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NUMERICAL TAXONOMIC STUDIES OF PARTHENOGENETIC AND BISEXUAL POPULATIONS OF *HAEMAPHYSALIS LONGICORNIS* AND RELATED SPECIES (ACARI: IXODIDAE)*

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ABSTRACT: The phenetic relationships of parthenogenetic and bisexual populations of *Haemaphysalis longicornis* and populations of several related species were investigated utilizing multivariate analyses. A total of 117 individual specimens (OTU's) representing 37 separate populations were analyzed. Fifty-five characters, 50 quantitative and 5 qualitative, were evaluated for each OTU. The mean of each character within the 37 populations was utilized in numerical taxonomic and factor analyses. Phenograms and graphs generated by these analyses illustrate phenetic relationships among the different species and populations. *Haemaphysalis lagrangei*, *H. bispinosa*, *H. hystricis*, and *H. mageshimaensis* are separated distinctly from each other and from *H. longicornis* by this taxonomic approach. This agrees with certain classical taxonomic studies which considered them as valid species. Multivariate numerical analyses indicate phenetic differences between parthenogenetic and bisexual populations of *H. longicornis*; parthenogenetic and bisexual populations usually segregate into 2 separable groups through cluster analysis.

Phylogenetic relationships are obscured among the approximately 800 species of ticks (Ixodoidea), both by lack of reported fossil ticks and by convergent evolution within most tick genera which has resulted in their comparative homogeneity. Moreover, the immatures of most species show great intergenetic similarities. The approximately 155 species of *Haemaphysalis* (Hoogstraal, pers. comm.) appear to be one of the best taxa for study of phylogenetic specialization and speciation in the Ixodoidea because a greater proportion of known associations of larvae, nymphs, males, and females are available than in any other tick genus. Hoogstraal, using classical methods, is currently revising the genus.

The *H. longicornis* Neumann, 1901, group is one of the most confusing and interesting species complexes in the genus. These ticks have been known most often as *H. bispinosa* Neumann, 1897, but also as *H. neumanni* Donitz, 1905. Recently Hoogstraal et al. (1968) clarified part of this confusion by distinguishing structural and biological differences between *H. bispinosa* and *H. longicornis* and designating *H. neumanni* as a synonym of the latter. However, Dr. Y. Saito (Niigata University School of Medicine, Niigata City, Japan) recently found (pers. comm.) several new species from Japan which are phenetically quite similar to *H. longicornis*.

H. longicornis is a vector of *Coxiella burneti* (Q fever), *Theileria sergenti*, *T. mutans*, and the virus causing Russian spring–summer encephalitis. Moreover, it is a serious pest of cattle, horses, and deer. Investigations in Australia, Japan, and Korea (Oliver and Bremner, 1968; Oliver et al., 1973, 1974) indicate that some populations of *H. longicornis* are bisexual and diploid whereas others are parthenogenetic and triploid ($2n = 21$ in males, 22 in females; $3n = 32$ or 33); reproduction in other populations may be parthenogenetic and bisexual according to the potential of different individuals which may be variously aneuploid (22 to 28). Hybridization attempts in the laboratory suggest that the triploid parthenogenetic races are reproductively isolated, but the aneuploid and bisexual races are not (Oliver et al., 1973).

Hoogstraal et al. (1968) tentatively considered the parthenogenetic and bisexual populations of *H. longicornis* as one species. Although differences do exist, parthenogenetic populations could not be distinguished from the bisexual using classical taxonomic methods. Parthenogenetic individuals are usually slightly larger in average size and have a slightly

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TABLE I. *List of analyzed female specimens (OTU's) of* Haemaphysalis longicornis *populations and related species.*

