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Phylogeny and putative hybridization in the subtribe Paranepheliinae (Liabeae, Asteraceae), implications for classification, biogeography, and Andean orogeny

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Abstract The nuclear ribosomal ITS region and the chloroplast *trnL-trnF* (*trnLF*) intergenic region were sequenced for 45 accessions of *Paranephelius* and six accessions of *Pseudonoseris*, the two genera of the subtribe Paranepheliinae (Liabeae, Asteraceae) distributed in the alpine regions of the Andes. This data set was used to estimate relationships between these genera and within each genus to aid in evaluating morphological variation and classification. Our results with both ITS and *trnLF* markers support the monophyly of subtribe Paranepheliinae, and place *Pseudonoseris discolor* as the first diverged taxon sister to the clade containing *Paranephelius*. *Pseudonoseris szyszylowiczii* exhibited intraspecific divergence supporting intergeneric hybridization between *Pseudonoseris* and *Paranephelius*. Within *Paranephelius*, genetic divergence is low and not adequate to fully resolve phylogenetic relationships at the species level, but two genetically and morphologically recognizable groups were revealed by the ITS data. Several accessions possessing multiple ITS sequences represent putative hybrids between the two groups. These putative hybrids have caused some taxonomic confusion and difficulties in establishing species boundaries in *Paranephelius*. The divergence time estimates based on ITS sequences indicated that the stem of subtribe Paranepheliinae dates to 13 million years ago, but the diversification of the crown clade of the extant members began in the early Pleistocene or late Pliocene, perhaps associated with the uplift of the Andes and the climatic changes of global cooling.

Key words Andes, Asteraceae, biogeography, hybridization, ITS sequences, Liabeae, Paranepheliinae, Peru, speciation, *trnL-trnF*.

Hybrid speciation in plants and the potential insights gained from molecular studies on this mode of speciation have been reviewed recently (Hegarty & Hiscock, 2005). Hybridization in general has been considered an important process in evolutionary biology (Mallet, 2007). Most examples in nature are interspecific hybridizations, whereas intergeneric hybrids have only been rarely documented in Asteraceae with examples reported from a few tribes, e.g., Cichorieae (Fehrer et al., 2007) and Gnaphalieae (Smissen et al., 2007). Hybridization has not been reported in the Liabeae.

The Andean Cordillera forms a continuous, high elevation chain (+3000 m), largely unbroken for over 7500 km along the Pacific side of South America, from Venezuela to Tierra del Fuego. In general, the Andean alpine environments are wetter and more fragmented in the north, which is referred to as "páramo" distributed from Venezuela to northern Peru (Cuatrecasas, 1968; Luteyn, 1999). To the south, between central Peru and northwestern Argentina, the high-elevation communities are termed "puna" and are more xeric and continuous. In northern Peru, between the páramo and puna, transitional alpine communities above 3000 m are locally known as "jalca" formations (Weberbauer, 1936, 1945; Sánchez & Dillon, 2006), and are interpreted as drier than páramo but wetter than puna (Sánchez, 1976; Bazán-Zurita et al., 1998; Luteyn, 1999). This region is well known for its high levels of endemism and taxon diversity in plant and animal groups originated through the climatic changes and continuous uplift of the Andean Cordillera beginning in the late Tertiary (Simpson & Todzia, 1990). Above 1000 m in elevation throughout the Andean Cordillera, the Asteraceae are well represented with the estimated diversity of over 300 genera and nearly 3500 species (Funk et al., 2007). The diversity of Asteraceae increases above 3000 m, where it becomes the largest family of flowering plants with ca. 110 genera and over 950 species recorded between

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Venezuela and northern Peru (Dillon, 2005). The tribe Liabeae is entirely neotropical in its distribution from Mexico to South America and has its greatest generic and species diversity in northern Peru (Robinson, 1983; Funk et al., 1995, 1996). The tribe consists of three well-supported subtribes: Liabinae, Munnoziinae and Paranepheliinae (Kim et al., 2003). Among the three subtribes, only Paranepheliinae, consisting of two genera (*Paranephelius* Poepp. ex Endl. and *Pseudonoseris* H. Rob. & Brettell), is endemic to the alpine region of Andes.

The subtribe Paranepheliinae is distributed from northern Peru to northwestern Argentina (Dillon, 2005; Fig. 1). The Huancabamba Depression (HD in Fig. 1), a region implicated as a biogeographic barrier in many groups (Ayers, 1999; Weigend, 2002), represents the northern limit of its distribution. Department of Cajamarca, the region directly south of the Huancabamba Depression is one of the centers of biodiversity in the Andes. Many plant groups show high levels of species diversity in this region, e.g., Iochrominae (Solanaceae, Smith & Baum, 2006) and *Nasa* Weigend (Loasaceae, Weigend, 2002; Weigend et al., 2004). This region is geographically complex and its extreme species diversity is considered to be a result of isolation and adaptive radiation in the small fragmented ranges and habitats within this area (Young et al., 2002; Sánchez-Baracaldo, 2004; Sánchez & Dillon, 2006). The subtribe Paranepheliinae also has its center of diversity in this region.

Six of the seven named species of *Paranephelius* along with *Pseudonoseris striata* (Cuatrec.) H. Rob. & Brettell and *Ps. szyszylowiczii* (Hieron.) H. Rob. & Brettell are found in this region. Within Dept. Cajamarca reside the type localities for *P. ferreyrii* H. Rob. (near Cumbemayo) and *P. jelskii* (Hieron.) H. Rob. & Brettell (near Cutervo). *Paranephelius wurdackii* H. Rob. was described from the Calla Calla region in western Dept. Amazonas directly east of Dept. Cajamarca and the Río Marañón. The type localities for *P. uniflorus* Poepp. & Endl. and *P. ovatus* Wedd. are thought to be in central Peru, and the type locality of *P. bullatus* A. Gray ex Wedd. is likely in Dept. Junin, south of La Libertad, an area where José Pavón, the collector of the type, is known to have explored in the late 1780's. *Paranephelius ovatus* is a wide-ranging and morphologically variable taxon recorded from 13 departments from northern to southern Peru and extending into northern Bolivia and northern Argentina. *Paranephelius uniflorus* has been cited from northern to southern Peru and northern Bolivia; and *P. asperifolius* (Muschl.) H. Rob. & Brettell is described from Bolivia and is reported from northwestern Argentina.

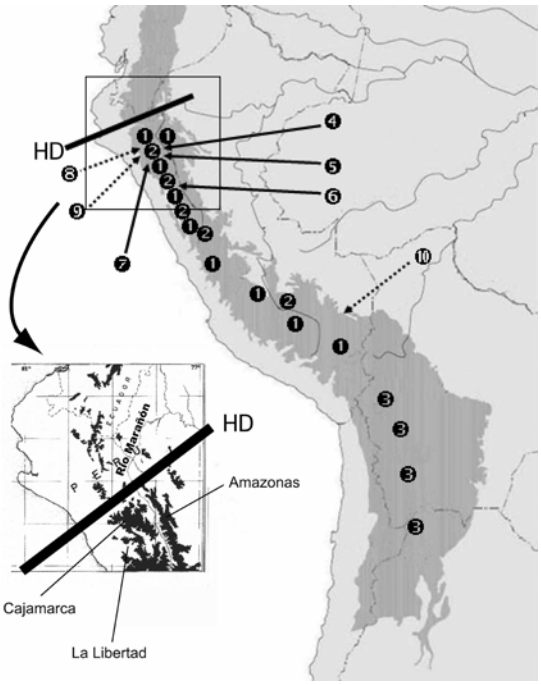

Fig. 1. Distribution map of *Paranephelius* (1–7) and *Pseudonoseris* (8–10). 1, *P. ovatus*; 2, *P. uniflorus*; 3, *P. asperifolius*; 4, *P. wurdackii*; 5, *P. ferreyrii*; 6, *P. bullatus*; 7, *P. jelskii*; 8, *Ps. striata*; 9, *Ps. szyszylowiczii*; 10, *Ps. discolor*. HD=Huancabamba depression.

