



HHS Public Access

Author manuscript

Zootaxa. Author manuscript; available in PMC 2015 December 30.

Published in final edited form as:

Zootaxa. 2012 March 1; 3218: 1–17.

***Anopheles (Kerteszia) lepidotus* (Diptera: Culicidae), not the malaria vector we thought it was: Revised male and female morphology; larva, pupa, and male genitalia characters; and molecular verification**

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Abstract

The name *Anopheles (Kerteszia) lepidotus* Zavortink, commonly used for an important malaria vector in the eastern cordillera of the Andes, is here corrected to *An. pholidotus* Zavortink. We discovered that *An. (Ker.)* specimens from Peru, and reared-associated specimens from Ecuador, had unambiguous habitus characters that matched those on the male holotype of *An. lepidotus*. However, the specimens do not exhibit characters of the female allotype and female paratypes of *An. lepidotus*, which are actually *An. pholidotus*. Our specimens are the first correctly associated females of *An. lepidotus*, which allow us to provide a new morphological interpretation for the adult habitus of this species. This finding is also corroborated by molecular data from a portion of the *Cytochrome Oxidase I (COI)* gene and ribosomal DNA Internal Transcribed Spacer 2 (rDNA ITS2). The pupal stage of *An. lepidotus* is described for the first time, and additional larval characters are also noted. Diagnostic morphological characters for the adult, pupal, and larval stages of *An. pholidotus* are provided to separate the two species. All stages of *An. lepidotus* are easily separated from other currently known species in subgenus *Kerteszia* and a new key to the females of *An. (Kerteszia)* is given. Previously published distribution, bionomics, and medical significance data are corrected and enhanced.

Keywords

Anopheles lepidotus; *Anopheles pholidotus*; redescription; key; morphology; ITS2; *COI*; malaria

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Introduction

Zavortink (1973) provided a preliminary review of current knowledge of the morphological diagnoses for species in subgenus *Kerteszia* Theobald, of genus *Anopheles* Meigen. In that publication he treated 10 species, including two new species, *An. lepidotus* Zavortink, and *An. pholidotus* Zavortink, and one provisional (unnamed) species called “Species 10. Auyan-Tepui Mesa form.” Subsequently three species have been described, i.e., *An. rollai* Cova Garcia, Pulido, & Escalante de Ugueto (1976, 1977b), *An. gonzalezrinconesi* Cova Garcia, Pulido, & Escalante de Ugueto (1977a), and *An. auyantepuiensis* Harbach & Navarro (1996) for “Species 10. Auyan-Tepui Mesa form” of Zavortink (1973). Of the two species described by Zavortink, *An. lepidotus* was based on distinctive characters he noted on two larval exuviae from two reared males collected in Restrepo, Meta, Colombia in 1935, which were previously identified as *An. boliviensis* Theobald by Komp & Orsono-Mesa (1936). Zavortink designated a female collected in 1943 in Villavicencio, Meta, Colombia as the allotype of *An. lepidotus* and 31 female paratypes from various collection dates from Meta, Colombia. *Anopheles pholidotus* was described based on a male holotype, female allotype, and paratypes including a male, a female, larval and pupal exuviae, and whole larvae collected in Bocas del Toro, Panama. He considered *An. lepidotus* and *An. pholidotus* as closely related to *An. boliviensis* because of dense scale patches on the abdominal terga. In a key to the females, he used only the width of scales on the proximal abdominal terga to separate his two new species. However, in discussing these two species Zavortink (1973, p. 18) qualified their differences and stated “Although *lepidotus* closely resembles *pholidotus* or is possibly even indistinguishable from it in the adult and male genitalia, I am recognizing it as a distinct species on the basis of the larval differences indicated in the key and description.” Subsequent malaria workers in Peru, Ecuador, Colombia, and Venezuela who used this female key couplet (p. 7) to separate the two species found judging whether the proximal tergal scales were “narrow to moderately broad” versus “moderately broad to broad” difficult, thus making identifications of females inconclusive. Specimens from one of these studies (Quiñones *et al.* 1984) were sent to one of us (BAH), who determined in 1987 that the species they reported from Tolima Department, Colombia, were actually *An. pholidotus*, not *An. lepidotus*. To our knowledge this unpublished determination, evidenced by a note in the unit tray in the NMNH collection containing them, was only recently researched and confirmed by Escobar *et al.* (2010).

In 1985–86, Hayes *et al.* (1987), working under the auspices of a National Research Council grant through the BOSTID (Board on Science and Technology for International Development) program, Washington, DC, collaborated with personnel from the Instituto Nacional de Salud, Ministerio de Salud, Lima, Peru, and the Walter Reed Biosystematics Unit (WRBU), Smithsonian Institution, and conducted two malaria surveillance trips in Junín Province, Peru. During those trips, three females of *Anopheles* (*Kerteszia*) were collected that initially were considered to be a new species (BAH) because of several obvious morphological characters not previously described in subgenus *Kerteszia*. However, resolving the correct identity and describing the three specimens was not considered appropriate at that time without additional specimens, particularly specimens reared with associated larval and pupal exuviae. In 1998, larvae were collected (RCW) from arboreal

bromeliads in Ecuador and reared, producing a limited number of males and females with associated exuviae. Based on a larval character (seta 3-C stout) given in the description and key of Zavortink (1973), these specimens were given a tentative field identification as *An. lepidotus*. Later, one of us (BAH) noted that the females from Ecuador had morphological characters that matched those of the three females collected 12–13 years earlier in Peru. This initiated a thorough study of the unknown species from Peru and the putative *An. lepidotus* from Ecuador, including an examination of type specimens and other *Kerteszia* specimens deposited in the Smithsonian Institution. The results of this study are provided below.

Material and methods

Morphological

Terminology follows Harbach & Knight (1980, 1982). Costal and apical wing spot designations follow Wilkerson & Peyton (1990), which unlike Zavortink (1973), means that the apical fringe spots on *Kerteszia* species are not homologous with the apical pale (AP) spots on other *Anopheles*. Other terminology follows Peyton *et al.* (1992), who revised the position of seta 3-VI in *Kerteszia* pupae. Abbreviations for specimens follow: ♀, female; ♂, male; G, genitalia; Pe, pupal exuviae; Le, larval exuviae, and L, fourth-instar larva. The use of an asterisk by a stage abbreviation means the life stage is illustrated. We agree with Reinert (1990) in the use of “Pa” instead of “P” as the designation for the pupal paddle, which eliminates confusion with the “P” used for the larval prothorax. Larval and pupal chaetotaxy tables are not included because of limited specimens. Genus and subgenus abbreviations follow Reinert (2009). Pinned adults and whole adults in alcohol were examined with a Leica S8APO® dissection microscope and a cold fiber dual optics light source with a blue filter. We follow Tanaka *et al.* (1979), who recognized that all true palpomeres have scales, and Wood *et al.* (1979) who stated “The palpus is typically five-segmented, which is the standard number in the Nematocera.” Harbach & Kitching (1998) agreed with Tanaka *et al.* (1979) and stated that mosquitoes can have five palpomeres. Forattini (2002), however, claimed mosquitoes have four palpomeres, but our data (see below) support Tanaka *et al.* (1979), with *Anopheles* (*Kerteszia*) having five palpomeres. The few apical pale scales that normally occur on the tip of palpomere 5 of *Kerteszia* species (except *An. bambusicolus*) may be rubbed and missing. When this occurs on *An. lepidotus*, the apical pale scales on palpomeres 1–4 are still evident and clearly demonstrate the presence of five palpomeres. The specimens studied here are deposited in the National Museum of Natural History (NMNH = USNM), Smithsonian Institution, Washington, DC.

Molecular

DNA was extracted from five specimens of *Anopheles lepidotus* (EC166-102; EC151-5; EC168a; EC168b; EC151-1) and three *An. pholidotus* (VZ7-102, VZ11-4, VZ7-101), using the commercially available DNeasy Blood & Tissue Kit (QIAgen®, Maryland, USA). Amplification of rDNA Internal Transcriber Spacer 2 (ITS2) was carried out following the protocol of Li & Wilkerson (2005). Barcode fragments (658bp) of the mtDNA *Cytochrome Oxidase I (COI)* gene were amplified using the standardized Mosquito Barcoding Initiative (MBI) protocol as described in Ruiz *et al.* (2010); the shorter internal barcode fragment (472bp) was amplified following Linton *et al.* (2001).

Sequencing reactions were carried out in both directions using the Big Dye Terminator Kit® on an ABI 3730 automated sequencer (PE Applied BioSystems, Warrington, England). Sequences were edited using Sequencher™ v.4.8 (Genes Codes Corporation, Ann Arbor, MI). The ITS2 sequences were aligned using MAFFT v.6.814b (Kato *et al.*, 2002) within Geneious Pro 5.0.2 software, and the *COI* sequences were aligned and translated into amino acids in MacClade v.4.06 (Maddison & Maddison 2003). Sequence similarities were compared with those available in GenBank using Basic Local Alignment Search Tool—BLAST (<http://blast.ncbi.nlm.nih.gov>). Sequence divergence was calculated in MEGA v.4.0 (Kumar *et al.* 2008) using the Kimura 2-parameter distance model (K2P) (Kimura 1980). A bootstrap Neighbor-Joining (NJ) tree (Saitou & Nei 1987) was generated in PAUP v.4.0 (Swofford 2003), using 1000 replicates of the K2P distance matrices.