Pop. No.	OTU code	Species	Population and sex Source	Collection locality	Collection date
1	HLG-V1	*H. lagrangei*	Field-collected F & M *Muntiacus muntjak*	Mt. Sontra, Danang, Vietnam	1 Oct. 1967
2	HLG-V2	*H. lagrangei*	Field-collected F & M *Paradoxurus hermaphroditus*	Mt. Sontra, Danang, Vietnam	30 Mar. 1966
3	HBS-M1	*H. bispinosa*	Field-collected F & M Goat	Selangor, Subang Res., Malaya	18 Sept. 1967
4	HBS-M2	*H. bispinosa*	Field-collected F & M Goat	Selangor, Meru Res., Malaya	13 Nov. 1967
5	HBS-M3	*H. bispinosa*	Field-collected F & M Goat	Malacca, Larang, Malaya	11 Jan. 1967
6	HYS-AK	*H. hystricis*	Lab-reared F	Amami Isl., Kagoshima, Kyushu, Japan	
7	HLC-A1	*H. longicornis*	Parthenogenetic F Lab-reared	C.S.I.R.O., Brisbane, Australia (derived from Mt. Tamborine, SW of Brisbane)	
8	HLC-JO	*H. longicornis*	Parthenogenetic F Lab-reared	Army 406th Lab., Tokyo, Japan (derived from Komochi, Japan)	
9	HLC-OP	*H. longicornis*	Parthenogenetic F Lab-reared	Hiruzon, Okayama, Honshu, Japan	
10	HLC-MK	*H. longicornis*	Parthenogenetic F Lab-reared	Miyazaki, Kyushu, Japan	
11	HLC-KK	*H. longicornis*	Parthenogenetic F Lab-reared	Kanagawa, Kanto, Japan	
12	HLC-SK	*H. longicornis*	Parthenogenetic F Lab-reared	Shibino, Kagoshima, Kyushu, Japan	
13	HLC-YK	*H. longicornis*	Parthenogenetic F Lab-reared	Yakushima, Kagoshima, Kyushu, Japan	
14	HLC-J5	*H. longicornis*	Parthenogenetic F Lab-reared	Kanagawa, Japan	
15	HLC-J6	*H. longicornis*	Field-collected F Vegetation	Shuzan, Kyohoku, Kyoto City, Japan	6 Apr. 1970
16	HLC-J7	*H. longicornis*	Field-collected F Vegetation	Hiyamagun, Hokkaido, Japan	23 June 1970
17	HLC-J48	*H. longicornis*	Field-collected F Cattle	Mori, Oshuma, Hokkaido, Japan	21 June 1967
18	HLC-J57	*H. longicornis*	Field-collected F Cattle	Sannohe, Aomori, Honshu, Japan	7 June 1967
19	HLC-J22	*H. longicornis*	Field-collected F Cattle	Kitaibaraki, Ibaraki, Honshu, Japan	2 July 1961
20	HLC-J31	*H. longicornis*	Field-collected F Cattle	Agatsuma, Gumma, Honshu, Japan	17 July 1965
21	HLC-J34	*H. longicornis*	Field-collected F Horse	Agatsuma, Gumma, Honshu, Japan	26 June 1965
22	HLC-J85	*H. longicornis*	Field-collected F Cattle	Miyake Isl., Tokyo, Honshu, Japan	24 June 1968
23	HLC-J14	*H. longicornis*	Field-collected F Cattle	Hushi, Ishikawa, Honshu, Japan	18 Sept. 1961
24	HLC-J16	*H. longicornis*	Field-collected F & M Cattle	Yosa, Kyoto, Honshu, Japan	14 Aug. 1962
25	HLC-J68	*H. longicornis*	Field-collected F & M Cattle	Oki Isl., Shimane, Honshu, Japan	15 June 1967
26	HLC-J75	*H. longicornis*	Field-collected F & M Cattle	Tomota, Okayama, Honshu, Japan	17 July 1967
27	HLC-J61	*H. longicornis*	Field-collected F Cattle	Ube, Yamaguchi, Honshu, Japan	21 June 1967
28	HLC-J106	*H. longicornis*	Field-collected F & M Cattle	Meisai, Tokushima, Shikoku, Japan	10 July 1968

TABLE I. (*Continued*).

Pop. No.	OTU code	Species	Population and sex Source		Collection locality	Collection date
29	HLC-J05	*H. longicornis*	Field-collected Cattle	F & M	Aso, Kumomoto, Kyushu, Japan	23 May 1962
30	HLC-J52	*H. longicornis*	Field-collected Cattle	F	Kitamorogat, Miyazaki, Kyushu, Japan	5 June 1967
31	HLC-J56	*H. longicornis*	Field-collected Cattle	F	Ara, Kagoshima, Kyushu, Japan	12 July 1967
32	HLC-K1	*H. longicornis*	Field-collected Cattle	F & M	Cheju City, Cheju DO, Korea	June 1970
33	HLC-JxK	*H. longicornis*	Bisexual hybrid Lab-reared	F & M	Army 406th Lab., Tokyo, Japan (Cheju Do, Korea Partheno-genetic × Oki, Japan Bisexual)	
34	HLC-NB	*H. longicornis*	Bisexual Lab-reared	F	Niimi, Okayama, Honshu, Japan	
35	HLC-SI	*H. longicornis*	Bisexual Lab-reared	F	Satomi, Ibaraki, Honshu, Japan	
36	HLC-TS	*H. longicornis*	Bisexual Lab-reared	F	Tokushima, Shikoku, Japan	
37	HSP-01	*H. mageshimaensis*	Bisexual Lab-reared (derived from cattle)	F & M	Mageshima, Kagoshima, Kyushu, Japan	9 April 1971

F = Females
M = Males

longer development cycle (Kitaoka, 1961). Only parthenogenetic individuals are known from Australia, New Zealand, New Caledonia, Fiji, New Hebrides, Tonga, most of Primorye (northeastern USSR), Hokkaido and Honshu Islands of Japan. Previously it was thought that the parthenogenetic races did not occur in southern Japan, but Saito (1972) found them on Kyushu and Yakushima. Bisexual populations are known from southern Honshu and Kyushu Islands of Japan, Korea, and extreme south of Primorye. Hoogstraal et al. (1968) suggest that *H. longicornis* appears to have been imported into Australia from northern Japan in the nineteenth century and thence to New Zealand, New Caledonia, and Fiji.