Paranephelius is a distinctive genus possessing several apomorphic characters for the Liabeae (Funk et al., 1996) such as acaulescent habit with a basal rosette of leaves, showy capitulescences of one or a few, large, essentially sessile radiate capitulae, each capitula with 21 or more yellow ray florets (Fig. 2: A–O) with tomentum on the outer surfaces, and long tubular, 5-lobed disc florets. Though the genus has long been recognized as distinct, delineation of species has been difficult due to random and confusing suites of character variations, such as the variable combinations of leaf shape, texture, and pubescence (Fig. 3), and characters of the phyllaries. Since these characters have been used in the delineation of species, variation at the population level has made classification difficult.

Pseudonoseris is a genus of three species with narrow distributional ranges in the central Andes of Peru with one species, *Ps. discolor* (Muschl.) H. Rob. & Brettell, confined to lower elevations of the "ceja de la montaña" of Dept. Puno in southern Peru and the other two species, *Ps. striata* and *Ps. szyszylowiczii*, from the western versant of the Depts. Cajamarca and Lambayeque in northern Peru, a disjunction of over

Fig. 2. Morphological variation of *Paranephelius*. **A,** Sessile capitula with acute phyllaries (*ASA 17557*). **B,** Short peduncles arising from basal rosette (*ASA 17557*). **C,** Basal rosette with multiple capitula (*ASA 17557*). **D,** Basal leaves (*ASA 17575*). **E,** Sessile capitula with densely tomentose, acute phyllaries. **F,** Abaxial tomentose leaf surface (*ASA 17557*). **G,** Basal leaves (*ASA 17578*). **H,** Fleshy roots (*ASA 17578*). **I,** Habit with solitary capitulum (*ASA 17573*). **G,** Basal leaf (*ASA 17573*). **K,** Fleshy roots and stolons (*ASA 17573*). **L,** Sessile capitula with oblong or ovate phyllaries (*ASA 17573*). **M,** Individuals, several are products of stolons (*ASA 17573*). **N,** Capitulum of bullate-leaved population (*ASA 17572*). **O,** Basal leaves with adaxial (left) and abaxial (right) surfaces (*ASA 17572*).

1200 km (Fig. 1). It possesses short or rosulate leafy stems, scapose capitulescences with 2–4 pedunculate capitula (Fig. 4: A–G), stipitate glandular phyllaries, and 15–20, red to orange ray florets. *Pseudonoseris* has been suggested to be the closest relative of *Paranephelius*, differing from the latter in possessing latex, erect and branching capitulescences (Robinson, 1977) and a suite of pollen ultrastructural characters (Feuer & Dillon, 1982; Robinson & Marticorena, 1986). Because the subtribe Paranepheliinae is restricted to the Andean Cordillera from high-elevation or alpine environments to adjacent, lower-elevation environments on both slopes (e.g., ceja de la montaña), its evolutionary history may have been influenced by orogeny of the Andes. In this study, we examined the genetic variation and status of the subtribe Paranepheliinae using nuclear ribosomal ITS sequences and a chloroplast intergenic region. These data allowed for testing the current classification and morphological characters used in delineation of species against the observed genetic differentiation.

1 Material and methods

1.1 Plant materials

Forty-five accessions of *Paranephelius*, six accessions of *Pseudonoseris*, and five outgroup species representing genera within tribe Liabeae were sampled in this study. Collections were made in the field and additional accessions were derived from sampling herbarium collections at HAO, CPUN, F, MO, NY, and US. Populations of *Paranephelius* were field sampled in Departments of Amazonas, Cajamarca, and La Libertad (Appendix I), including the type localities of *P. ferreyrii* and *P. wurdackii* (Robinson, 1977). Sampling included populations along altitudinal gradients, and in areas of secondary sympatry where morphological variability was evident. Some sequences used in the analyses were retrieved from the data bank of DDBJ (DNA Data Bank of Japan) (Appendix II). For the accessions of *Pseudonoseris*, about 20 PCR products from a single individual were amplified by cloning and then sequenced. All new sequences generated in this study have been registered with DDBJ (DNA Data Bank of Japan, Appendix I).

1.2 DNA extraction and PCR amplification

Total DNA was extracted from dried leaves using a modified CTAB method of Doyle and Doyle (1987). The primers used for amplification were ITS1 and ITS4 (White et al., 1991) for the internal transcribed region of the nuclear ribosomal DNA (ITS), and *trnL* and *trnF* (Taberlet et al., 1991) for the intergenic region between *trnL* and *trnF* in the chloroplast DNA (designated as *trnLF* hereafter). Amplification reactions followed Wen and Zimmer (1996); 95 ℃ for 3 min, followed by 38 cycles at 94 ℃ for 20 s, 50 ℃ for 30 s, and 72 ℃ for 40 s, and 72 ℃ for 5 min. PCR products were sequenced in both directions by cycle-sequencing using the Big-Dye version 3 chemistry (Perkin-Elmer), with a Prism 3100 Genetic Analyzer (ABI).

Sequences were edited and aligned with Sequencher version 4.1 (Gene Codes), Clustal X version 1.81 and SeqPup/PPC version 0.6, followed by manual alignment.

1.3 Phylogenetic analyses

The ITS and *trnLF* sequence data were analyzed phylogenetically with PAUP* (version 4.0b10, Swofford, 2003), using maximum parsimony and neighbor-joining (Saitou & Nei, 1987) methods in which gaps were treated as missing data or as new characters. Maximum parsimony analyses were performed by heuristic searches with 1000 random sequence additions and tree bisection-reconnection (TBR) branch swapping. No character was weighted. The bootstrap support (BS) for the clades (Felsenstein, 1985) revealed in the maximally parsimonious tree(s) (MPTs) was examined with 1000 bootstrap replicates and the heuristic search options. Each data set was also analyzed under the Bayesian inference using MrBayes (Ronquist & Huelsenbeck, 2003) with the model estimated with Modeltest version 3.6 (Posada & Crandall, 1998; Posada & Buckley, 2004). Four simultaneous chains were run with trees sampled every 100 generations. The number of generations needed to reach stationarity was determined by plotting likelihood scores against generations.

1.4 Divergence time estimates

Because there are no fossils for Paranepheliinae to calibrate the molecular clock, a rate of ITS evolution of other plant lineages from the literature was used to estimate divergence times in Paranepheliinae. We used the nucleotide substitution rate of the Hawaiian silversword alliance (Baldwin & Sanderson, 1998), a group of Asteraceae known as a good example of insular adaptive radiation. The rate of the Hawaiian silversword alliance is considered to be equivalent with that of *Espeletia* Mutis ex Humb. & Bonpl., an Andean endemic genus of Asteraceae (Rauscher, 2002).

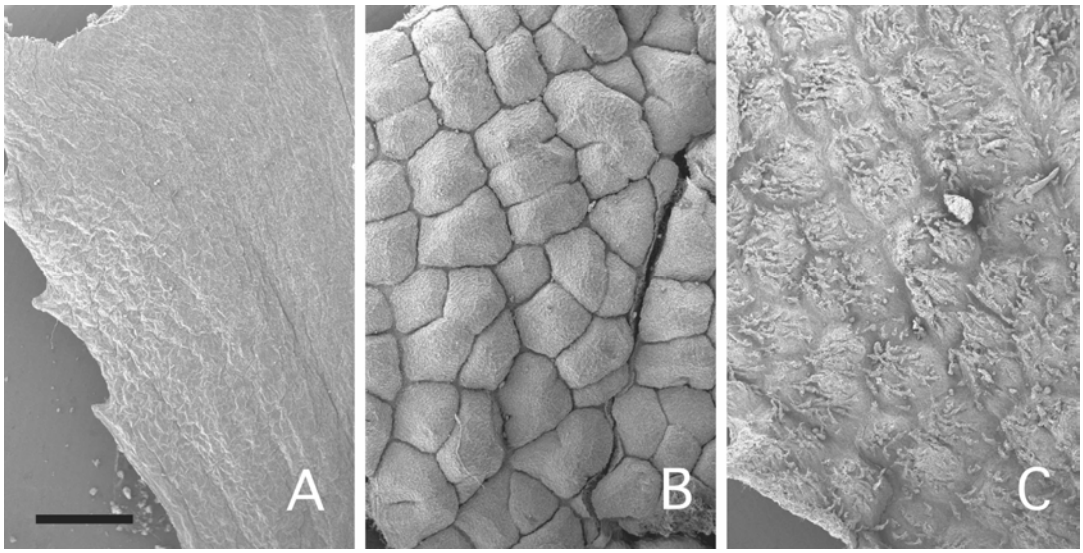

Fig. 3. Scanning electron micrographs (SEM) of upper leaf surfaces of *Paranephelius.* **A,** Smooth and hairless surface (ISV 11397). **B,** Strongly bullate and hairless surface (ISV 2778). **C,** Bullate and dense tomentose surface (ISV 11843). Bar=1 mm.