Results

Morphological

Currently, there are 12 recognized species of *An. (Kerteszia)*, and 10 of these were examined during this study. Specimens of *An. gonzalezrinconesi* and *An. rollai* were not available, thus we relied on the original descriptions and illustrations for those two species. All *Kerteszia* specimens deposited in the NMNH were examined regardless of species determinations, and no additional females were found that match the females of putative *An. lepidotus* collected in Peru and Ecuador. However, examination of the primary types of *Kerteszia* deposited in the NMNH revealed that the male holotype of *An. lepidotus* possesses the same unique habitus characters found on the Peru and Ecuador females (Table 1), as well as on the Ecuador males. Also, the larval exuviae of the Ecuador females and males possess the same distinct characters that Zavortink (1973) found on the larval exuviae of the holotype and single male paratype, and used as the basis for describing *An. lepidotus*. The many shared unique adult and larval characters among the Peru and Ecuador males, females, and larvae with the male and larval exuviae of the holotype of *An. lepidotus*, infers the specimens originally collected in Peru and Ecuador are *An. lepidotus*, and not a new species. As a consequence of this finding, 175 female specimens in the NMNH that Zavortink (1973) identified as *An. lepidotus* (including the allotype and 31 paratypes he designated as *An. lepidotus*) were assigned incorrectly and are not *An. lepidotus*. After examining the Colombian, Panamanian, and Venezuelan *An. pholidotus* specimens and finding considerable variation in wing spots and the width of abdominal scales, we are reassigning the females that Zavortink designated as *An. lepidotus* to *An. pholidotus*. At this point, since we do not have DNA from specimens of *An. pholidotus* from the type locality, we are not investigating the possibility introduced by Zavortink (1973) that *An. pholidotus* may represent more than one species. The pupa of *An. lepidotus* is described for the first time, and several diagnostic characters were found that will separate this stage of *An. lepidotus* from pupae of other *Kerteszia* species. Examination of larval and pupal exuviae of *An. lepidotus* from Ecuador not only confirmed the larval characters that Zavortink used to describe this species, but also revealed additional new diagnostic characters. Additional male genitalia characters were also found that will assist in the identification of *An. lepidotus*.

Anopheles lepidotus possesses many unique morphological characters on females and males and is probably the easiest species in subgenus *Kerteszia* to identify in the adult stage. Although *An. lepidotus* and *An. pholidotus* share several unique characters not found on other *Kerteszia* species, morphological characters on females, males, larvae, and pupae will also easily differentiate *An. lepidotus* from *An. pholidotus* (see below).

Since the descriptions of *An. lepidotus* and *An. pholidotus* Zavortink (1973), differentiating females of these two species has been impossible because the original females assigned to *An. lepidotus* were incorrectly identified *An. pholidotus*. In addition to the unique female characters of *An. lepidotus* (Table 1), diagnostic characters for *An. pholidotus* are presented (Table 2) that will solve the problem of identifying females and other life stages of these two species. To help alleviate any problems with the identification of the *An. (Kerteszia)* females, a key is presented below.

Key to the females of *Anopheles (Kerteszia)*

1	Mesepimeron with 1 long, large and curved (C-shaped) scale-patch that extends ventrally from upper setae	2
–	Mesepimeron with 1 or 2 small scale-patches	3
2(1)	Proboscis, pedicel, and palpomere 1 with white scales; hindtarsomeres 1 and 2 without apical pale band (from dorsal view) (Fig. 3)	<i>lepidotus</i>
–	Proboscis, pedicel, and palpomere 1 without white scales; hindtarsomeres 1 and 2 with narrow apical pale band (from dorsal view)	<i>pholidotus</i>
3(1)	Mesepimeron with 1 small scale-patch next to upper setae	4
–	Mesepimeron with 2 small scale-patches (upper and middle)	7
4(3)	Abdominal terga II–VII with numerous dark decumbent scales; sterna with few white scales	<i>boliviensis</i> , <i>gonzalezrinconesi</i> , <i>rollai</i> (currently cannot be separated)
–	Abdominal terga and sterna without scales (except possibly on VII, VIII and cerci)	5
5(4)	Hindtarsomere 5 entirely white-scaled; wing without pale fringe spot at tip of wing	<i>bambusicolus</i>
–	Hindtarsomere 5 with base dark, apical 0.35–0.60 pale; wing with large pale fringe spot at tip or rarely divided into 2 small pale fringe spots	6
6(5)	Scutum with white scales on acrostichal area from anterior promontory to near prescutellar setae; hindtarsomeres 2–4 with narrow white band on distal 0.15–0.5	<i>ayantepuiensis</i>
–	Scutum without white scales on acrostichal area; hindtarsomeres 2–4 with broad white band on distal 0.5–0.7	<i>neivai</i>
7(3)	Hindtarsomeres 2–4 with narrow apical pale band, 0.3 or less length of tarsomeres; hindtarsomere 5 usually entirely dark	<i>bellator</i>
–	Hindtarsomeres 2–5 with broad apical pale bands, 0.4–0.7 length of tarsomere	8
8(7)	Scutum with anterior 0.3–0.4 of acrostichal and dorsocentral areas and middle of scutellum with few white scales; vein M entirely or mostly white-scaled basal to level of Cu fork	<i>laneanus</i>
–	Scutum without pale scales on acrostichal, dorsocentral, and scutellum; vein M with dark scales basal to level of Cu fork	9
9(8)	Scales on palpomeres 3 and 4 predominately decumbent, those on base of 3 may be slightly erect	<i>cruzii</i> ^a

– Scales on palpomere 3 covered with slightly erect scales, palpomere 4 with slightly erect to decumbent scales *homunculus**

*This character is only useful if the specimens are in good condition. Other previously described characters are too variable because *An. cruzii* is a sibling species complex of 2 or 3 undescribed species (Ramirez & Dessen 2000a,b; Rona et al. 2009, 2010), as is *An. homunculus* (Sallum et al. 2009).

Molecular

The ITS2 sequence analysis showed the presence of two divergent sets of sequences; *An. lepidotus* (447 bp, $n = 5$) and *An. pholidotus* (496 bp, $n = 3$). DNA alignment showed 343 identical sites (66.5%), 89 gaps, 45 transversions, and 39 transitions (Fig. 1). The nucleotide frequencies (%) were T: 26.2 %, C: 26.6 %, A: 19.6 % and G: 27.6 %, and the mean of K2P genetic divergence was 0.228.

The *COI* barcode region (658 bp), and the shorter region (520 bp), showed seven different haplotypes for the samples analyzed ($n = 7$), with a mean intra-specific differentiation of 0.009 (*An. lepidotus*) and 0.003 (*An. pholidotus*), and a mean inter-specific divergence of 0.082 (range 0.076–0.087). Mean nucleotide frequencies were T: 39.0 %, C: 16.9 %, A: 28.7 % and G: 15.5 %. An analysis of nucleotide frequencies over all samples showed an average of 508 identical nucleotide pairs (174 first position; 177 second position; 157 third position), and 13 differences (ten transitions and three transversions). The NJ-K2P tree supported the presence of *An. lepidotus* and *An. pholidotus* as different lineages, with a 100% bootstrap value (Fig. 2). No stop codons were observed in the *COI* sequences, eliminating the presence of possible pseudogenes. All changes were synonymous except for the one at position 62 (G/A) [*An. pholidotus* (Valine) and *An. lepidotus* (Isoleucine), respectively].

COI sequences generated formed part of the activities of the Mosquito Barcoding Initiative (project leaders Yvonne-Marie Linton & Richard Wilkerson) and complete specimen records and *COI* sequence data, including electropherograms, are available through the Barcode of Life database website at <http://boldsystems.org/>. GenBank accessions for *COI* and ITS2 sequences generated in this study are in Table 3.

Taxonomy: *Anopheles lepidotus* Zavortink, 1973

Anopheles (Kerteszia) boliviensis Theobald, 1905, of Komp & Osorno-Mesa, 1936: 415 (♂*, L*); Komp, 1937: 500 (♂*, L); Lane, 1953: 279 (♂*, L*); Komp, 1956: 40 (♂, L, biology and distribution, in part); Stone, Knight & Starcke, 1959: 35 (♂, L, taxonomy, distribution, in part); Forattini, 1962: 448 (♂*, L); Aragão, 1964: 76 (biology and distribution, in part).

Anopheles (Kerteszia) lepidotus Zavortink 1973: 17 (♂*, ♀* [misidentification], L, key to females incorrect, biology and distribution, in part); Knight & Stone, 1977: 58 (only ♂, L, distribution, in part).