The objective of this study is to clarify phenetic relationships among various populations of *H. longicornis* and several related species through the techniques of numerical taxonomy (Sokal and Sneath, 1963; Sneath and Sokal, 1973). Perhaps these analyses may provide insights into species relationships among the *H. longicornis* group and a preliminary categorization of possible intranspecific units represented by the various populations of *H. longicornis*.

Classical numerical taxonomic analyses have been utilized primarily in studies dealing with species, genera, and higher categories, but not often in studies of intraspecific variation. Herrin (1969) found numerical taxonomy useful in intraspecific studies of geographic populations of *Hirstionyssus* mites. He included 20 replicates of *H. isabellinus* and 10 replicates of *H. talpae* from throughout the Holarctic region. In both species the Palearctic and Nearctic OTU's tended to cluster in two more or less separate groups or subclusters.

MATERIALS AND METHODS

Materials utilized

Populations of ticks included in the multivariate numerical analyses are listed in Table I.

Attempts were made to include specimens from various geographic areas throughout Japan and other localities in Asia and Australia. Random selection of four replicates from each of the 37 populations was impossible in 16 cases because of insufficient numbers of specimens. Any specimen for which all characters could not be scored (measurements made accurately) was discarded and another selected in its place so that no NC values appear in the basic data matrix. Data were recorded for 117 specimens (OTU units).

Each specimen was placed in a numbered vial of 70% ethyl alcohol and a small amount of glycerine. Specimens were examined at 60× magnification while submerged in 70% ethyl alcohol. Measurements were made using an American Optical filar micrometer. These units were used directly in the

TABLE II. *List of characters utilized for* Haemaphysalis *numerical taxonomic and factor analyses.*

Dorsal Measurements

1. Length of trochanter I.
2. Length of femur I.
3. Length of tibia I.
4. Length of pretarsus I.
5. Length of tarsus I.
6. Width of dorsal plate of trochanter I.
7. Greatest width of femur I.
8. Greatest width of tibia I.
9. Greatest width of pretarsus I.
10. Basal width of tarsus I.
11. Length of femur II.
12. Length of tibia II.
13. Length of pretarsus II.
14. Length of tarsus II.
15. Length of femur III.
16. Length of tibia III.
17. Length of pretarsus III.
18. Length of tarsus III.
19. Length of femur IV.
20. Length of tibia IV.
21. Length of pretarsus IV.
22. Length of tarsus IV.
23. Length of basis capitulum (tip of cornua to palp segment 2).
24. Length of capitulum (base to tip of cheliceral sheath).
25. Length of cornua.
26. Width of cheliceral sheath (base of hypostome).
27. Width of basis capitulum at base of palp article I.
28. Distance between tips of cornua.
29. Length of palpal segment 2 and 3 combined.
30. Length of palpal segment 3 including dorsal spur.
31. Greatest width of palpal segment 2.
32. Length of scapulae.
33. Length of scutum (medially).
34. Greatest width of scutum.

Ventral Measurements

35. Length of hypostome.
36. Length of basis capitulum (posterior edge to hypostome).
37. Length of ventral spur of palpal segment 3.
38. Width of basis capitulum at palpal segment 2.
39. Diagonal length of coxa I including spur.
40. Median length of coxa I.
41. Median length of coxa II.
42. Width of coxa II at level of spur and including spur.
43. Greatest width of coxa III.
44. Width of coxa III at level of spur and including spur.
45. Median length of coxa III.
46. Median length of coxa IV.
47. Width of coxa IV.
48. Width of coxa IV at level of spur and including spur.

TABLE II. (*Continued*).

49. Length of spiracular plate.
50. Greatest width of anal plate.

Qualitative Characters

51. Shape of spur on coxa II:
    1—broadly rounded
    2—narrowly rounded or blunt
    3—acute
52. Shape of spurs on coxae III and IV:
    0—spur on coxae III and IV broadly rounded
    1—spur on coxa III narrowly rounded; spur on coxa IV broadly rounded
    2—spur on coxae III and IV narrowly rounded or blunt
    3—spurs on coxae III and IV acute
53. Denticle formula of hypostome:
    1—4/4
    2—5/5
54. Projection of dorsal plate on trochanter I:
    1—broadly rounded
    2—narrowly rounded or blunt
    3—acute
55. Posteroventral spur or projection on trochanters I and II.
    0—absent
    1—present and broadly rounded
    2—present and narrowly blunt

analysis although they can be converted into microns by a multiplication factor of 1.46.

Fifty-five characters from all areas of the body were used in the analyses (Table II). Characters which might vary excessively as a function of developmental factors (i.e., degree of engorgement) were excluded. Fifty of the characters were quantitative (measurements) and five were qualitative. Of 50 quantitative characters, 34 were measured ventrally and 16 dorsally. Thirty-six characters were from the legs, 14 from the capitulum, and 5 from other body areas.