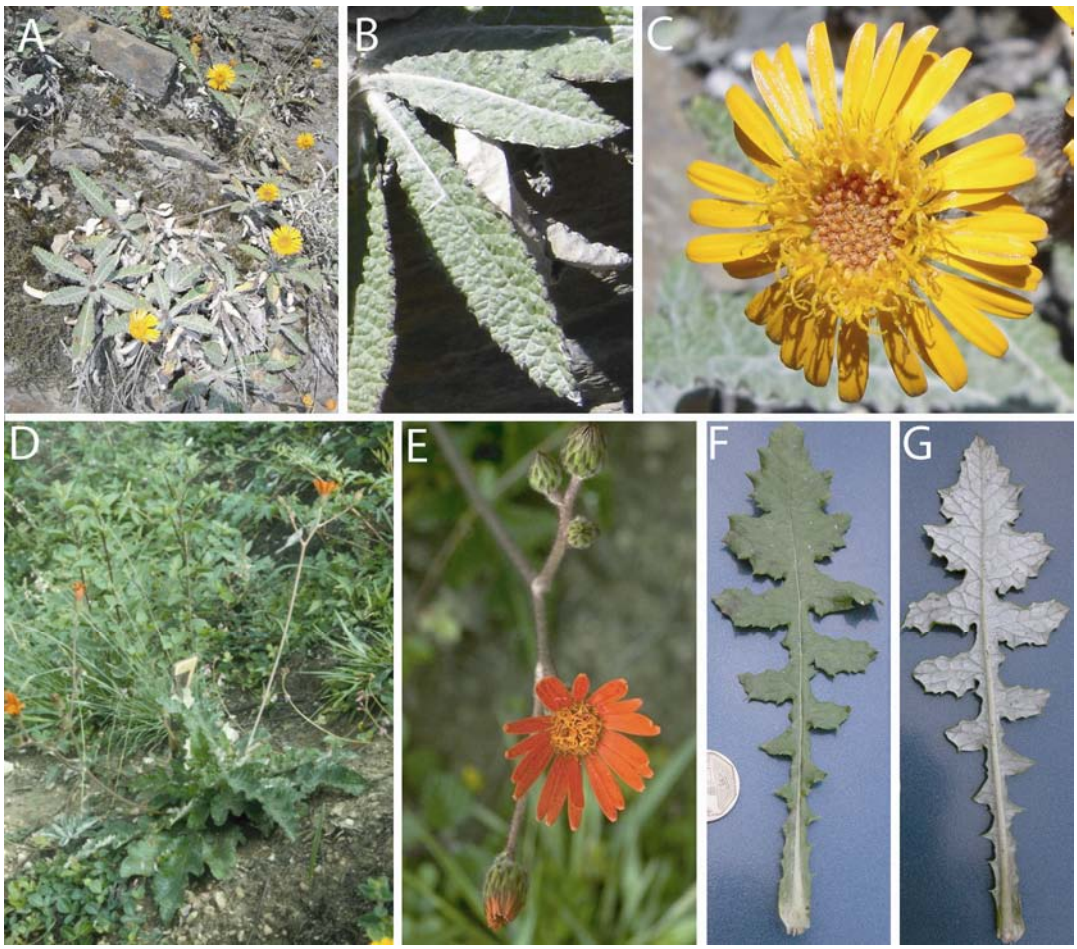

Fig. 4. *Pseudonoseris.* **A–C,** *P. discolor.* **A,** Plants growing in rocky soils in full sun (*Quipuscoa 3338*). **B,** Basal leaves with thin arachnoid tomentum (*Quipuscoa 3338*). **C,** Capitulum (*Quipuscoa 3338*). **D–G,** *P. szyszylowiczii.* **D,** Habit along disturbed roadside (*ASA 17548*). **E,** Capitulum (*ASA 17548*). **F,** Basal leaf, adaxial surface (*ASA 17548*). **G,** Basal leaf, abaxial surface (*ASA 17548*).

2 Results

2.1 Nuclear ribosomal ITS data

The lengths of the ribosomal DNA regions of the subtribe Paranepheliinae were 239 or 240 bp for the ITS1, 205 or 206 bp for the ITS2, and 165 bp for the 5.8S. Additionally, the 5′-end of the 26S region (26 bp) was also included in the analyses. The sequence boundaries between the two ITS regions and three coding regions (18S, 5.8S, and 26S) of rDNA were determined following Baldwin (1992). Three accessions (Kunkel 965, Wood 8345, and Wood 16192) could not be amplified with the primers for this region. There were 28 variable sites and two indels within the subtribe Paranepheliinae, 11 of which were phylogenetically informative within the subtribe (Fig. 5). After the alignment with the outgroups, the matrix had 705 nucleotide sites with 99 informative sites. Bayesian analysis of the ITS data resulted in stabilization of the likelihood scores at ca. 350,000 generations, and 875 trees were discarded as burn-in.

Pairwise distance (HKY85) among the sequences within *Paranephelius* ranged from 0.00 to 2.17% with the maximum between Zapata 06 and Funk 12088. Between *Paranephelius* and *Pseudonoseris*, the maximum distance was 3.33% (between ISV 2778 and Ps discolor 1i). Comparing Paranepheliinae and the other genera of tribe Liabeae, the pairwise distance ranged from 7.78% (between Ps discolor 2d and *Erato* DC.) to 21.29% (between ISV 2778 and *Chrysactinium* Wedd.).

On the ITS tree, *Ps. discolor* and *Paranephelius* formed a clade (BS=PP=100%), and *Ps. discolor* was sister to the clade of *Paranephelius* (BS=61%, PP<80%). Two groups can be roughly recognized in *Paranephelius*, designated here as Group A and Group B (Figs. 5, 6). These two groups diagnostically differed in four nucleotide substitutions (the sites 208, 492, 634, and 656). In addition to these diagnostic sites, there were sequence variations within each group. In Group A, two subgroups (A1 and A2) were recognizable, and at least seven subgroups were found in Group B (B1-7). In some accessions, direct sequencing detected multiple ITS sequences as single-site additive polymorphisms with the occurrence of overlapping double peaks on both complementary strands for these diagnostic sites. Eight accessions (ISV 8136, Dillon 2884, ISV 12100, Zapata 13A, Zapata 10, Zapata 12A, Dillon 2843, ASA 16384) were heterozygous on some of the four sites. These heterozygosity could be interpreted as combinations of any of the two haploid groups. In the phylogenetic analysis of *Paranephelius*, these eight accessions were excluded because of these polymorphisms.

Direct sequencing of the ITS regions of some accessions of *Pseudonoseris* yielded many double peaks. We thus sequenced about 20 PCR products of the ITS regions for each accession by cloning all accessions of *Pseudonoseris*. The NJ method was adopted to show similarities between closely related sequences. Figure 7 shows an NJ tree based on data from the direct sequencing of *Paranephelius* and the cloned sequences of *Pseudonoseris*. We found multiple ITS sequences in all accessions of *Pseudonoseris.* The sequences of the two accessions of *Ps. discolor* formed a clade sister to *Paranephelius*, while all sequences of the four accessions of *Ps. szyszylowiczii* were nested in the clade of *Paranephelius*.

2.2 Intergenic region between *trnL* and *trnF* (*trnLF*) of chloroplast DNA

Based on the ITS results, 19 *Paranephelius* and five *Pseudonoseris* accessions were selected to be sequenced for the *trnLF* region. Three taxa, *Chionopappus benthamii* S. F. Blake, *Munnozia annua* (Muschl.) H. Rob. & Brettell, and *Philoglossa purpureodisca* H. Rob., were used as outgroups. The lengths of the sequenced *trnLF* regions of the subtribe Paranepheliinae were either 869 bp or 863 bp. Bayesian analysis of the *trnLF* data resulted in stabilization of the likelihood scores at ca. 200,000 generations, and 500 trees were discarded as the burn-in. No node was supported with more than 80% posterior probability in the *trnLF* tree. *Paranephelius* and *Pseudonoseris* formed a clade with relatively low support (BS=68%, PP=80%). There were only two parsimony informative sites out of six variable sites within *Paranephelius*, and the relationships within *Paranephelius* was thus not well resolved (Fig. 8). However, *Ps. discolor* was sister to the clade of the remaining accessions in the strict consensus tree. It was noted that the four accessions of *Ps. szyszylowiczii* were nested in the clade of *Paranephelius*, although the bootstrap value of this clade was less than 50%.