Overview

Because Komp & Orsono-Mesa (1936) assigned two males with larval skins to *An. boliviensis*, numerous publications before Zavortink (1973) were unknowingly addressing *An. lepidotus* instead of *An. boliviensis*. Numerous articles following Zavortink (1973) that addressed *An. lepidotus* were correct, in part, as the male, larva and genitalia are *An.*

lepidotus. However, post-1973 articles addressing *An. lepidotus* females and their structures were incorrect and should now be interpreted as *An. pholidotus*. Also, post-1973 published discussions of the biology, distribution, medical significance, and the phylogenetics of *An. lepidotus* are only partially correct. A good example of the above can be found in Gonzalez & Carrejo (2009) where, as in Zavortink (1973), the larval and male genitalia keys and descriptions correctly separate the two species, but the adult female key and associated characters in the text do not. As in other publications, the distribution records in this report need to be re-assessed. Country records are revised here, but within country provincial and local records for the two species need to be re-evaluated.

Male and female diagnostic characters. (Figs. 3, 4A; Table 1)

Maxillary palpus with apical pale scales on all 5 palpomeres; palpomere 1 with apical white scales; pedicel with dorsal and ventrolateral patches of small white spatulate scales; mid-portion of proboscis with large area of variable pale scaling on dorsum, laterally, and/or on venter; postspiracular area with small ventral patch of white scales (may be present or absent on males); wing normally with 7 pale fringe spots; M_{1+2} fork with white scales (may be 1–3 scales or obscure on males); hindtarsomeres 1 and 2 without apical pale scales on dorsum; terga I, III–VIII normally with white scales; female cerci with erect white scales; male gonocoxite with erect white scales to apex; accessory setae on gonocoxite of unequal length; internal seta of gonocoxite with long gradually attenuated tip.

FEMALE (Fig. 3, Table 1)—Head. Pedicel with dorsal and ventrolateral patches of small white spatulate scales; palpomeres 1–5 with white scales on distal half or apex, palpomere 1 with several apical white scales, palpomere 2 with apical 0.6 pale-scaled, distal half of palpomere 3 with long white area ventrolaterally and long dark area dorsomesally before white apex, palpomeres 4,5 with very small white-scaled area at apex; palpomeres 1,2 with erect black scales; proboscis dark-scaled on basal 0.1, with white or translucent scales dorsally, laterally and/or ventrally (variable) on median 0.5, dark-scaled on apical 0.3.

Thorax (Fig. 3). Scutal integument with 2 submedium and 2 lateral longitudinal dark lines; scutum with pale scales on acrostichal and dorsocentral rows on anterior 0.4; long erect setae on scutum pale except for stout dark setae at anterior ends of acrostichal and dorsocentral rows (rarely extending posteriorly) and dark setae over wing root and infrequently in prescutellar area; scutellum may have pale and dark setae, with several black scales on mid-region and infrequently laterally; antepronotum with long white scales, occasionally with small patch of black scales at mesal end; postpronotum with pale pruinose area posteroventrally; proepimeron and subspiracular areas with pale pruinose areas in line with that on postpronotum; postspiracular area with small ventral patch of white scales in line with anterior pruinose areas and white scales on upper mesokatepisternum; pruinose areas and connecting white scales form upper lateral white line on side of thorax from postpronotum to mesokatepisternum; lower lateral pale line on side of thorax incorporates pruinose areas on the metameron, lower mesepimeron, middle of mesokatepisternum, and propleuron; propleuron without scales, usually with 1 long seta; prespiracular area with setae and several white scales; mesepimeron with 1 long vertical “C-shaped” row of long white scales that extends downward from upper mesepimeral setae to middle of mesepimeron.

Wing. (Fig. 3). Wing with 5 pale spots on costa—a small humeral spot that starts at or just

distal to humeral crossvein and 4 large sector, accessory sector, subcostal, and preapical pale spots, preapical pale spot extends to apex of R_1 ; pale subcostal spot extends to R_{2+3} ; pale fringe spots at wing apex usually consist of two small spots located at tips of R_2 and R_3 , occasionally merged into one larger spot (combined R_2 and R_3 fringe spots); posterior fringe spots occur at tips of M_1 , M_2 , M_{3+4} , CuA, and 1A, usually increasing in size from M_1 to 1A; vein R_{4+5} with small pale spot at base, apex with dark fringe scales; base of vein M dark-scaled, pale-scaled at M_{1+2} fork; base of vein CuA usually dark-scaled before pale-scaled mcu crossvein; 1A dark-scaled to distal end, or rarely with 1–3 pale scales at tip. **Legs.** (Fig. 3). Fore- and midcoxae with white scales; foretarsomeres 4,5 normally dark-scaled, 4 occasionally with small dorsal pale patch; hindtarsomere 1 with small basal pale spot, small postbasal dark band, dark-scaled dorsally to apex, but pale-scaled ventrally from postbasal dark band to apical dark band; hindtarsomere 2 dark dorsally from base to apex, with long pale area on venter from near base to near apex; hindtarsomere 3 dark dorsally except for small apical pale band, pale ventrally from near base to apex; hindtarsomere 4 dark-scaled on basal 0.3–0.4 and pale-scaled to apex; hindtarsomere 5 dark-scaled; hindtarsomeres 1–3 dark from dorsal view except for small basal pale spot on Ta-III₁ and small apical pale spot on Ta-III₃; Ta-III₄ dark basally and pale apically, and Ta-III₅ dark; from ventral view Ta-III₁ is nearly all pale from near base to small dark tip, Ta-III₂ pale except for small dark areas at base and apex, Ta-III₃ pale from near base to apex; Ta-III₄ and Ta-III₅ as described for dorsal view. **Abdomen** (Fig. 3). Terga and sterna with long, erect, spatulate, white and brown scales; tergum I with several long erect white scales on apicomesal area, sternum I without scales, tergum II with apicomesal patch of brown partially erect scales, sternum II with curved mesal row of long erect white scales, terga III–VII with long erect white scales either laterally (III–V) or lateral and basal (VI–VII), terga III–V with mesal patch of brown erect scales beyond base (III) or extending from base to apex (IV, V), those on V forming basal and apical transverse rows of long erect scales, sterna III–V with postbasal row of erect white scales, tergum VI with small patch of brown basomesal erect scales and distinct apical row of brown erect scales, sternum VI with basal row of erect white scales and apical row of erect brown scales, tergum VII with basal row of erect white scales and apical row of mixed erect white and brown scales, sternum VII nearly covered with erect long brown scales and few white lateral scales, tergum and sternum of VIII covered with long erect white scales.

MALE—Males possess the same diagnostic characters (Table 1) as females and generally have a habitus matching the females. Occasionally characters on males may be less evident or absent, these include the posterior extension of white scales in the acrostichal row on the scutum, absence of a small ventral patch of scales on the postspiracular area, fewer (1–3) white scales on wing fork M_{1+2} , scales not so dense on wing veins, and very faint posterior fringe spots on the wing. Certain characters like the costal pale spots on the wing may appear larger on males. **Genitalia.** (Fig. 4A). Tergum VIII with median broad erect spatulate white scales; gonocoxite with erect white scales to apex; 2 accessory setae on gonocoxite of unequal length, longest seta flattened and broadened apically with a pointed tip, shortest seta with broad rounded tip; internal seta of gonocoxite with long gradually attenuated tip; aedeagus long slender without leaflets; ventral lobe of claspette with dense long spicules mesally near rounded bare apex, with shorter more scattered spicules basally and laterally,

lateral margin with broad narrowing and bluntly rounded lobe joining basal stem at emarginate angle; dorsal lobe composed of 2 stems, each with 3–4 sinuous flattened setae.

Pupal diagnostic characters (Fig. 4B,C)

Pinna exceptionally long, 0.41–0.55 (mean 0.46) trumpet length; seta 1-IX short, very thick, acutely pointed; lateral margin of paddle (Fig. 4C) exceptionally thick, usually sclerotized and pigmented on basal portion; paddle with midrib absent or weakly developed; paddle asymmetrical, with relatively straight apical margin at seta 1-Pa, and without long filamentous spicules on distal 0.33 of lateral margin.

Pupa

Integument light to medium brown, most pigmentation on trumpet and segments I–IV; setae very thin and weak, single or with few branches. **Cephalothorax.** Trumpet medium brown, angusticorn, without meatal cleft, with wide opening, pinna exceptionally long, 0.41–0.55 (mean 0.46) trumpet length (n = 12); seta 13-CT or alveolus present. **Abdomen.** Setae 0-VII, VIII long, frail, nearly as long as seta 1-VII; 1-II–VII short, frail, single or with few distal branches; 1-IX very short, thick, sharply pointed; 9-I very short, 9-II, III weakly developed, 9-III slightly longer than 9-II, 9-IV, V length ratio 0.19–0.50 (mean 0.33), 9-V, VI length ratio 0.38–0.61 (mean 0.52), 9-V–VIII well developed, spine-like, darkly pigmented, with long sharp dorsolateral aciculae; 10-VI present; 14-III absent. **Paddle.** Base with distinct darkly pigmented transverse line; paddle asymmetrical, lateral half considerably longer than mesal half, paddle index (L/W) 1.72–2.06 (mean 1.92), apical margin straight near seta 1-Pa on both lateral and mesal sides; lateral edge exceptionally thick, sclerotized and pigmented, extending to near tip; midrib absent or very weakly developed; spicules on lateral margin small, wide, and acute, beginning on basal 0.33, with larger and longer stout spicules on distal 0.33 approximately equal length of 1-Pa; lateral spicules in 2 rows, one directed dorsally and the other ventrally, except in most distal row of longer stout spicules; distal 0.33 of lateral margin of paddle without long sinuous or straight spicules that are much longer than 1-Pa; seta 1-Pa short, stout, sharp pointed, not filamentous, inserted mesal to tip of paddle; 2-Pa short, filamentous, usually inserted cephalad and some distance from 1-Pa.