Computational and analytical methods

The Numerical Taxonomy System of Multivariate Statistical Programs (NT-SYS) prepared by F. James Rohlf and associates, State University of New York, Stony Brook, was used for most analyses. Programs for the computation of population means and the condensation transformation (Crovello, 1968) were written by one of us (C.S.H.). The factor analysis program utilized was the BMDO 3M, General Factor Analysis, prepared by the UCLA Health Science Computing Facility (Dixon, 1968). All computations were performed by the I.B.M. 360 model 65 computer at the University of Georgia Computer Center via a terminal at Georgia Southern College Computer Center equipped with N.C.R. Century 100. A copy of the NT-SYS programs was obtained on magnetic tape and loaded onto a data cell (I.B.M. 2321) at the University of Georgia Computer Center.

The first basic data matrix of 99 OTU's and 55 characters was analyzed using classical numerical

taxonomic methods (Sokal and Sneath, 1963; Sneath and Sokal, 1973). That is, the data matrix was standardized by characters prior to computation of the similarity coefficients (Pearson's product moment correlation, r, and Sokal's taxonomic distance, d). Each of the two similarity matrices was analyzed by UPGMA (Unweighted Pair Group Method with Arithmetic Averages) cluster analysis, resulting in 2 phenograms.

Following this preliminary analysis, the number of OTU's included in the analysis was reduced because OTU relationships were difficult to analyze and clarify. In an effort to alleviate this problem the mean of each of the initial 28 populations was computed and these data were then used as the basic data. This matrix of 28 population means was transformed by variance standardization and condensation methods. For each transformed data matrix, 2 similarity coefficients (correlation and taxonomic distance) were computed and 4 similarity matrices were produced: (1) standardized correlation, (2) standardized distance, (3) condensed correlation, and (4) condensed distance. Each of these 4 similarity matrices was then analyzed by 5 methods of cluster analysis: UPGMA, WPGMA (Weighted Pair Group Method with Arithmetic Averages), Complete Linkage, Single Linkage, and WPGMS (Weighted Pair Group Method with Spearman's Sums of Variables) which yielded a total of 20 different phenograms. For descriptions and discussion of the various methods of cluster analysis see Sokal and Sneath (1963) and Sneath and Sokal (1973). From each phenogram, matrices of cophenetic values were computed and compared with the original matrices of similarity values. This comparison, together with the computation of a cophenetic correlation coefficient for each phenogram, provided an estimate of the proportion of information transferred to the phenogram from the similarity matrix (Sokal and Rohlf, 1962). Subsequent to the analysis of the 28 population means, 9 populations were added to the data matrix of population means. Means of these 37 populations were then analyzed exactly as the 28, except that only the UPGMA cluster analysis was utilized because in previous analyses it gave the best cophenetic correlations.

An R-type factor analysis was used as a second method of analyzing the correlation coefficient matrix. The UCLA Biomedical Computer Program, General Factor Analysis (BMDO 3M), was used in these computations. The R-type factor analysis consists of computing character correlations rather than OTU correlations, then performing a principal component factor analysis of characters followed by an orthogonal rotation of the factor matrix. Finally, a matrix of projections of each OTU on each rotated factor is produced. These OTU factor scores are then graphed to illustrate OTU relationships based on 2 factors at a time. In this study 4 such factor analyses were performed. The first 2 analyses utilized the 99 original OTU's, but the data were standardized only for the second. The third analysis was based on the unstandardized matrix of 28 population means, and the fourth analysis was based on the unstandardized matrix of 37 population means. Graphs were prepared and analyzed for each of these factor analyses.

RESULTS AND DISCUSSION

Classical numerical taxonomic analyses

Figures 1 and 2 summarize OTU relationships given by the UPGMA cluster analysis of the condensed distance matrix and the condensed correlation matrix, respectively, based on the data matrix of 28 population means. Of the 20 phenograms produced in the analyses of the 28 population means only these two are chosen for presentation because they illustrate OTU relationships best. Since an analysis of the different methods of numerical taxonomy is not an objective of this study, a comparison of all the cluster analyses is only summarized here. This selection is based on the computation of cophenetic correlation coefficients between similarity matrices and phenograms, between correlation matrices and distance matrices, and between correlation phenograms and distance phenograms. All cluster analyses of the standardized correlation matrix give rather low cophenetic correlation values (below 0.770). The coefficients for all WPGMS phenograms of standardized and condensed distance and condensed correlation matrices are between 0.800 and 0.900, whereas most remaining phenograms (UPGMA, WPGMA, Complete and Single linkage) produce high cophenetic values (above 0.900). A table of these data can be obtained by requesting it from authors. The UPGMA, WPGMA, and complete linkage phenograms of standardized and condensed distance matrices are identical in the formation of clusters and quite similar in the placement of OTU's within the clusters. All phenograms of standardized and condensed correlation matrices gave much less pleasing results. However, the UPGMA phenogram of the condensed correlation matrix (Fig. 2) is selected for comparison with the phenogram of the condensed distance matrix (Fig. 1). Our primary interest in these phenograms is the grouping of OTU's rather than the relative levels at which OTU's link with others. Thus, the clusters and clustering levels are important rather than the vertical ordering of the clusters and OTU's.