2.3 Morphological variation

Species of *Paranephelius* (Robinson, 1977) have been differentiated based on a combination of characters including leaf shape, adaxial leaf surface morphology, and floral characters. The phyllary shape and pubescence are variable and unreliable for differentiating the two groups revealed in the ITS sequences. However, the overall leaf shape and lobing are rather uniform in Group A, which has pinnately dentate leaves (Fig. 9), generally ovate or oblong phyllaries (Fig. 2: L), and bullate adaxial leaf surfaces (Fig. 2: D,

Accession No.	Site position 208	492	634	656	92	293	532	645	659	617	222	
ASA16162	G	A	T	A	C	T	G	C	G	C	T	A1
ISV4052	G	A	T	A	C	T	G	C	G	C	T	A1
ISV5577	G	A	T	A	C	T	G	C	G	C	T	A1
Zapata04	G	A	T	A	C	T	G	C	G	C	T	A1
ISV10487	G	A	T	A	C	T	G	C	G	C	T	A1
Zapata07	G	A	T	A	C	T	G	C	G	C	T	A1
Zapata06	G	A	T	A	C	T	G	C	G	C	T	A1
Zapata01	G	A	T	A	C	T	G	C	G	C	T	A1
ASA17554	G	A	T	A	C	T	G	C	G	C	T	A1
ASA17557A	G	A	T	A	C	T	G	C	G	C	T	A1
ASA17563	G	A	T	A	C	T	G	C	G	C	T	A1
ISV11843	G	A	T	A	T	C	G	C	G	C	T	A2
ASA16455	G	A	T	A	T	C	G	C	G	C	T	A2
ASA17575A	G	A	T	A	T	C	G	C	G	C	T	A2
ASA17578A	G	A	T	A	T	C	G	C	G	C	T	A2
Dillon6472	A	T	C	G	T	C	T	T	A	T	A	B1
ISV10414	A	T	C	G	T	C	T	T	A	T	A	B1
ISV2778	A	T	C	G	T	C	T	T	A	T	T	B2
Dillon4600	A	T	C	G	T	C	T	T	A	T	T	B2
Wurdack1240	A	T	C	G	T	C	T	T	A	C	T	B3
ISV6904	A	T	C	G	T	C	T	C	G	C	T	B4
Zapata03	A	T	C	G	T	C	G	C	G	C	T	B5
Zapata05	A	T	C	G	T	C	G	C	G	C	T	B5
ISV10493	A	T	C	G	T	C	G	C	G	C	T	B5
Zapata02	A	T	C	G	T	C	G	C	G	C	T	B5
ASA17572A	A	T	C	G	T	C	G	C	G	C	T	B5
ASA17573A	A	T	C	G	T	C	G	C	G	C	T	B5
ASA17177	A	T	C	G	T	C	G	C	G	C	T	B5
ASA17340	A	T	C	G	T	C	G	C	G	C	T	B5
Funk11314	A	T	C	G	T	C	G	C	G	C	T	B5
ISV11397	A	T	C	G	T	C	G	C	G	C	C	B6
Funk12088	A	T	C	G	T	C	G	C	G	T	T	B7
ISV10335	A	T	C	G	T	C	T	C	A/G	C	T	B3xB4
Zapata11A	A	T	C	G	T	C	G	C/T	A/G	C	T	B3xB5
Zapata13A	A	T	C	A/G	T	N	G/T	C	A/G	C	T	(A1, A2)xB3
Zapata10	A	T	C	A/G	T	N	G/T	C	A/G	C	T	(A1, A2)xB3
ISV8136	A	A/T	C	A/G	T	C	G/T	C	A/G	C	T	(A1, A2)xB3
Zapata12A	A	A/T	C	A/G	T	C	G/T	C	G	C	T	(A1, A2)xB4
Dillon2884	A	A/T	C/T	A/G	C/T	C	G	C	G	C	T	A1xB5
ISV12100	A	A/T	C/T	A/G	C/T	C/T	G	C	G	C	T	A1xB5
ASA16384	G	A/T	C/T	A/G	T	C	G	C	G	C	T	A2xB5
Dillon2843	G	A	C/T	A	T	C	G	C	G	C	T	A2xB5

Fig. 5. The ITS informative sites among *Paranephelius*.

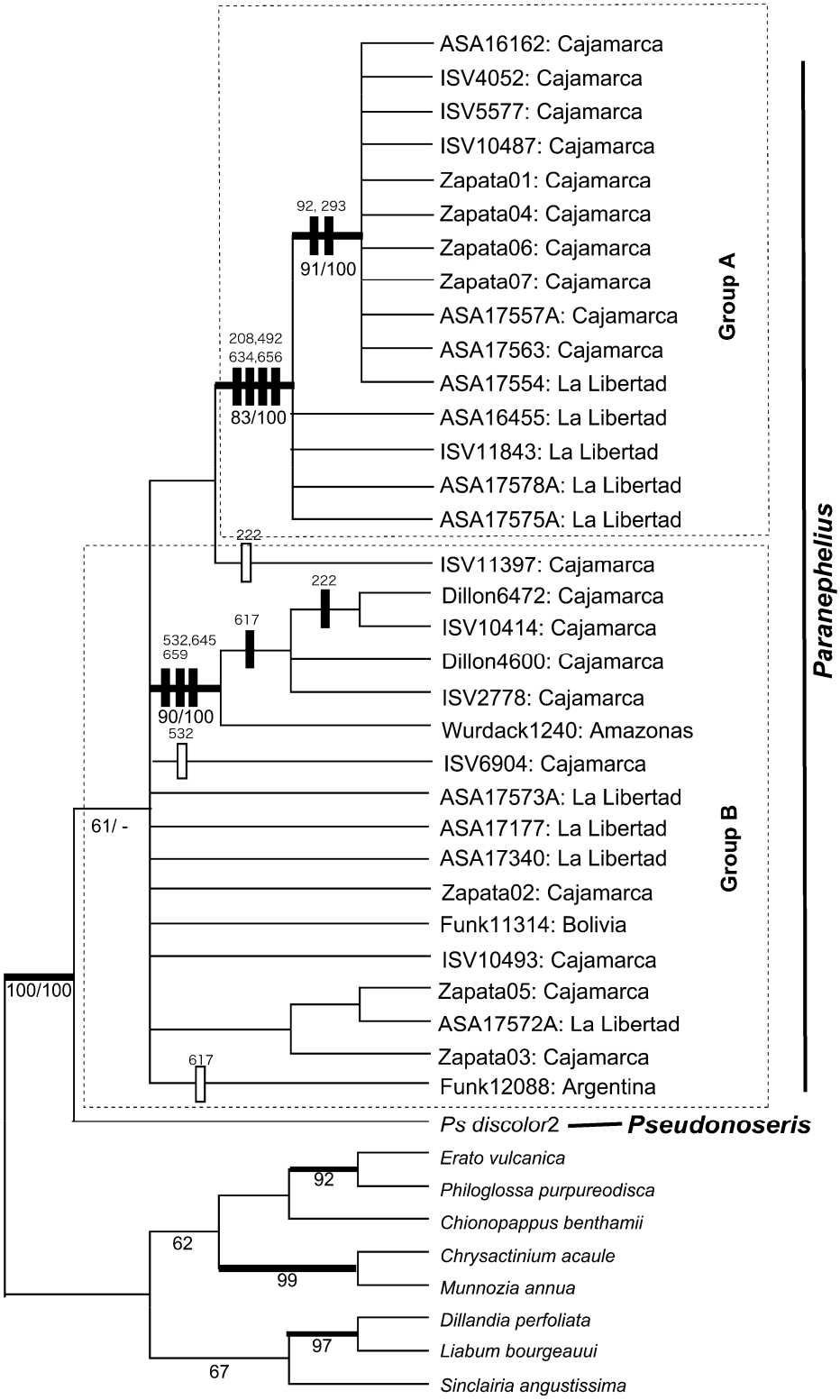

Fig. 6. The 50% majority-rule consensus tree derived from the maximum parsimony analysis of ITS sequences of Paranepheliinae (*CI*=0.760, *RI*=0.773). Bootstrap support (>50%) and Bayesian posterior probabilities (>80%) are shown below the branches (BS/PP), but PP is not shown in the outgroups. The clades which are not collapsed in the strict consensus tree are indicated with thickened branches. Black box designates a site change, white box indicates a site change presumed to be a parallel evolution, and the numbers above the boxes indicate the site positions designated in Fig. 5. The collection locality is cited by the name of the department for the accessions collected in Peru.