Larval diagnostic characters (Fig. 4D,E)

Seta 3-C very thick, short, usually sharply pointed; 11-P very short; 1-I small with 4 or 5 simple branches; palmate setae (1-II–VI) only on 5 segments, moderately open, not brush-like; 2-IV exceptionally long, equal in thickness to 6-IV, usually simple, rarely with aciculae; 3-VIII with few branches near base; 6-VI stout, long, with median length aciculae on basal 0.33 and shorter aciculae more distal, without strong basal branches; 9-IV–VI nearly equal length of 7-IV–VI, with 2–5 branches; pecten spines from side view with single mesal row of stout aciculae extending out to tip.

Larva

Integument light brown on head, abdominal plates, and sclerotized structures on segments VIII and X. **Head.** Antennal seta 3-A as long as or only slightly longer than 2-A; 3-C very

thick, short, usually sharply pointed, infrequently tip split or with small aciculae; distance between both 2-C, measured between the outer adjacent margins of the two alveoli, narrow, not more than 2.5 times distance between outer adjacent alveoli margins of 2-C and 3-C on one side; 5-C simple; 11-C usually with 2–6 small distal branches. **Thorax.** Seta 11-P very short. **Abdomen.** Seta 1-I with 4–5 simple branches; palmate setae on five segments (II–VI); palmate setae moderately open and well formed, not brush-like; 1-VII short with 2 or 3 simple branches; 2-IV exceptionally long, stout, equal thickness to 6-IV, usually simple, rarely with aciculae; 3-VIII with 2 or 3 large branches on basal 0.33; 5-II–V plumose, with branches along main stem; 6-VI as stout and long as 6-V, with frail median length aciculae on basal 0.33, and short aciculae more distally, without strong branches near base; 9-IV–VI nearly equal length of 7-IV–VI, with 2–5 branches; 8-S present; pecten spines (18–23) of nearly equal length, from side view with single row of long stout aciculae; seta 1-X very long, attenuated, approximately 2 times saddle length; most anterior ventral brush seta (4a-X) short, usually <0.33 length of seta 4b-X; ventral brush (4-X) with 9 pairs of setae.

Egg

Unknown.

Specimens Examined (*Anopheles lepidotus*)

Seventy specimens were examined (19♀, 13♂, 14Le, 17Pe, 7G) from three countries as follows. COLOMBIA: Meta Department, Restrepo, *holotype* (specimen B, pinned), ♂, with slides of associated larval exuviae and genitalia, from leaf axil of bromeliad, XII-1935, Orsono-Mesa, in NMNH; *paratype* (specimen A mounted on slide), 1♂, with slides of associated larval exuviae and genitalia, from same locality and collection as *holotype*. ECUADOR: Napo Province, Yasuni National Park, Tiputini Biodiversity Station, EC104, -2, -3, 1♀, 2LePe, from bromeliad, 29-X-1998, R. Wilkerson; EC126(1), 1♀, biting human, 1-XI-1998, R. Wilkerson; EC151, -1 through -4, 4♂, 4LePe, 2G, from bromeliad, 4-XI-1998, R. Wilkerson; EC166, -101, -102, -2 through -4, -6, 3♀, 3♂, 3Le, 4Pe, 3G (2M in ETOH), from bromeliad, 7-XI-1998, R. Wilkerson; EC168, 5♀ (3 in ETOH), biting human, 7-XI-1998, R. Wilkerson; EC256, -100, -103, -105 through -109, -1 through -3, 6♀, 4♂, 3Le, 7Pe, from bromeliad, 26-V-1999, R. Wilkerson. PERU: Junín Province, Mission Cutivireni, PE349, 1♀, biting human in hut, 20-III-1985, Falcone and others; PE359, 1♀, biting human in canopy, 22-III-1985, Hayes, Harrison & Savage; Junín Province, Puerto Ocopa, PE346, 1♀, biting human in hut, 26-II-1986, Calderón & Hayes.

Bionomics

Anopheles lepidotus is a true “bromelicolus” species that occurs at relatively low elevations in remote or semi-remote tropical forests on the Amazonian slopes of the Andes in South America. It has been collected at elevations between 234 and 483 m, but not at elevations of 1,700 m like *An. auyantepuiensis* (Harbach & Navarro, 1996), or above 2,000 m like *An. boliviensis*, *An. gonzalezrinconesi*, *An. pholidotus*, and *An. rollai* (Navarro et al., 2010). Specimens were collected as larvae in arboreal bromeliads in Colombia and Ecuador and reared to adults with associated exuviae of the immature stages, and in human landing collections (HLC) in Ecuador and Peru at ground and canopy levels. To date, nine host-

seeking *An. lepidotus* females were captured (out of 19 known females) in human landing collections. Seven of those females were captured in the canopy on open platforms at 15 m height (Peru) and at 34 m height (Ecuador), whereas two females were captured in Peru at ground level inside unscreened houses. One of the last two specimens was collected engorged while feeding on a human. The three females collected (1 canopy, 2 at ground level) in Peru in 1985–86 came to humans in February and March shortly after dark (1800–1900 hr), although HLC continued at ground level throughout the night. The remaining six females captured by HLC were taken in November 1998 at 34 m in the canopy in Ecuador between 1800–1900 hr. These collections during the dry summer months differ from 1942–43 collections of *An. boliviensis* in Villavicencio, Colombia, which were most common during the wet season between April and October (Bates, 1945). However, Bates qualified those data by stating, “The capture data must be viewed with some suspicion because the species has predominantly late diurnal and crepuscular habits, and the captures were made at midday.” Larval collections from bromeliad axils (species unknown) at varying heights up to 34 m resulted in 11 males and 16 females that were reared in Ecuador during October and November 1998, plus the two males (holotype and paratype) collected during December 1935 from bromeliad axils at an unspecified height in Colombia. Larvae collected during the Ecuador trip survived well after collection, but development of the successive instars and stages was lengthy.

Species associated with *An. lepidotus* and captured by HLC between 1600–2000 hr during a canopy (15 m) collection (PE359) in Mission Cutivireni, Peru (12° S, 74° W) in 1985 were: *Aedes* sp.; *Ochlerotatus (Chrysoconops) fulvus* (Wiedemann) (as *Aedes (Och.) fulvus* (Wiedemann); see Reinert *et al.* (2008)); *Anopheles (Anopheles) fluminensis* Root; *An. (Nyssorhynchus) oswaldoi* (Peryassú); *An. (Nys.) rangeli* Gabaldón, Cova Garcia & Lopez; *Chagasia ablusa* Harbach; *Ch. bonnae* Root; *Haemagogus (Conopostegus) sp.*; *Hg. (Haemagogus) sp.*; *Psorophora (Janthinosoma) ferox* (von Humboldt); *Ps. (Grabhamia) dimidiata* Cerqueira; and *Sabethes (Sabethoides) chloropterus* (von Humboldt). Species associated with *An. lepidotus*, captured at ground level by HLC between 1800–0600 hr inside a house (PE349) in Mission Cutivireni, Peru in 1985 were: *Ochlerotatus fulvus* (as *Ae. fulvus*); *An. fluminensis*; *An. (Ano.) intermedius* (Peryassú); *An. (Nys.) nuneztovari* Gabaldón; *An. (Nys.) oswaldoi*; *An. (Nys.) rangeli*; *An. (Nys.) trinkae* Faran; *An. (Nys.) sp.*; *Ch. bonnae* Root; *Ps. (Gra.) cingulata* (Fabricius); *Ps. (Jan.) ?horrida* (Dyar & Knab); and *Ps. (Psorophora) lineata* (von Humboldt). Taxa associated with *An. lepidotus* in larval collections from bromeliad axils at 34 m height in Napo, Ecuador (0° 38' 17" S, 76° 08' 42" W) in 1998 were: EC104, *Wyeomyia* sp.; EC151 and EC166, *Culex* spp.

Distribution

Anopheles lepidotus is only known from four localities in three countries on the Amazonian slopes of the Andes. Those sites are Colombia (Meta, Restrepo, and Buena Vista), Ecuador (Napo, Yasuní National Park, Tiputini Biodiversity Station), and Peru (Junín, Mission Cutivireni, and Puerto Ocopa). The Ecuador and Peru collections represent the first confirmed records of this species in those two countries. The female previously identified as *An. lepidotus* from Cochabamba, Bolivia (Zavortink, 1973) is actually *An. pholidotus*.