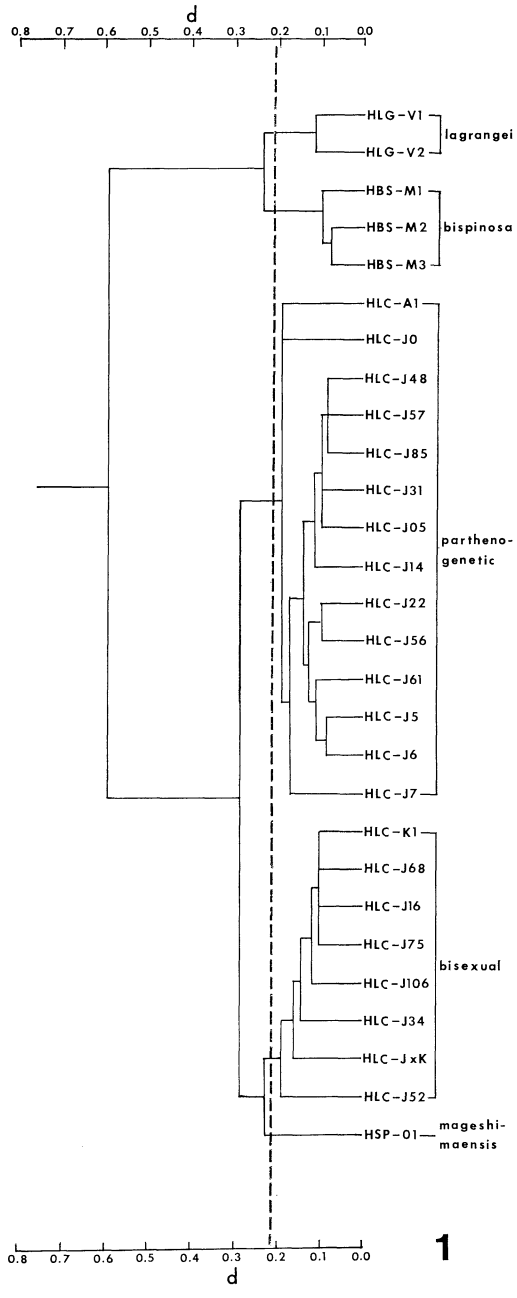


FIGURE 1. Phenogram obtained from the UPGMA cluster analysis of the condensed distance matrix based on the 55 character by 28 OTU data matrix. Cophenetic correlation coefficient = 0.924.

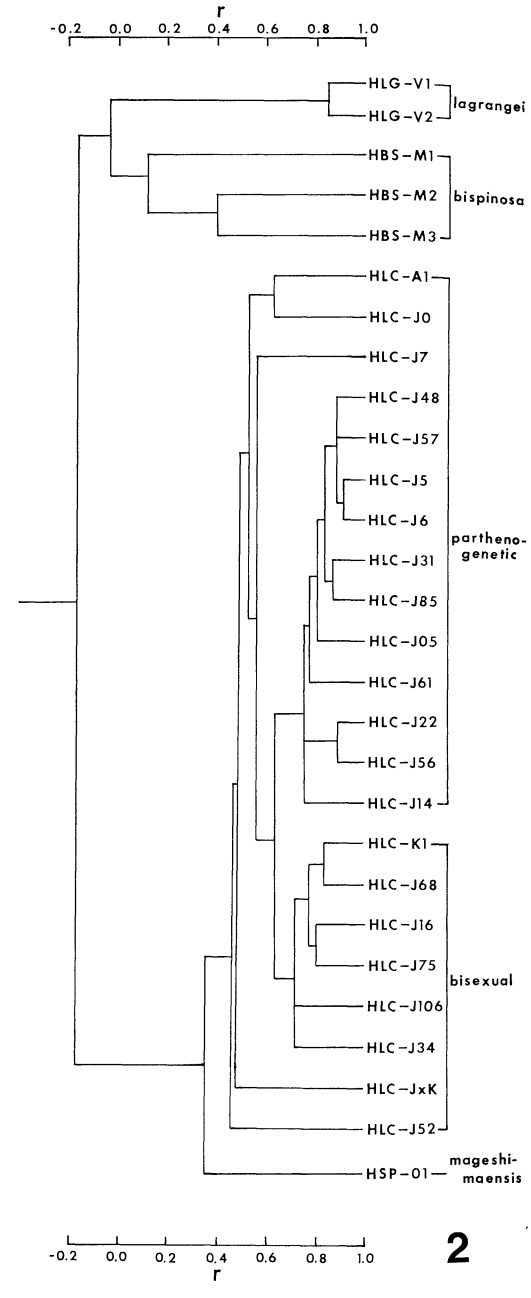


FIGURE 2. Phenogram obtained from the UPGMA cluster analysis of the condensed correlation matrix based on the 55 character by 28 OTU data matrix. Cophenetic correlation coefficient = 0.921.