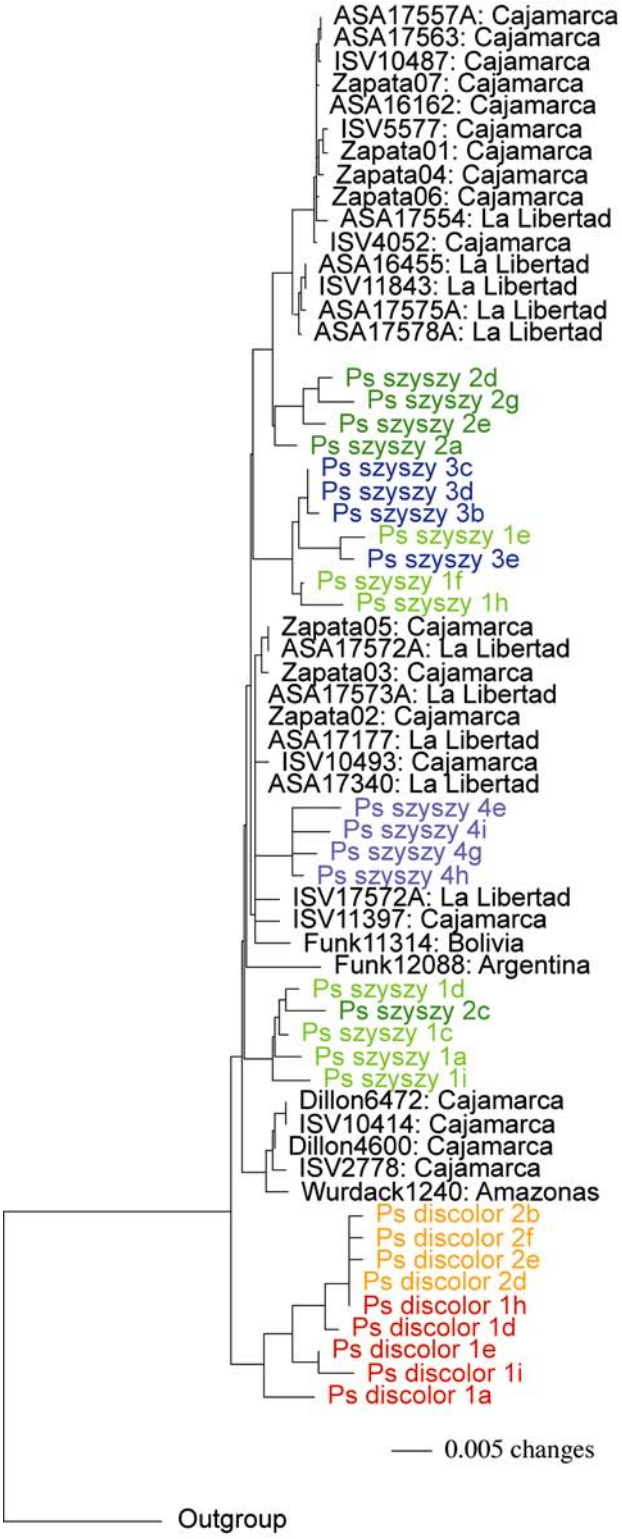

Fig. 7. The neighbor-joining tree based on the ITS data including cloned sequences of *Pseudonoseris*. The alphabets at the end of the names of *Pseudonoseris* indicate each of the cloned sequences.

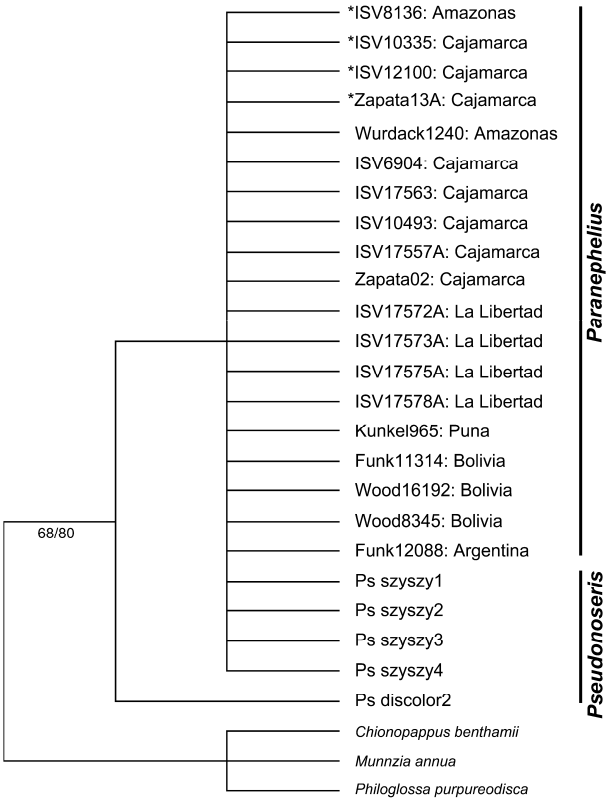

Fig. 8. The strict consensus tree derived from maximum parsimony analysis of *trnLF* sequences of Paranepheliinae (*CI*=1.000, *RI*=1.000). Bootstrap support (>50%) and Bayesian posterior probabilities (≥ 80%) are shown below the branches (BS/PP). The collection locality is cited by the name of the department for the accessions collected in Peru. Asterisks show hybrids presumed from ITS sequences.

F, G; Fig. 3: C) with or without dense pubescence. The leaves of Group B are variable from unlobed ovate (Fig. 2: J) to dentately-lobed elliptic in shape (Fig. 2: O), but predominately ovate (Fig. 10). Its adaxial leaf surfaces may range from smooth (Fig. 2: J) to bullate (Fig. 2: O; Fig. 3: B) and glabrous to pubescent (Fig. 3: A, C).

3 Discussion

3.1 Monophyly of the subtribe Paranepheliinae

Within the tribe Liabeae, *Paranephelius* and *Pseudonoseris* constitute subtribe Paranepheliinae and they share the specialized morphological characters such as acaulescent habits, rosulate or basally aggregated leaves, corolla tubes with long discs, and pollen ultrastructure of pseudocaveate tectum, i.e., not strictly caveate, but rather with thin basal columellae under the spines (Feuer & Dillon, 1982; Funk et al.,

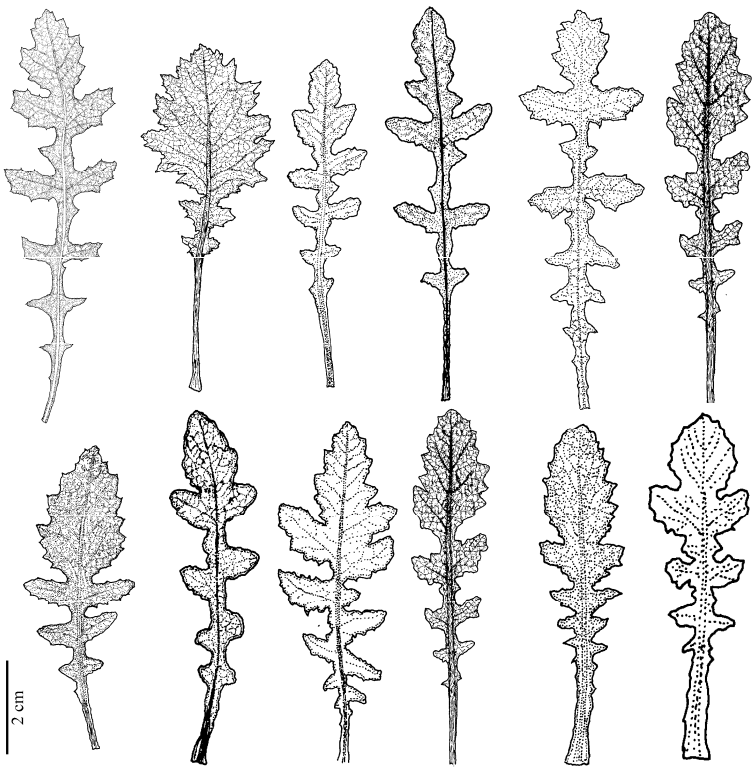

Fig. 9. Leaf shapes in Group A.