Medical significance

Prior to this study, *An. lepidotus* was considered a fairly abundant species in areas of Colombia where malaria transmission occurred. Quiñones *et al.* (1984) proposed that *An. lepidotus* females were the primary *Kerteszia* species biting humans and the probable vector in an area of high malaria endemicity in Colombia. However, subsequent to that study specimens were sent to us for examination (courtesy of Dr. Suarez, SNEM, Colombia) and they were not *An. lepidotus*, but *An. pholidotus*. Reared specimens from the above 1984 study area and examinations of dissected male genitalia also confirmed that the correct species was *An. pholidotus*, not *An. lepidotus* (Escobar *et al.*, 2010). Since the holotype and paratype male of *An. lepidotus* were reared from larvae in 1935 in Meta, Colombia, there have been no other confirmed specimens of this species collected and preserved from the type locality or Colombia. During the last 28 years only an additional 30 specimens of *An. lepidotus* have been collected in three sites in Peru and Ecuador. Zavortink (1973) thought that *An. lepidotus* was the dominant and most important species in the Meta Department of Colombia, but we found that only two specimens (types) of *An. lepidotus* are known from Meta (and Colombia). Thus, the biological information and medical importance for the specimens previously identified as *An. boliviensis* and subsequently assigned to *An. lepidotus* by Zavortink actually apply to *An. pholidotus* or *An. boliviensis* (to a lesser extent). For all of the above reasons, we consider *An. lepidotus* an uncommon (or inaccessible in the forest canopy) or rare species, and unlikely to be involved in the transmission of human malaria parasites on a large scale. However, *An. lepidotus* was the only *Kerteszia* species collected biting humans in Ecuador and Peru, and it obviously has an affinity for human blood in the canopy and at ground level. This suggests it may become infected with malaria by feeding on primates in the canopy and then transmit the parasites to humans at a later time. Deane (1967, 1988) determined that in the State of Amazonas, Brazil, adjacent to Peru, *Plasmodium malariae* was the major simian malaria parasite, which occurred in 25 monkey species in Brazil. Sulzer *et al.* (1975) and Sulzer *et al.* (1978) conducted three malaria surveys in Mission Cutivireni, our 1985–86 collection site, and declared the locality a hyperendemic area for *Plasmodium vivax* and *P. malariae*, with 97.2% of the Ashaninka Amerindians infected in the initial studies. Hayes *et al.* (1987) conducted vector studies at Mission Cutivireni and determined that *An. trinkae*, a ground feeding species, was the primary malaria vector at the Mission and in Puerto Ocopa, but the *Plasmodium* species were not identified and no *Kerteszia* species dissected. Forattini (2002) and Collucci & Sallum (2003) discussed the role of several arboreal *Kerteszia* species in the transmission of malaria parasites, particularly *P. malariae* (= *P. brasilianum*) in primates and humans in Brazil and other areas of South America. Thus, humans living in or moving into areas like Mission Cutivireni on the eastern slopes of the Andes might be exposed to *P. malariae* in a simian/arboreal *Anopheles*/human cycle like those described above by Forattini (2002) in Brazil, or similar to the macaque/*Anopheles*/human *P. knowlesi* cycles now known in peninsular Malaysia, Malaysian Borneo, and other parts of southeast Asia (Vythilingam *et al.*, 2008; Tan *et al.*, 2008).

Morphological discussion

Adult

Based on the adult diagnostic characters (Table 1), *An. lepidotus* is morphologically unique among species in subgenus *Kerteszia*. Those characters are visible using a 60–80 × magnification dissection microscope, even when examining adults stored in alcohol. At least two characters appear unique in genus *Anopheles*, i.e., white scales on the apex of palpomere 1, and white scales on the ventral portion of the postspiracular area. Linthicum (1988) mentioned scattered pale scales on palpomeres 1 and 2 of species in the Argyritarsis Section of subgenus *Nyssorhynchus*, but not at the apex of palpomere 1. Although the wings and legs of *An. lepidotus* are dark compared to some other *Kerteszia* species, this species has striking tendencies toward leucism as it displays more white scales and setae in places where they do not occur on any other species in the subgenus. Examples of leucism are: (1) white apical scales on all five palpomeres, (2) white scales on the proboscis, (3) two patches of white scales on the pedicel, (4) white scales on the ventral portion of the postspiracular area, (5) white erect scales on the abdominal terga (mixed with dark scales) and sterna, (6) small white scales on the anterior third of the acrostichal row on the scutum, (7) white setae covering most of the scutum, (8) more white fringe spots on the wing, (9) white scales on the cerci, and (10) white scales on the gonocoxite. Some of these characters suggest evidence of a relationship with subgenus *Nyssorhynchus* (3, 5, 6, 7), whereas the others (2, 4, 7, 9, 10) suggest a relationship with subgenus *Cellia* (2, 7, 9) or are apomorphic (1, 4, 10).

A major goal of this study was to clearly differentiate *An. lepidotus* from *An. pholidotus*. In this regard, adults of *An. lepidotus* possess five secondary diagnostic characters that are shared with only one other species in subgenus *Kerteszia*. *Anopheles lepidotus* and *An. pholidotus* share four of those characters: (1) one long vertically curved, white scale-patch on the mesepimeron; (2) four or more palpomeres with white apical scales (*An. lepidotus* with white apical scales on palpomeres 1–5, *An. pholidotus* with white apical scales on palpomeres 2–5, whereas the remaining species do not have white apical scales proximal to palpomere 3); (3) transverse apical rows of erect dark spatulate scales on terga V–VII; and (4) wing with a pale fringe spot next to the tip of vein M₁ [Harbach & Navarro (1996) found this fringe spot uncommon on *An. auyantepuiensis*]. Based on the above four diagnostic characters shared by *An. lepidotus* and *An. pholidotus*, they likely share a common distant ancestor (see Molecular Discussion). Character (5), totally dark hindtarsomere 5, is shared with *An. bellator* Howard, Dyar, & Knab [Wilkerson & Peyton (1991) found one specimen of *An. cruzii* with hindtarsomere 5 entirely dark].

Both *An. lepidotus* and *An. pholidotus* possess small white scales along the anterior 0.4 of the acrostichal row, as do *An. auyantepuiensis*, *An. laneanus*, *An. gonzalezrinconesi*, and *An. rollai* (Harbach & Navarro, 1996; Sallum *et al.*, 2000). Thus, this character occurs on half of the known *An. (Kerteszia)* species and is not unique on *An. auyantepuiensis* as originally stated in Harbach & Navarro (1996). Also, this study determined that *An. gonzalezrinconesi*, and *An. rollai* are not morphologically similar to *An. lepidotus* and *An. pholidotus*, but are much more similar to *An. boliviensis*, provided one or both of the two former species are not synonymous with *An. boliviensis*. Without reared specimens of *An. gonzalezrinconesi*, *An.*

rollai, and the unknown males and unknown immature stages of *An. boliviensis*, more precise relationships of these three species cannot be determined. Komp & Orsono-Mesa (1936) described two males and their larval skins and identified them as *An. boliviensis*, which Zavortink (1973) subsequently used as the holotype and paratype for describing *An. lepidotus*. Thus, only the female of *An. boliviensis* is currently known. As noted above, the long vertically curved white scale-patch on the mesepimeron is found only on *An. lepidotus* and *An. pholidotus*. The other *Kerteszia* species, i.e., *An. bellator*, *An. cruzii*, *An. homunculus* Komp, and *An. laneanus* Correa & Cerqueira, have two small white patches of scales on the mesepimeron, one adjacent to the upper setae and a middle anterior patch, whereas *An. auyantepuiensis*, *An. bambusicolus* Komp, *An. boliviensis*, *An. gonzalezrinconesi*, *An. neivai* Howard, Dyar, & Knab, and *An. rollai* only have a single white scale-patch adjacent to the upper setae. The significance of these three different scale patterns on the mesepimeron cannot be overemphasized as they quickly (compared to variable hindtarsal and wing patterns) can be used to identify the three *Kerteszia* species assemblages mentioned above. The two unique patches of white scales on the pedicel of *An. lepidotus* are lacking on other species of *Kerteszia*. Also, two patches of scales on the pedicel are not found on species in the Albimanus or Argyritarsis Sections of subgenus *Nyssorhynchus*, which only have one patch of white scales on the pedicel (Faran, 1980; Linthicum, 1988). The presence of white scales on the proboscis, white scales at the M₁₊₂ fork, numerous spotting differences on the wing veins and pale fringe spots, variable hindtarsal color patterns, are all characters that occur in some species of other subgenera of *Anopheles*. However, the presence of white erect scales covering the cerci and gonocoxites is much more unusual in *Anopheles*.