In the formation of well-structured clusters the distance phenogram (Fig. 1) produces much more pleasing results than the correlation phenogram (Fig. 2). There are, however, definite similarities in the two. In both, two major clusters are represented: (1) the cluster including *H. lagrangei* (HLG-V1 and HLG-V2) and *H. bispinosa* (HBS-M1, HBS-M2, and

HBS-M3), and (2) the very large cluster including all populations of *H. longicornis* (parthenogenetic and bisexual) and *H. mageshimaensis* (HSP-O1). Within these two major clusters the subdivisions are structured much better in the distance phenogram than in the correlation phenogram. In the first major cluster, *H. lagrangei* and *H. bispinosa* are separated quite nicely only in the distance phenogram. In the correlation phenogram the structuring of subclusters within the second major group (*H. longicornis* population) is rather poor, but in the distance phenogram (Fig. 1) two very definite groups are formed. The larger subcluster includes parthenogenetic populations (with a few exceptions) whereas the smaller subcluster contains bisexual populations. Within the parthenogenetic subclusters the two laboratory-reared populations (HLC-A1 and HLC-JO) are linked with each other prior to both joining the primarily field-collected populations. Three of the population clustering with the parthenogenetic group are assumed bisexual because of the presence of males or because of the collection locality (southern Japan). Males are present only in the HLC-JO5 population and it undoubtedly is bisexual. The other two, HLC-J56 and HLC-J61, are from southern Japan, Kyushu, and southern Honshu, respectively. Populations from these areas were considered previously to be bisexual; however, Saito (1972) reported parthenogenetic races from these areas also. Within the bisexual subcluster the HSP-01 population (*H. mageshimaensis*) seems to represent a distinct species phenetically closely related to *H. longicornis*. It is linked to that subcluster at its lowest level in the distance phenogram. Moreover, in the correlation phenogram this population is separated from all other *H. longicornis* populations. During the course of the present study the HSP-01 species was described by Saito and Hoogstraal (1973). Only one population (HLC-J34), which had been assumed to be parthenogenetic *a priori*, clusters in the bisexual group. The central Honshu collection locality of this population is identical to that of the HLC-J31 population which clusters with the parthenogenetic group. The HLC-J52 population clusters with the bisexual group although no males are contained in the sample. The collection locality for this population is adjacent to that of the HLC-K56 population which clusters with the parthenogenetic group, although it was considered *a priori* to be bisexual.

Although the placement of phenon lines is subjective, a phenon line drawn vertically across the distance phenogram (Fig. 1) at the 0.21 level defines five clusters as follows: (1) *H. lagrangei* (HLG-V1 and HLG-V2); (2) *H. bispinosa* (HBS-M1, HBS-M2, and HBS-M3); (3) *H. longicornis* basically parthenogenetic populations (HLC-A1 through HLC-J7); (4) *H. longicornis* basically bisexual populations (HLC-K1 through HLC-J52); and (5) *Haemaphysalis mageshimaensis* (HSP-01).

The clustering of parthenogenetic and bisexual populations is not as clear-cut in the correlation phenogram (Fig. 2) as in the distance phenogram. The HLC-A1, HLC-JO, and HLC-J7 populations are joined at a lower level than that joining the major parthenogenetic and bisexual subclusters. The bisexual HLC-JxK and HLC-J52 populations are linked at an even lower level. The placement of all other OTU's is basically the same as in the distance phenogram.

Figure 3 summarizes OTU relationships of 37 population means given by the UPGMA cluster analysis of the condensed distance matrix. This phenogram produces more pleasing results than the UPGMA cluster analysis of the standardized distance matrix, the standardized correlation matrix, or the condensed correlation matrix. The initial 28 populations cluster very nearly the same in this phenogram as in the previous UPGMA condensed distance phenogram (Fig. 1). However, the nine added populations fail to cluster as expected. All eight *H. longicornis* populations cluster closer to the bisexual group than to the parthenogenetic group even though only three were determined *a priori* to be bisexual. Seven of these eight populations clustered very closely together. The *H. hystricis* population (HYS-AK) is very clearly separated from other *Haemaphysalis* species included in the analyses. A phenon line drawn vertically across the phenogram (Fig. 3) at the 0.21 level defines six clusters as follows: (1) *H. lagrangei* (HLG-V1 and HLG-V2); (2) *H. bispinosa* (HBS-M1, HBS-M2, and HBS-M3); (3) *H. hystricis* (HYS-AK); (4) *H. longicornis* basically parthenogenetic popu-