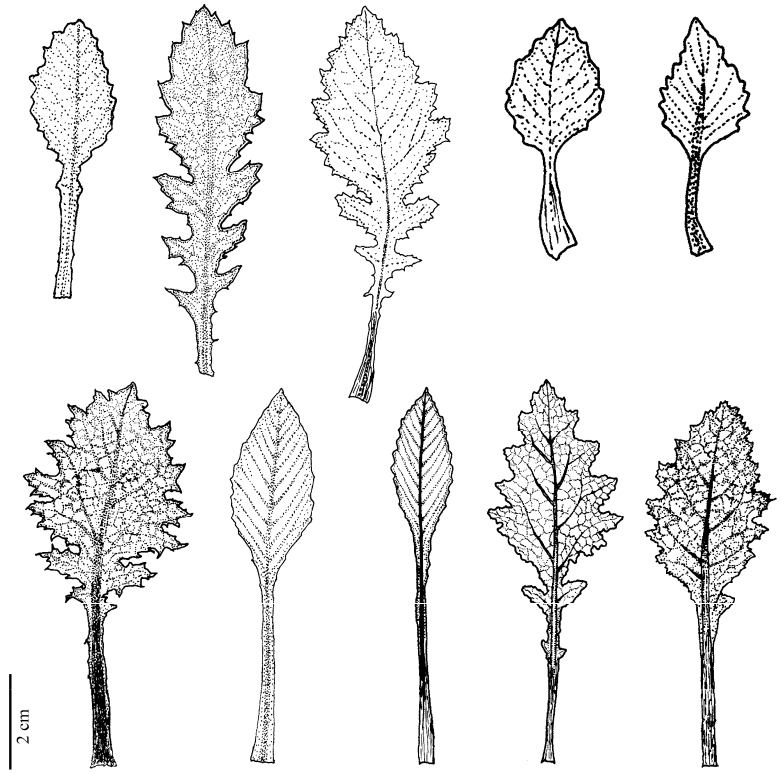

Fig. 10. Leaf shapes in Group B.

2007; Robinson & Marticorena, 1986). The monophyly of the subtribe Paranepheliinae was also supported by a cladistic analysis using morphological characters (Funk et al., 1996). In this study, molecular phylogenetic analyses of both ITS and *trnLF* sequences showed *Paranephelius* and *Pseudonoseris* form a well supported clade (BS=PP=100% in the ITS tree, BS=68%, PP=80% in the *trnLF* tree).

3.2 Intergeneric relationship between *Paranephelius* and *Pseudonoseris*

Although the phylogenetic resolution within the subtribe Paranepheliinae is relatively low, some of our results are consistent with the previous phylogenetic hypotheses based on morphology.

The majority of *Paranephelius* accessions used in this study are from northern Peru with a few from Bolivia and Argentina, representing the northern and southern ends of its distribution range. Although the species boundaries within *Paranephelius* are still not well defined, our samples cover the range of morphological variation within the genus. *Pseudonoseris* has three species, with *Ps. discolor* from Dept. Puno on the eastern versant and the other two from about 1250 km to the north on the western versant of the Andes (*Ps. striata* in Dept. Lambayeque and *Ps. szyszylowiczii* in Dept. Cajamarca). Four accessions of *Ps. szyszylowiczii* and two of *Ps. discolor* were included in this study.

Morphologically, *Pseudonoseris* is easily distinguished from *Paranephelius* by containing latex in its vegetative organs, erect branching inflorescences (Fig. 4), simple low-alveolate surface of the receptacle, and shortened outer series of the pappus. These distinctive characters of *Pseudonoseris* were considered to be a less specialized condition than those of *Paranephelius*, and the lower elevation of its distribution is also considered to indicate ancestral features of *Pseudonoseris* (Robinson, 1983; Funk et al., 1996). Our results showed that *Ps. discolor* is sister to the clade of *Paranephelius* in both the ITS and *trnLF* trees.

However, the positions of the *Ps. szyszylowiczii* accessions in the *trnLF* tree require further consideration. In the *trnLF* tree, although the bootstrap and posterior provability values are not high, all four *Ps. szyszylowiczii* accessions are nested in the clade of *Paranephelius*. This pattern may be the result of chloroplast capture due to hybridization between the two genera. Actually, the ITS sequences of these accessions of *Ps. szyszylowiczii* showed several double-peaked sites which indicated the existence of multiple sequences. Several (15–24) cloned rDNA fragments were thus sequenced for each accession of *Pseudonoseris*, and we found up to ten ITS sequences within an individual slightly different from each other. When all of them were included in the phylogenetic analysis, the trees obtained were quite messy. But some of them had 5.8S sequences with a few site changes or deletions compared to other 5.8S sequences common to *Paranephelius*. The analysis excluding those with different 5.8S yielded a tree shown in Fig. 7. Most of the sequences from an individual formed a clade except for those of Ps szyszy 1 and 2 which appear in separated clades. On the other hand, all cloned sequences of *Ps. discolor* formed a clade sister to the clade of *Paranephelius* (Fig. 7).

Because *Ps. discolor* is sister to the *Paranephelius* clade in both the ITS and the *trnLF* trees, it is likely that *Paranephelius* and *Pseudonoseris* separated before the divergence of *Paranephelius*. Concerning the positions of *Ps. szyszylowiczii*, two hypotheses are available. One is that *Ps. szyszylowiczii* originated within *Pseudonoseris*, and subsequently hybridized with *Paranephelius* as the maternal parent. Both the ITS and *trnLF* trees indicate that all four accessions used in this study are not pure *Ps. szyszylowiczii* but progenies of hybridization. The ITS sequences from the *Pseudonoseris* hybrids might be homogenized to some degree by concerted evolution. Another hypothesis is that *Ps. szyszylowiczii* itself originated via hybridization between *Pseudonoseris* and *Paranephelius*. The scattered positions of each accession of *Ps. szyszylowiczii* on the trees suggest multiple origins of these plants. The non-monophyly of the accessions Ps szyszy 1 and 2 in the ITS tree may even implicate backcrosses with *Paranephelius*. Nevertheless, both hypotheses assume multiple, complicated intergeneric hybridization. It is noted that *Pseudonoseris szyszylowiczii* is morphologically intermediate between *Ps. discolor* and *P. uniflorus*. Further studies are necessary to test the hypothesis of this intergeneric hybridization event.

Multiple ITS sequences can also arise via gene duplication through polyploidization. The only chromosome count available for *Pseudonoseris* is n=12 for *Ps. szyszylowiczii* (Dillon & Turner, 1982; Robinson et al., 1985), whereas *Paranephelius* has the counts of n=9, 14, and 29 (Robinson et al., 1985; Sundberg & Dillon, 1986). With the lack of chromosomal data of the plants we used in this study, we cannot further consider the possible effects of polyploidy.

3.3 Phylogeny and taxonomic implications in *Paranephelius*

The multiple ITS sequences detected in *Pseudonoseris* have made the phylogenetic analysis within *Paranephelius* using this marker difficult. We attempted another analysis using a data set excluding *Ps. szyszylowiczii* to infer the infrageneric relationships in *Paranephelius*. This analysis suggested two genetically differentiated groups, which can be recognized by four site changes in *Paranephelius* (Fig. 5). The two groups are designated as Group A and Group B (Figs. 6, 9, 10).

The clade of Group A is composed of accessions from northern Peru with strong support (BS=83%, PP=100%) and can be distinguished by the four site changes (Fig. 5). Group B includes the remaining accessions from Peru, Bolivia, and Argentina. Taxa of Group A and Group B are sympatric in Dept. Cajamarca, where the greatest diversity of this genus is detected (Fig. 1). The accessions of Group B are more diverse morphologically than those of Group A. The leaf characters such as shape, texture of surfaces, and hair density, as well as phyllary shape have been considered to be important in classifying *Paranephelius* (Robinson, 1977). The accessions of Group A have pinnately lobed leaves (Fig. 2: D, F, G; Fig. 9) and bullate adaxial surfaces with dense tomentose pubescence (Fig. 3: C). The accessions of Group B are more variable; but the leaves are usually ovate, with varying degrees of lobing (Fig. 10) and the adaxial surfaces are variable from smooth (Fig. 2: J; Fig. 3: A) to strongly bullate (Fig. 2: O; Fig. 3: B).