Pupa

The pupa of *An. lepidotus* is described for the first time. Generally, the pupa is lightly pigmented with the trumpet light brown, and has setae that are very fragile, short, with few branches. The trumpet of this species appears unique in the subgenus because the average length of the pinna is nearly half the trumpet length (occasionally >0.5). All other described *Kerteszia* species, including *An. pholidotus*, have a pinna approximately 0.15–0.33 the length of the trumpet. However, Wilkerson & Peyton (1991) found the pinna was variable on *An. cruzii* Dyar & Knab, with some specimens having a longer pinna. This noted difference on *An. cruzii sensu lato*, may be of value in differentiating the provisional molecular sibling species of *An. cruzii* currently recognized in Brazil (Collucci & Sallum, 2003, Malafronte *et al.*, 2007, Rona *et al.* 2009, 2010). The presence of seta 1 immediately adjacent to the posterior margin of segment VII on *An. lepidotus* is similar to most *Kerteszia* species, except *An. pholidotus* which has 1-VII removed from the posterior margin. Seta 1-IX is very short, thick, sharply pointed on *An. lepidotus*, whereas it is longer, thin, and more visible on *An. pholidotus*. *Anopheles lepidotus* apparently has the largest paddle length/width ratio in the subgenus, i.e., $x = 1.92$, range 1.72–2.06. The midrib on the paddle is weakly developed (or absent), hence the development of a stronger, thick, sclerotized, and pigmented lateral margin needed for support. The two rows of small basal spicules on the lateral paddle margins become one row of more stouter, longer spicules on the apical 0.33, which are no longer than two times seta 1-Pa length. The longer spicules on the apical 0.33 do not extend to 1-Pa and are much shorter than the very long apical spicules occurring on

the paddle of *An. pholidotus*. Seta 1-Pa on *An. lepidotus* is short and thick, whereas 2-Pa is frail, thin, and not inserted adjacent to, but usually cephalad of, 1-Pa.

Larva

Zavortink (1973) used seven characters found on larval exuviae of the male holotype and one male paratype as the basis for describing *An. lepidotus*, i.e., seta 3-C short, thick, usually pointed; seta 11-P weakly developed and short; palmate setae moderately developed, not brush-like; seta 5-II-V plumose; seta 5-VII short; seta 9-IV-VI usually branched, but only near the base; and pecten spines with one row of spicules restricted to basal portion of the external edge. Examination of the Ecuadorean larval specimens confirmed the seven characters described by Zavortink (1973), and also provided additional valuable characters for identifying *An. lepidotus*. The new characters include setae 2,3-A approximately equal in length (compare *An. pholidotus*); seta 1-II-VI palmate (five pairs), shared with *An. pholidotus* and uncommon specimens of *An. bambusicolus*; seta 1-I with 4 or 5 simple branches (compare *An. pholidotus*); seta 1-VII short, with 2 or 3 simple branches; seta 2-IV exceptionally long, development equal to 6-IV, rarely with aciculae; seta 6-VI long, without strong basal branches, with frail aciculae; seta 8-S present; and pecten with one row of spicules on spines, usually pointed mesally (compare *An. pholidotus* with two rows of spicules per spine).

Specimens examined (*Anopheles pholidotus*)

A total of 304 life stages and structures of *An. pholidotus* in NMNH were examined (256♀, 9♂, 14Le, 17Pe, 6L, 2G) from Bolivia, Colombia, Costa Rica, Ecuador, and Venezuela during this study. PANAMA (Type series): Bocas del Toro, La Zorra, PA173-110, *holotype* ♂, LePeG, collected in axil of terrestrial bromeliad, 5-IV-1963, A. Quiñones. Bocas del Toro, Caldera-Chirique Trail, 1,000m, *allotype* ♀, biting human in upper canopy of deep forest, 31-X-1955, P. Orguela. *Paratypes* PA173-104, ♂LePeG; PA173-111, Le5L; PA176, 1L, 6-IV-1963, collected in axil of epiphytic bromeliad, with same data as *holotype* (except PA176); ♀ with same data as *allotype*. COLOMBIA (Specimens misidentified as *An. lepidotus* by Zavortink (1973): Meta, Cuchilla, E. of Villavicencio, *allotype* ♀, 16-III-1943; Meta, near Buena Vista, paratype ♀, 4-VI-1942, W.H.W Komp; Meta, Restrepo. *Paratypes* with following data – 4♀, VIII-1935, W.H.W Komp; 3♀, 1935, W.H.W Komp; ♀, 20-XI-1936, W.H.W Komp; 2♀, KO-121A-10, W.H.W Komp. Other misidentified specimens follow: Meta, Restrepo, Retiro, 7♀, 10-VIII-1935, W.H.W Komp; Tolima, Villarrica, 12♀, various 1982–83 dates and collectors; 174♀, J.A. Kerr. Additional correctly identified specimens from Colombia include: Tolima, Villarrica, 8♀, various 1981,82,86 dates and collectors; Meta, Restrepo, 4♀, 20-XI-1936; Cajete, Cauca, ♀, 23-VI-1991, M. Barreto; Tolima, Icononzo, 6♀, X-XII-1982, C.M.; Tolima, Villarrica, 7♀, X-XII-1982, C.M. BOLIVIA: Camata, 3♀, 15-IV-1949; Cochabamba, Yungus d. Palmar, ♀, 1,200m, (second label – 4133); Cochabamba, Chapare, ♀, 30-IV-1944, Torres M.; Chapare, Palmar, 2♀, 1-V-1944. ECUADOR: Yumaza, Morone Santiago, 5♀, VIII-1967, Duret. VENEZUELA: Merida, 2♀, 10-VI-1938, P. J. Anduze; Tachira, El Tama National Park, near Mata Mula, 7°38.04 N, 72°25.81W, 1,727m elevation, VZ7, 8♀, 7♂, 11Le, 15Pe, from axils of bromeliad (*Tillandsia fendleri*), 13-I-2001, Wilkerson & Navarro, WRBU Acc. No. 1721.

Molecular discussion

DNA sequence divergences and bootstrap support confirmed *An. lepidotus* and *An. pholidotus* as two different taxa. These results corroborate the clear separation of these two species using morphological characters. The mean *COI* divergence (8.2%) is more than two-fold the threshold reported by Hebert *et al.* (2003) for species delimitation (3%), reinforcing the use of barcode sequence for effective species delimitation. Sequence of rDNA ITS2 gives a similar result as can be seen on the NJ K2P tree (Fig. 2), which has 100% support. The presence of indels in ITS2, resulting in a 49 bp size difference, could even be used in a simple PCR, or PCR/RFLP diagnostic for species identification without sequencing. In addition, a BLAST search in GenBank (<http://blast.ncbi.nlm.nih.gov>) did not reveal homologous sequence to ours for either species, which confirms our results as the first record of DNA sequences for these species. Sequences for ITS2 are available in GenBank for some species belonging to the subgenus *Kerteszia* – (*An. homunculus*, *An. cruzii s.l.*, *An. laneanus*, *An. neivai*, and *An. bellator*) but these have less than 83% similarity with our sequences. *Anopheles bellator* and *An. cruzii* have been characterized at the *COI* locus by Sallum *et al.* (2002). However, the region used by them only partially overlaps at the 3'-terminal of the barcode region used here, for this reason no comparison can be made.

Conclusion

In summary, we, along with Escobar *et al.* (2010), have shown that the name, *An. lepidotus*, historically given (e.g. Quiñones *et al.*, 1984) to the primary malaria vector in the Department of Tolima, Colombia, and most likely throughout this part of Colombia, is incorrect and should instead be *An. pholidotus*. Also, an entirely new adult habitus is described for *An. lepidotus*, and new characters and a key to the subgenus *Kerteszia* are provided to distinguish it from *An. pholidotus*. Furthermore, this study has affirmed the benefits of combined morphological and molecular studies, continued field collections of new material, preserved morphological voucher specimens that can be sub-sampled for DNA analysis, examining morphological type specimens when working on complex taxonomic problems, and the need for adequate time to resolve complex species identification problems. This study also demonstrated the risks or potential risks taken by not making certain that a holotype, allotype, and paratypes are confirmed as con-specific, relying on identified museum specimens without confirming them, and relying on only one methodology or life stage, whether it be morphology or molecular genetics.

Dedication

This paper is dedicated to Dr. Jack Hayes (deceased), a co-principal investigator on the BOSTID grant. Jack helped organize both trips to Peru, was devoted to helping local indigenous people suffering from mosquito-borne diseases, was very friendly, compassionate, an excellent traveling companion, and a tireless worker on those trips.

Acknowledgments

We gratefully acknowledge the National Research Council, National Academy of Science, Washington, DC, for support with BOSTID Grant No. MVR-PE-4-84-35, the Walter Reed Biosystematics Unit (WRBU), National Museum of Natural History, Smithsonian Institution, Washington, DC, National Institutes of Health grant

2R01AI054139 to Jan Conn, the Instituto Nacional de Salud, Ministerio de Salud, Lima, Peru, the Naval Medical Research Institute (NAMRID), Lima Detachment, Lima, Peru, and to Kelly Swing and the staff at the Tiputini Biodiversity Station, Universidad San Francisco de Quito, who made collections of crucial specimens for this study possible. We are very grateful to LCDR Chris H. Gardiner, for local assistance in Lima and in Junín Province. The hard field and laboratory work of Roberto Falcone and Victor Zambrano in Peru was of great value to the study. Special thanks are also due Taina Litwak for the beautiful illustrations, Judith Stoffer for the photomicrographs and their preparation for publication. We also appreciate the helpful and constructive edits and comments provided by Ralph Harbach, Anice Sallum, and Yvonne-Marie Linton.