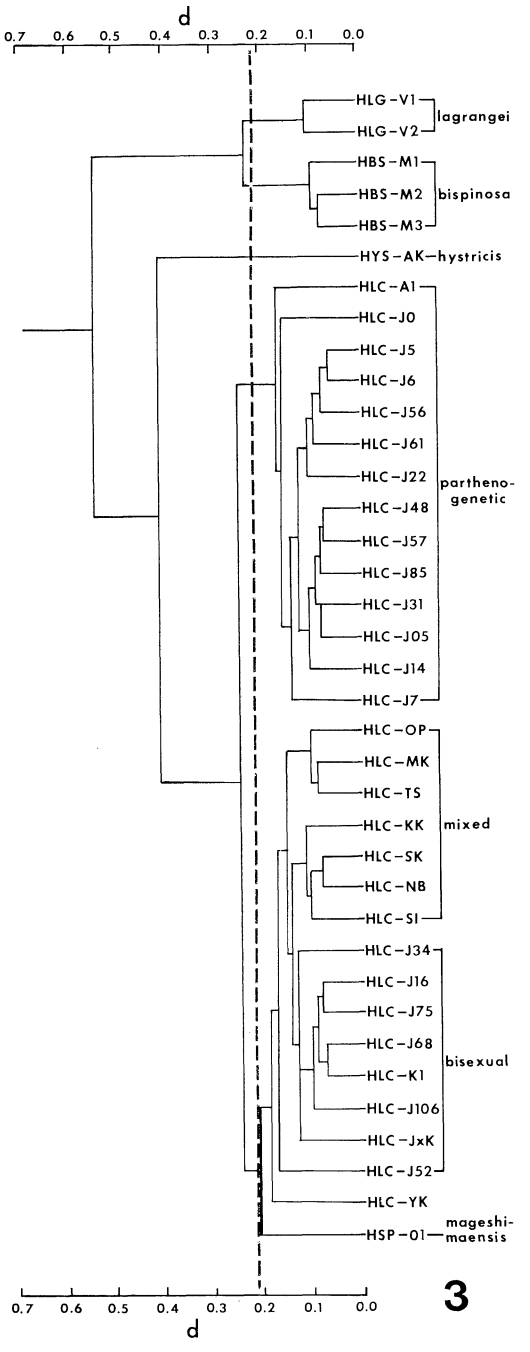


FIGURE 3. Phenogram obtained from the UPGMA cluster analysis of the condensed distance matrix based on the 55 character by 37 OTU data matrix. Cophenetic correlation coefficient = 0.913.

TABLE III. *Summary of rotated factor matrix of factor loadings on the characters for the analysis of 28 population means.*

Factors	Characters with 1st- and 2nd-order loadings
I	1st order—1, 3, 16, 17, 20, 21, 24, 26, 31, 33, 35, 40, 41, 45, 46, 47, 49
	2nd order—2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 19, 22, 28, 29, 38, 48, 50
II	1st order—2, 4, 5, 11, 12, 13, 14, 15, 18, 19, 22, 23, 25, 29, 30, 36, 39, 42, 43, 44, 48
	2nd order—1, 3, 6, 9, 16, 17, 20, 21, 24, 27, 31, 34
III	1st order—32
	2nd order—none
IV	1st order—6, 7, 8, 9, 10, 27, 28, 34, 37, 38, 50
	2nd order—39, 40

lations (HLC-A1 through HLC-J7); (5) *H. longicornis* mixed parthenogenetic and bisexual populations subcluster and the bisexual subcluster (HLC-OP through HLC-YK); and (6) *H. mageshimaensis* (HSP-01).

Factor analysis

Results of the first two factor analyses utilizing the 99 original OTU's are not presented here. The large number of OTU's included in these analyses results in cumbersome graphs which are difficult to analyze. Only selected graphs of the other two factor analyses based on 28 and 37 population means are presented. Four factors are extracted in each of these two analyses. The factor matrix is rotated orthogonally, and a matrix of projections of each OTU on each factor are produced.

In examining the rotated factor matrix of factor loadings on the variables for the analysis of 28 population means, factors I, II, and IV are considered to be most significant (Table III). To determine the high (first order) factor loadings for the characters, the rotated factor matrix was examined, and, for each character, the factor yielding the largest numerical value was considered to give the high or first-order loading. The high factor loadings were always greater than 0.5000. For some characters two factors yielded factor loadings greater than 0.5000; however, the higher value of the two was always considered to be the first-order loading and the second high the second-order loading. Factor III gives a high (first order) loading on only one character

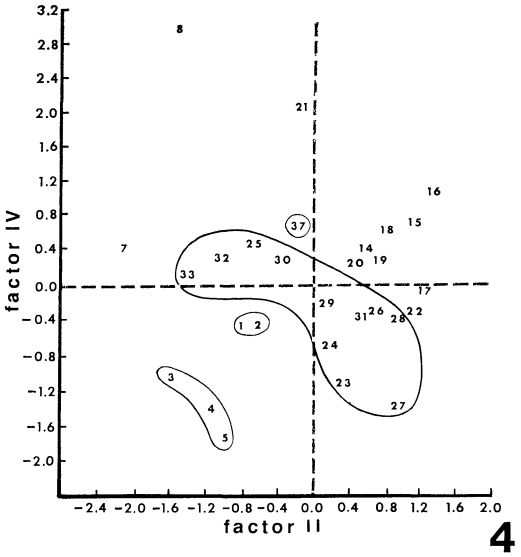


FIGURE 4. Factor analysis scatter diagram of factor II versus factor IV for the analysis of 28 population means.