Paranephelius is one of the most easily recognizable genera in the high-elevation, alpine communities of the central Andes (2500–4500 m), but the species delimitations are notoriously difficult (Robinson, 1977). Authors have used a combination of characters to diagnose species but the variation detected within a population is often so considerable that it defies recognition of species boundaries. The morphological features of Group B show wide variation across their geographic ranges and correspond to the taxonomic descriptions of *P. ovatus*, *P. bullatus*, *P. jelskii*, and *P. wurdackii*. The molecular analysis suggests that *P. asperifolius*, a species with pinnately lobed leaves and distributed from Bolivia to northwestern Argentina is included in Group B (Funk12088, Funk11314). The descriptions of *P. uniflorus* and *P. ferreyrii*, which have pinnately lobed, bullate surface leaves and dense pubescence, correspond to Group A. Assigning names to herbarium material was not adopted in this study because a detailed taxonomic revision of *Paranephelius* is in preparation. The described species will be evaluated in light of the molecular results.

3.4 Evidence of hybridization in *Paranephelius*

Biparentally inherited ITS may be polymorphic due to hybridization. It is known that such polymorphism can be homogenized rapidly by concerted evolution (Graur & Li, 1999). However, there are some examples of additive heterozygosity in ITS, which could be regarded as results of recent hybridization between different ITS sequence types (e.g., *Krigia* Schreb., Kim & Jansen, 1994; *Miscanthus* Andersson, Hodkinson et al., 2002; *Callicarpa* L., Tsukaya et al., 2003; *Mitchella* L., Yokoyama et al., 2003; and *Cardamine* L., Lihová et al., 2004).

Group A and Group B are distinguished by four diagnostic sites (Fig. 5). Among the 45 accessions of *Paranephelius*, eight (ISV 8136, Dillon 2884, ISV 12100, Zapata 13A, Zapata 10, Zapata 12A, Dillon 2843, ASA 16384) are heterozygous for the diagnostic sites of the two groups. Direct sequencing of the ITS region without cloning may not detect all heterozygous sites (Gravendeel et al., 2004). The homozygous state of certain sites such as site 208 does not necessarily argue against their hybrid origin, and the sequences of these eight accessions support that these accessions are hybrids between Group A and Group B. Furthermore, morphological characters also support this hypothesis of hybrid origins. The division of the leaves is the most discernable character between Group A and Group B; and the leaves of the eight accessions are intermediate between simple-unlobed and pinnately-lobed conditions. The accessions ISV 10335 from western Cajamarca (Prov. Chota) and Zapata 12A from Calla Calla, Dept. Amazonas in Group B showed heterozygosity at some sites (Fig. 5). Their multiple sequences implicate that hybridization between subgroups of Group B may also have occurred.

Both Group A and Group B are distributed sympatrically in northern Peru. For example, in Cumbemayo, Zapata 01 and Zapata 04 of Group A were collected in the same area as Zapata 02, Zapata 03, and Zapata 05 of Group B. Pollen exchange between such morphologically and genetically differentiated plants may not be difficult. Andean grasslands have been used as pastureland for an extended period of time, in addition to environmental disturbance such as road construction and large-scale mining. Such human activities might be followed by secondary contact between the once isolated species, and could have resulted in hybridization. No putative hybridization

has been found outside northern Peru.

3.5 Evolutionary history of Paranepheliinae in relation to Andean geologic history

The diversity of ITS sequences in Paranepheliinae is compared with that of other plants in previous reports. The maximum pairwise sequence difference within *Paranephelius* is 1.7% (11 sites of the 636 aligned characters) found between ASA 17554 and ISV 2778. In a previous study on the evolutionary rate of ITS sequences, Baldwin and Sanderson (1998) estimated the rate of diversification of the Hawaiian silverswords (Asteraceae: Heliantheae) using paleoclimatic and fossil data. According to their result, the maximum within-group divergence is about 4.5% for the group with the divergence with its most recent common ancestor estimated to be about 5 mya. Rauscher (2002) shows that the maximum diversity in the *Espeletia* complex, an Andean highland group of Asteraceae (Heliantheae), is about 3.9%. The *Espeletia* complex is distributed in the upper montane forest and the páramo of the northern Andes which are thought to have existed only for the last 2–4 million years. Therefore, Rauscher (2002) considers that the evolutionary rates of ITS sequences of the *Espeletia* complex and silversword are similar. Compared with these results, the diversity of *Paranephelius* is 1.7%, less than half of the other two reports. This level of divergence may suggest that modern *Paranephelius* may have diverged much later, perhaps in the early Pleistocene or possibly the late Pliocene. Outside the Asteraceae, the intraspecific genetic divergence of *Saxifraga oppositifolia* L. is comparable to that of *Paranephelius*. Holderegger and Abbott (2003) estimated the genetic divergence of *S. oppositifolia* as 1.5%. Although they did not determine the divergence time, the two major lineages of *S. oppositifolia* are thought to have become isolated during the Pleistocene. Our result estimated the divergence time of Paranepheliinae as in the early Pleistocene is consistent with that of the circumpolar species of *Saxifraga* L.

The subtribe Paranepheliinae is morphologically quite distinctive in the Liabeae (Feuer & Dillon, 1982; Robinson & Marticorena, 1986; Funk et al., 1996). The present molecular analyses confirm the monophyly of Paranepheliinae, but do not resolve its close sister group. *Microliabum* Cabrera, a small genus in Argentina, is presumed to be the sister to the subtribe (V. Funk, pers. comm.), however, the sequences of *Microliabum* were not available to test this hypothesis.

Although the low genetic diversity of Paranepheliinae found in this study suggests a relatively recent radiation, the morphological distinctiveness of Paranepheliinae within the tribe Liabeae seems to indicate its ancient origin. In comparison with the other two subtribes in the Liabeae (Liabinae, 9 genera and ca. 90 spp.; and Munnoziinae, 4 genera and ca. 50 spp.), the taxonomic diversity of Paranepheliinae is relatively small. It seems that the ancestor of Paranepheliinae originated in the alpine region of the Andes and has remained poorly diversified for a long time unless extinctions may have eliminated many members of the group. The average of pairwise divergence of ITS sequences between Paranepheliinae and the outgroup is 11.8%. Using the nucleotide substitution rate in the silverswords, the origin of Paranepheliinae is estimated to be about 13 mya during the middle Miocene. During this period, the climate was deteriorating and the central Andes were actively uplifting. The distribution of Paranepheliinae is from Peru through Bolivia and into northwestern Argentina, and most diverse in the Department of Cajamarca, Peru, near the northern limit of their distribution. The Department of Cajamarca, the region directly south of the Huancabamba Depression is one of the centers of biodiversity in the Andes (Young et al., 2002; Sánchez-Baracaldo, 2004; Sánchez & Dillon, 2006). The Andes is older in the south, and younger in the north. The ancestor of Paranepheliinae may have originated in the southern mountainous area of the Andes. The uplift of the Andes accompanied by climatic changes of getting drier and colder may have led to the expansion of distribution toward the north. In general, the Andean alpine environments are wetter in the north (páramo) and more xeric in the south (puna). The expansion perhaps stopped at the Huancabamba Depression (HD in Fig. 1), a major biogeographic barrier for many plants (Ayers, 1999; Weigend, 2002). The wet and fragmented environment there may have stimulated the adaptive radiation and diversification of Paranepheliinae.

For the alpine region of the central Andes, Weigend et al. (2004) also showed little genetic differentiation in *Nasa* ser. *Grandiflorae* (Loasaceae). In their analysis of the *trnL* intron, the seven species of the series *Grandiflorae* constituted a clade, but most of them formed a polytomy within the clade. Their results along with our observations on Paranepheliinae seem to support a model of recent speciation and geographic differentiation in the central Andes.