This research was performed under a Memorandum of Understanding between the Walter Reed Army Institute of Research and the Smithsonian Institution, with institutional support provided by both organizations. The material to be published reflects the views of the authors and should not be construed to represent those of the Department of the Army or the Department of Defense.

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An. pholidotus ATGCTTAAAT TTAGGGGGTA GTCACACATT ATTTGAGGCC CACTATAGAT TTCGTGTGTC 60
An. lepidotus .....A.A 60

An. pholidotus ATGTTGTGGT AACGCCGGCC GGTGCGTTCG CGCTCTCCAG CGATATGCGC GGTCTCTCTG 120
An. lepidotus .CTG..CTAA G..CG..AT. C.C.A.CCT. ...----- --...C..A. ..CGC.----- 120

An. pholidotus CCTGCTGTGT CTTCTCCTCT AACTCTCTAT CGTCAACAGC ACTCGCGAGC CACCGAACGC 180
An. lepidotus ----- - - - - - .ACT..C.. AT...CGGC. ....T..... 180

An. pholidotus GTAG---TTT CGTTCGATAT GGATTTGTTC TGCACCATCG TATCTCGTGT TGGTGAGAGT 240
An. lepidotus ..GAGCTG.. .....A.AGG ..T.C.----- - - - - - - - - - - - C .....A 240

An. pholidotus CGTAAGGTGG CTCAGCCGTG ACGGAAGACA CTCGCGAAAAG CGGACGCTCC GGT----- 300
An. lepidotus .....T..... .T....T..T T.G..ACTT. ..CG...AA. .C.CCCCGGT 300

An. pholidotus --CGGCCCTT CCTACGCACA CAGTCTTTCA ATGATTGACC AGCCGG---- ----GGCCGG 360
An. lepidotus CG..... .G..... .G..... GA....GCGG CGCC...T.A 360

An. pholidotus GCAACGCATC ATAATGCATC GGGAAGCGCA TGGCGCGTTC CCCGATAGTT GGCTATGCGG 420
An. lepidotus T.....T..... .CCGGAG.- -..AC.TCCG AA..... 420

An. pholidotus TGAGGATGGG CACTCAAAAA TGTGTACATC GCACTGTGTC GTGCGATGCG CAATATGCGT 480
An. lepidotus ..... 480

An. pholidotus TCAACGTATC GGTGTTTCATG TGTCCTGCAG TTCACA 516
An. lepidotus ..... 516

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FIGURE 1.

The ITS2 (rDNA) sequence alignments of *Anopheles (Kerteszia) pholidotus* (n = 3, Venezuela) and *An. lepidotus* (n = 5, Ecuador), using MAFFT (Katoh *et al.*, 2002). A total of 343 nucleotides were identical; 45 transversions, 39 transitions, and 89 gaps were observed. Underlined bases show the ITS2 primers.

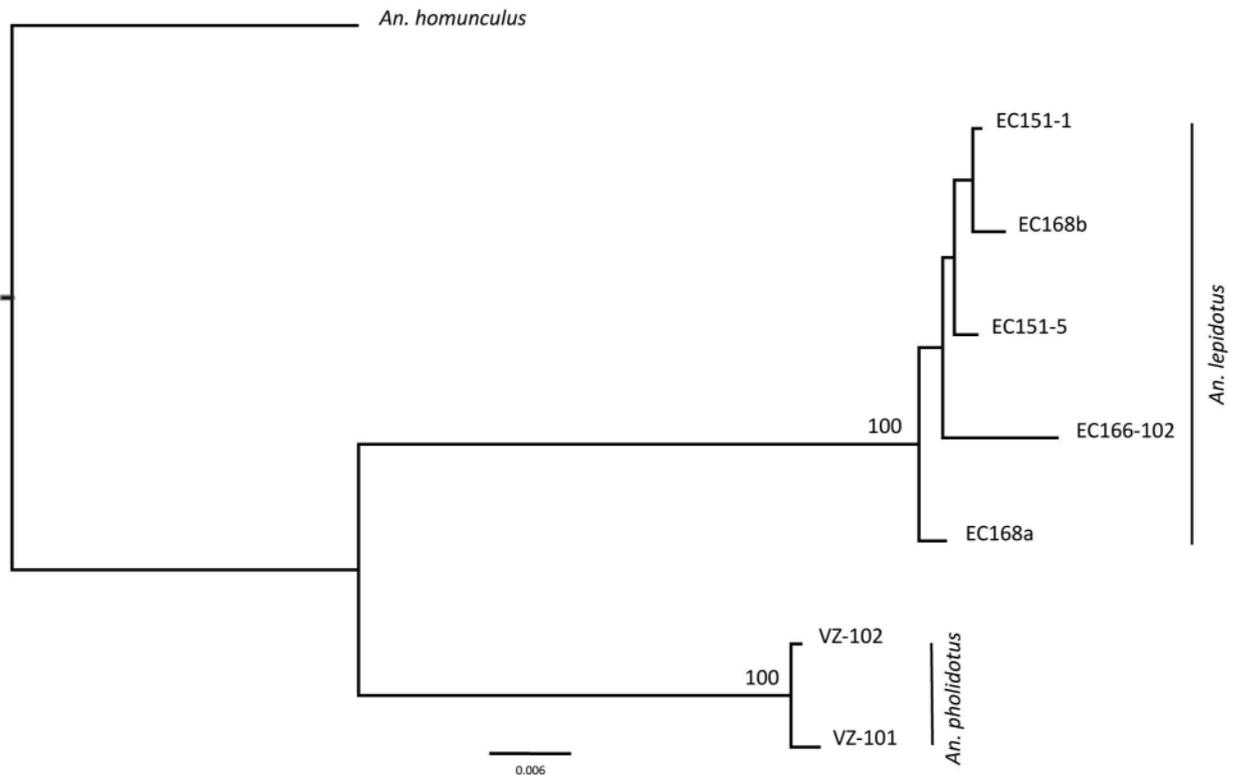


FIGURE 2. Bootstrap NJ-K2P tree of *COI* sequences belonging to *Anopheles (Kerteszia) lepidotus* and *An. pholidotus* from Ecuador (EC) and Venezuela (VZ). Bootstrap values below 70 % are not shown. Outgroup: *An. (Ker.) homunculus* Komp.

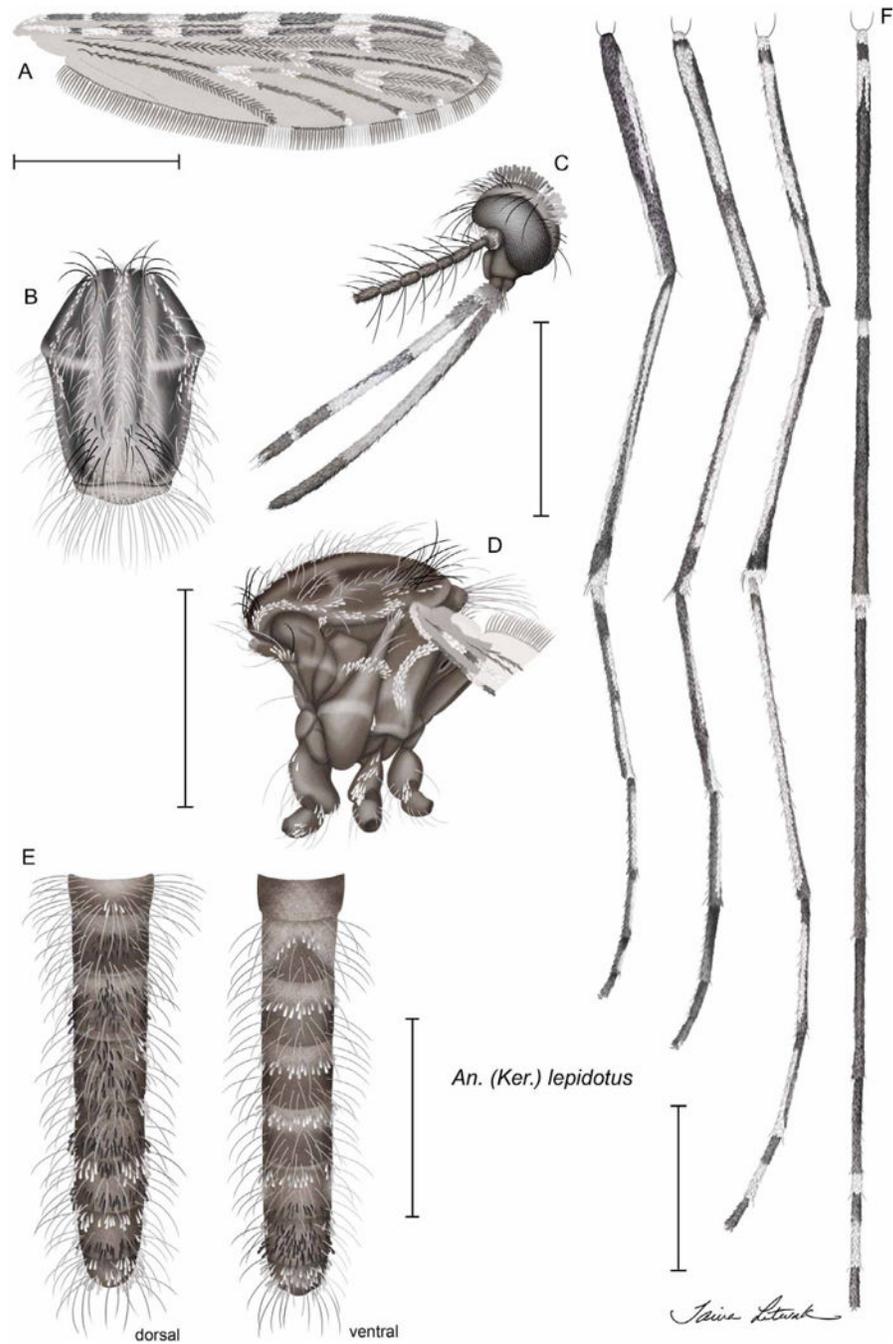


FIGURE 3.

