are large gaps in our understanding for holoparasitic plants. Thus *in situ* transdermal water loss was estimated in *Hydnora* and nutrient profiles and $\delta^{13}C$ and $\delta^{15}N$ signatures were measured for *Hydnora* and hosts in southern Africa and Madagascar. For comparison, $\delta^{13}C$ and $\delta^{15}N$ signatures were measured for aerial hemiparasites at the same sites. Transdermal water loss in *Hydnora* ranged from 0.14±.02 to 0.38±.04 mg cm^{-2} hr^{-1} and was comparable to transpiration rates for water conservative xerophytes. Concentrations of P and K were higher in *Hydnora* relative to CAM hosts; other mineral concentrations were significantly lower in the parasite or were not different. $\delta^{13}C$ signatures of holoparasites and hemiparasites relative to their hosts reflected host metabolism and differences in commitment to heterotrophic C gain. Holoparasite $\delta^{13}C$ values were significantly enriched (0.55‰ ± 0.23) compared to host shoot and depleted compared to host root tissues (-0.97‰ ± 0.12). Holoparasite $\delta^{13}C$ values were not significantly different compared to the estimated whole host $\delta^{13}C$ value. $\delta^{15}N$ values for holoparasites and hemiparasites were significantly correlated with hosts. The water conservative nature of *Hydnora* spp. was demonstrated using *H. africana* and *H. triceps* rhizomes. These results, combined with parasite-host mineral nutrition profiles showing differential concentration of P and K relative to other nutrients are suggestive of active processes of solute uptake. Stable isotope fractionation in host tissues dictated significant differences between parasite and host (shoot and root) $\delta^{13}C$ signatures. The confirmation of complete heterotrophy and the lack of a confounding transpiration stream may make *Hydnora* a promising model organism for the examination of parasite solute uptake.

The first phylogeny of Hydnoraceae using DNA sequences from plastid (*rpoB*) and nuclear regions (ITS) was generated using maximum parsimony and Bayesian inference methods. The ITS, *rpoB*, and combined analyses each supported the monophyly of *Hydnora* and *Prosopanche* as sister genera. The earliest diverging lineage of *Hydnora* was the Madagascar endemic *Hydnora esculenta.* The ancestral state of *Prosopanche* and *Hydnora* host preference appears to be Fabaceae. Moreover, a well supported Fabaceae parasitizing clade was resolved as sister to an exclusively *Euphorbia* parasitizing clade, indicating a single host shift from Fabaceae to *Euphorbia.* Angular rhizomes are plesiomorphic in the family; derived terete rhizomes were present only in the *Hydnora*-Fabaceae parasitizing clade (*Dorhyna*). Pink-Red internal perianth color and recessed osmophores are symplesiomorphic for the most derived *Hydnora-Euphorbia* parasitizing clade. In the section *Euhydnora* floral morphometric data were congruent with the combined data phylogeny, revealing three cryptic taxa within *Hydnora africana sensu lato*, (1) *Hydnora africana,* (2) *Hydnora longicollis,* a valid but largely forgotten taxon, and (3) a new *Hydnora* sp. from southwestern Nambia and the Northern Cape of South Africa. The *Hydnora* subgeneric sections: *Dorhyna, Euhydnora, Neohydnora,* and *Tricephalohydnum* were supported by the phylogeny, with no changes except the proposed addition of the putative new taxon to *Euhydnora* and the recently described *Hydnora sinandevu* to *Dorhyna.*

The results showing evidence of a plastid genome in *Hydnora* are compelling and immediately raise questions regarding the function of such a plastome. Complete plastome sequencing for *Hydnora* and *Prosopanche* would contribute to our

understanding of non-photosynthetic plastome functions. Moreover, additional sequence data and taxon sampling would facilitate dating of important nodes in the combined phylogeny. The absence of a known Hydnoraceae fossil record will complicate dating excercises, however, this could be circumvented by using Piperalean fossils, assuming family level relationships in Piperales can be resolved. A remarkable Hydnoraceae node worthy of dating is the split between *Hydnora* and *Prosopanche*, to test if long distance dispersal or Gondwanan vicariance played a role in the evolution of the genera. Other nodes that should be dated are the apparent host shift from mainly Fabaceae to *Euphorbia* and the divergence of the Malagasy taxon. It would be fascinating to examine if ancient events such as southern African aridification (Jürgens 1997) and the retreat of *Acacia*-savanna vegetation may have caused a subsequent radiation of *Euphorbia* coupled with a cospeciation based radiation of *Hydnora.* These questions should be addressed with a more robust phylogeny of *Hydnora* and a phylogeny of *Euphorbia* host and non-hosts for comparison of branching patterns. Working on the Hydnoraceae first requires a shovel, however difficult; the study of the Hydnoraceae will continue to unearth botanical treasures if one should look.

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VITA

Jay Francis Bolin

Education:

- B.S. Environmental Science. 2000. Minor: Entomology. Virginia Tech, Blacksburg VA.

- M.S. Biology. 2004. Old Dominion University, Norfolk VA. Mentor: Dr. Lytton Musselman.

- Ph.D. Ecological Sciences. 2009. Old Dominion University, Norfolk VA. Mentors: Dr. Lytton Musselman (primary), Dr. Rebecca Bray, Dr. Tim Motley, and Dr. Erika Maass (University of Namibia).

Professional Experience:

- 2000-2001. **Environmental Scientist**: Apex Environmental, Rockville, MD.

- 2001-2006. **Teaching Assistant**: Old Dominion University, Dept. of Biological Sciences. Instructed laboratories in Botany, Plant Physiology, General Biology, and General Biology for non-majors.

- 2008-2009. **Fulbright Student Fellow to Namibia**: Research in the ecology and evolution of the Hydnoraceae and lecturing at the University of Namibia.

Publications:

- Nine peer-reviewed publications as of Sept. 2009.