4 Conclusions

1. *Paranephelius* and *Pseudonoseris* comprise a monophyletic group endemic to the central Andean Cordillera, and *Pseudonoseris discolor* is sister to *Paranephelius*.

2. Complicated intergeneric hybridization between *Paranephelius* and *Pseudonoseris* may explain that *Ps. szyszylowiczii* is nested within the *Paranephelius* clade.

3. Two genetically distinguishable groups were recognized in *Paranephelius*. Multiple ITS sequences suggest that hybridization in *Paranephelius* has occurred in northern Peru and may be responsible for the taxonomic confusion and difficulties.

4. The low genetic diversity in *Paranephelius* (1.7%) suggests their recent radiation and speciation in the high Andes.

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Appendix I Classification and voucher data on geographic distribution and origin of voucher accessions of the ingroup, *Paranephelius*, *Pseudonoseris*, hybrids, and outgroups. The application of species names follows the discussion within the manuscript; no formal synonymy changes are implied. Sequences were deposited in DDBJ (DNA Data Bank of Japan). Sagástegui=ASA, Sánchez=ISV.

Ingroup: ***Paranephelius***—Argentina. Prov. Salta, *Funk 12088* (US), AB355482 (ITS), AB355569 (*trnLF*). Bolivia. Prov. Chuquisaca, *Wood 8345* (US), AB355570 (*trnLF*); *Wood 16192* (US), AB355570 (*trnLF*). Prov. La Paz, *Funk 11314* (US), AB355483

(ITS), AB355572 (*trnLF*). Peru, Dept. Amazonas, Chachapoyas, *Wurdack1240* (type: *P. wurdackii*, US), AB355484 (ITS), AB355573 (*trnLF*); *Zapata 11A* (F) AB359077. Dept. Cajamarca, Prov. Cajamarca, *Dillon 4600* (F), AB355485 (ITS); *Dillon 6472* (F), AB355486 (ITS); *Sánchez 2778* (F), AB355487 (ITS); *Sánchez 6904* (F), AB355488 (ITS), AB355574 (*trnLF*); *Sánchez 10414* (F), AB355489 (ITS); *Sánchez 10493* (F), AB355490 (ITS), AB355575 (*trnLF*); *Sánchez 11397* (F), AB355491 (ITS); *Sánchez 10487* (F), AB355492 (ITS); *Zapata 02* (F), AB355493 (ITS), AB355576 (*trnLF*); *Zapata 03* (F), AB355494 (ITS); *Zapata 05* (F), AB355495 (ITS), *Sánchez 4052* (F), AB355500 (ITS); *Sánchez 5577* (F), AB355501 (ITS); *Zapata 01* (F), AB355502 (ITS); *Zapata 04* (F), AB355503 (ITS); *Zapata 06* (F), AB355504 (ITS); *Zapata 07* (F), AB355505 (ITS). Prov. Chota, *Sagástegui 17563* (F), AB355506 (ITS), AB355580 (*trnLF*). Prov. Contumazá, *Sagástegui 16162* (F), AB355507 (ITS); *Sagástegui 17557A* (F), AB355508 (ITS), AB355581 (*trnLF*). Dept. Junin, Prov. Junin, *Kunkel 965* (US), AB355577 (*trnLF*). Dept. La Libertad, Prov. Gran Chimú, *Sagástegui 17554* (F), AB355509 (ITS). Prov. Otuzco, *Sagástegui 17177* (F), AB355496 (ITS), *Sagástegui 17573A*, AB355497 (ITS), AB355578 (*trnLF*). Prov. Sánchez Carrión, *Sagástegui 17340* (F), AB355498 (ITS), *Sagástegui 16455* (F), AB355510 (ITS), *Sagástegui 17575A* (F), AB355511 (ITS), AB355582 (*trnLF*), *Sagástegui 17578A* (F), AB355512 (ITS), AB355583 (*trnLF*). Prov. Santiago de Chuco, *Sagástegui 17572A* (F), AB355499 (ITS), AB355579 (*trnLF*), *Sánchez 11843* (F), AB355513 (ITS).

***Paranephelius* hybrids**—Peru. Dept. Amazonas, Prov. Chachapoyas, *Sánchez 8136* (F), AB355514 (ITS), AB355584 (*trnLF*); *Zapata 10* (F), AB355515 (ITS); *Zapata 12A* (F), AB355516 (ITS). Dept. Cajamarca, Prov. Cajamarca, *Dillon 2884* (F), AB355517 (ITS). *Sánchez 12100* (F), AB355518 (ITS), AB355585 (*trnLF*). Prov. Celendín, *Zapata 13A* (F), AB355519 (ITS), AB355586 (*trnLF*). Prov. Chota, *Sánchez 10335* (F), AB355520 (ITS), AB355587 (*trnLF*). Dept. La Libertad, Prov. Pataz, *Sagástegui 16384* (F), AB355521 (ITS). Prov. Sánchez Carrión, *Dillon 2843* (F), AB355522 (ITS).

Pseudonoseris discolor (Muschl.) H. Rob. & Brettell—Peru. Dept. Puno, Prov. Sandia, *Quipuscoa 3338A* (F), AB355529 (ITS-1a), AB355530 (ITS-1d), AB355531 (ITS-1e), AB355532 (ITS-1h), AB355533 (ITS-1i), *3338C* (F), AB355523 (ITS), AB355588 (*trnLF*), AB355534 (ITS-2b), AB355535 (ITS-2d), AB355536 (ITS-2e), AB355537 (ITS-2f).

Pseudonoseris szyszylowiczii (Hieron.) H. Rob. & Brettell—Peru. Dept. Amazonas, Prov. Chachapoyas, *Wurdack 467* (US, szyszy4), AB355554 (ITS-4e), AB355555 (ITS-4g), AB355556 (ITS-4h), AB355557 (ITS-4i), AB355592 (*trnLF*). Dept. Cajamarca, Prov. Celendín, *Sánchez 3822* (F, szyszy1), AB355538 (ITS-1a), AB355539 (ITS-1c), AB355540 (ITS-1d), AB355541 (ITS-1e), AB355542 (ITS-1f), AB355543 (ITS-1h), AB355544 (ITS-1i), AB355589 (*trnLF*); *Sagástegui 17548* (F, szyszy2), AB355545 (ITS-2a), AB355546 (ITS-2c), AB355547 (ITS-2d), AB355548 (ITS-2e), AB355549 (ITS-2g), AB355590 (*trnLF*); *Zapata 20* (F, szyszy3), AB355550 (ITS-3b), AB355551 (ITS-3 c), AB355552 (ITS-3d), AB355553 (ITS-3e), AB355591 (*trnLF*).

Outgroup: ***Chionopappus benthamii*** S. F. Blake—Peru, Dept. La Libertad, Prov. Gran Chimú, *Sagástegui 17543* (F), AB355524 (ITS), AB355593 (*trnLF*). ***Chrysactinium acaule*** (Kunth) Wedd.—Peru. Dept. La Libertad, Prov. Pataz, *Sagástegui 16381* (F), AB355525 (ITS). ***Erato vulcanica*** (Klatt) H. Rob.—Costa Rica. Prov. Cartago, *Wilbur 30775* (F), AB355526 (ITS). ***Munnozia annua*** (Muschl.) H.Rob. & Brett.—Peru. Dept. La Libertad, Prov. Gran Chimú, *Sagástegui 17550* (F), AB355527 (ITS), AB355594 (*trnLF*). ***Philoglossa purpureodisca*** H. Rob.—Peru. Dept. Cajamarca, Prov. Contumazá, *Dillon 4512* (F), AB355528 (ITS), AB355595 (*trnLF*).

Appendix II Accession numbers for the sequence data obtained from the DNA Data Bank of Japan (DDBJ)

Taxon	Accession No.	Reference
Liabum bourgeaui Hieron.	AF539922	Kim et al., 2002
Dillandia perfoliata (S. F. Blake) V. A. Funk & H. Rob.	AF539937	Kim et al., 2002
Sinclairia angustissima (A. Gray) B. L. Turner	AF539953	Kim et al., 2002
Chrysactinium acaule (Kunth) Wedd.	AF539939	Kim et al., 2002
Munnozia campii H. Rob.	AF539927	Kim et al., 2002
Philoglossa mimuloides (Hieron.) H. Rob. & Cuatrec. f.	AF539950	Kim et al., 2002
Erato polymnioides DC.	AF539946	Kim et al., 2002