Anopheles (Kerteszia) lepidotus Zavortink, female habitus: A, wing; B, thorax, dorsal view; C, head, lateral view; D, thorax, lateral view; E, abdomen, dorsal and ventral views; F, (left to right) foreleg, anterior view; midleg, anterior view; hindleg, anterior view; hindleg, dorsal view.

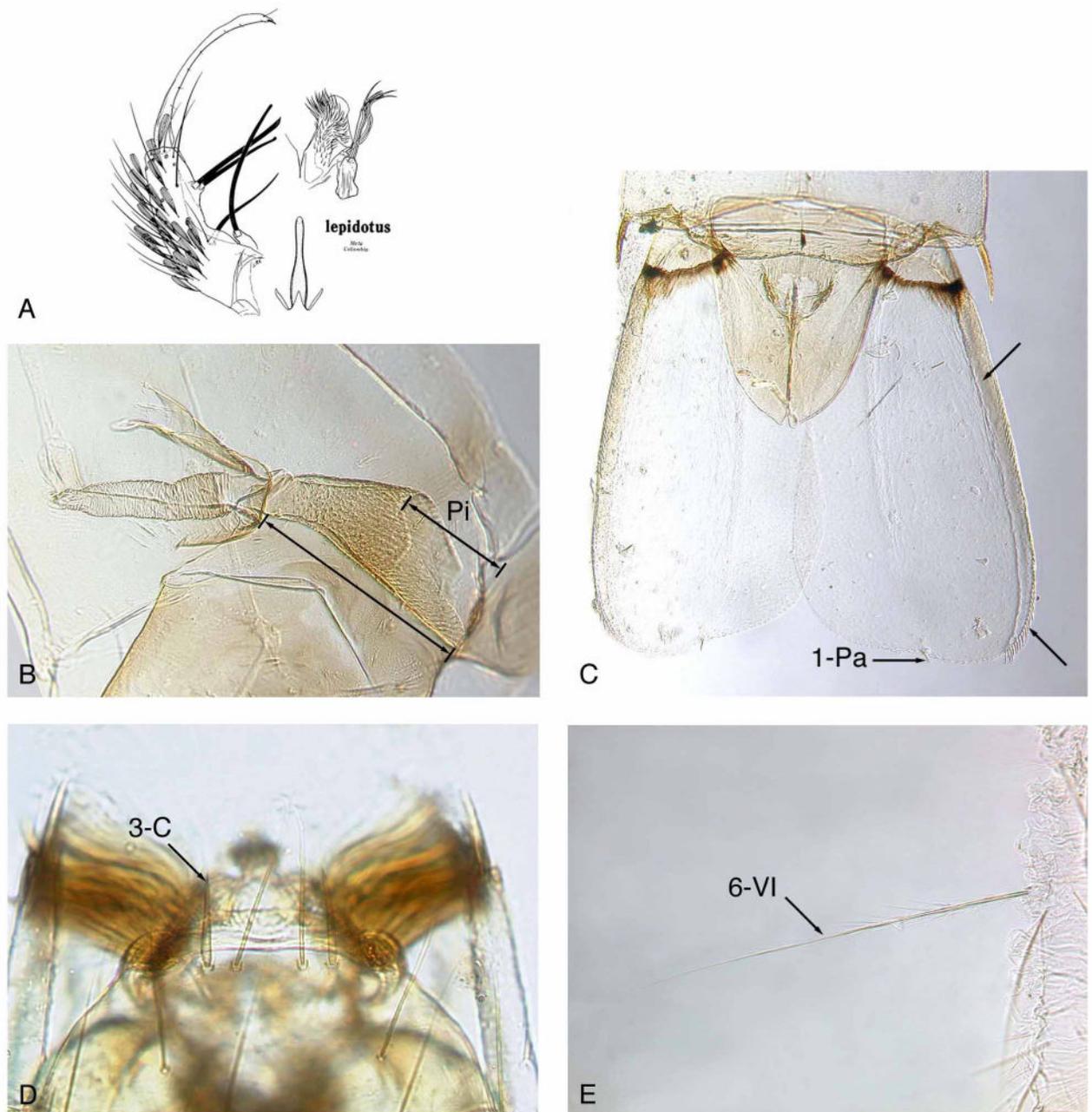


FIGURE 4. *Anopheles (Kerteszia) lepidotus*

A, male genitalia (from Zavortink, 1973); B, *An. lepidotus* pupal trumpet, pinna (Pi) long, about 0.5 trumpet length; C, pupal paddle showing lateral margin exceptionally thick, lateral margin without long filamentous spicules and relatively straight apical margin at 1-Pa; D, seta 3-C very thick and short; E, seta 6-VI stout, long, with median length aciculae on basal 0.33 and shorter aciculae more distal, without strong basal branches.

TABLE 1

Diagnostic external morphological characters differentiating adults (both sexes) of *An. lepidotus* from other *Anopheles* (*Kerteszia*) species.

Structure	Character
Head	Palpomere 1 with small patch of apical white scales
	Palpus with white apical scales on all 5 palpomeres
	Antennal pedicel with small dorsal and lateral patches of white scales
	Median portion of proboscis with white or translucent scales
Thorax	Ventral portion of postspiracular area with small patch of white scales
Wing	Vein M ₁₊₂ fork with small patch of white scales
	Wing with 7 pale fringe spots (R ₂ , R ₃ , M ₁ , M ₂ , M ₃₊₄ , CuA, and 1A)
Leg	Hindtarsomeres 1 and 2 without apical white bands from dorsal view
Genitalia	Cerci covered with erect white scales
	Gonocoxite covered with erect white scales

TABLE 2

Diagnostic characters differentiating *An. pholidotus* from other *Anopheles* (*Kerteszia*) species.

Stage	Character
Female	Palpomeres 2–5 with apical white scales (4 pale bands on palpi)
	Mesepimeron with one long ventrally projecting row of long white scales
	Vein M ₁ with pale fringe spot adjacent to tip
	Terga V–VII with distal transverse rows of long erect dark scales
	Hindtarsomeres 2 and 3 with narrow apical white bands from dorsal view
Male	Internal spine of gonocoxite with flattened tip
	Anterior lobe of ventral claspette broadly rounded, not bent downward
Pupa	Trumpet pinna short (<0.33 of trumpet length)
	Seta 1-VII inserted cephalad of segment posterior margin
	Seta 9-V approximately 0.5 length of 9-VI
	Apical 0.5 of lateral margin of paddle with spicules gradually increasing in length, most apical spicules very long and straight, not wavy
Larva	Seta 3-A long and tapered, much longer than 2-A
	Seta 3-C moderately long, slightly more stout than 2-C, with long attenuated tip
	Seta 11-P moderately developed, long
	Seta 1-I long, single
	Seta 1-II–VI with brush-like palmate setae (5 pairs)
	Seta 6-VI with several strong basal branches
	Seta 9-IV–VI single
Pecten with two rows of spicules on each spine	

Specimens from Venezuela and Ecuador sequenced with *COI* and ITS2 DNA markers with their locality data and GenBank accession numbers.

TABLE 3

Species	Country, locality	Coordinates	Date	Collection	GenBank ITS2	COI
<i>An. lepidotus</i>	Ecuador, Napo, Tiputini Biodiversity Station	-0.63806, -76.14500	4 Nov 1998	EC151-1	JN967763	JQ041286
				EC151-5	JN967764	JQ041283
			7 Nov 1998	EC166-102	JN967765	JQ041285
<i>An. pholidotus</i>	Venezuela, Táchira, El Tama National Park	7.63444, -72.43917		EC168a	JN967766	JQ041282
			13 Jan 2001	EC168b	JN967767	JQ041284
			14 Jan 2001	VZ7-102	JN967768	JQ041287
			14 Jan 2001	VZ7-101	JN967769	JQ041288
				VZ-11-4	JN967770	-