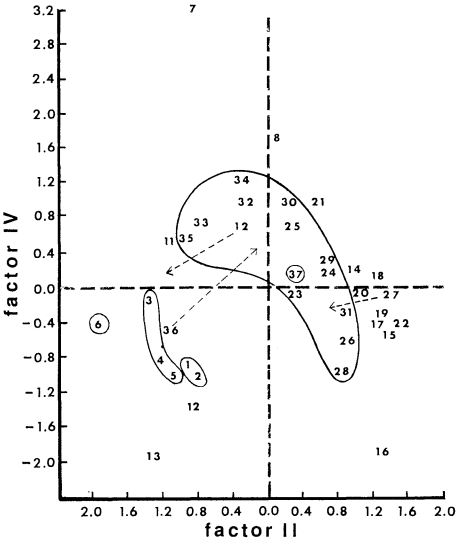
(No. 32), whereas each of the other three factors give high loadings on more than 11 characters. As is usual in a factor analysis, factor I is a size factor accounting of the largest proportion of total variance (89.07%). Factor II, which accounted for 2.31% of variance, seems to be predominantly a factor of length and width of structures, and factor IV (1.11% of variance) primarily of width of structures. The factor scores (matrix of projections of each OTU on each of the four factors) for the analysis of 28 population means were used in plotting six scatter diagrams or graphs (each of the four factors versus each other factor). The graph of factor II versus IV yields the most pleasing results (Fig. 4). As shown in this plot of factors II versus IV, the *H. bispinosa* (Pop. Nos. 3, 4, and 5) and the *H. lagrangei* (Pop. Nos. 1 and 2) populations form separate clusters apart from each other and from the *H. longicornis* populations. The *H. longicornis* bisexual populations (as determined *a priori*) are somewhat dispersed but form a broad cluster. The parthenogenetic populations are scattered. One population (No. 23-HLC-J14), presumed to be parthenogenetic, clusters in this analysis with the bisexual populations. The two laboratory-reared parthenogenetic populations (Nos. 7-HLC-A1 and 8-HLC-JO) separate some

TABLE IV. *Summary of rotated factor matrix of factor loadings on the characters for the analysis of 37 population means.*

Factors	Characters with 1st- and 2nd-order loadings
I	1st order—1, 2, 3, 4, 7, 8, 9, 11, 12, 13, 15, 16, 17, 19, 20, 21, 24, 26, 27, 28, 32, 34, 35, 38, 41, 42, 43, 44, 45, 46, 47, 48
	2nd order—6, 10, 18, 22, 29, 31, 33, 39, 40, 49
II	1st order—6, 23, 25, 31, 36, 50
	2nd order—1, 5, 7, 8, 14, 24, 26, 30, 42, 43, 44
III	1st order—5, 10, 14, 18, 22, 29, 30, 33, 37, 39, 40, 49
	2nd order—2, 3, 4, 9, 11, 12, 13, 15, 16, 17, 19, 20, 21, 23, 25, 27, 28, 32, 35, 47, 48
VI	1st order—none
	2nd order—41, 45, 46, 50

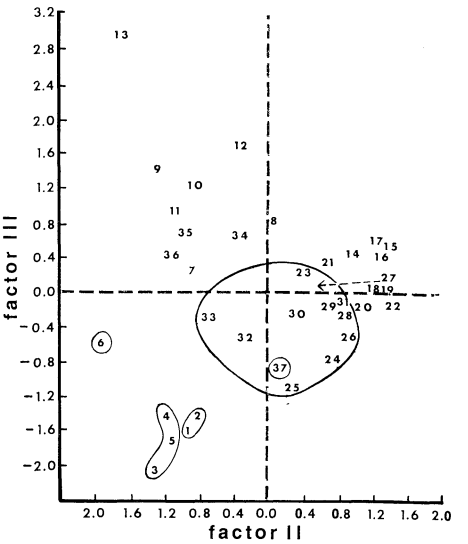
distance from other parthenogenetic populations. *Haemaphysalis mageshimaensis* (Pop. No. 37-HSP-01) does not cluster with the *H. longicornis* bisexual populations nor with the parthenogenetic populations even though it is located on the graph in the general area of parthenogenetic populations.

The analysis of 37 population means gives less conclusive results. The 28 populations of the previous analysis, however, cluster very nearly the same. The *H. longicornis* populations added in this analysis again fail to cluster according to *a priori* assumptions. The summary of the rotated factor matrix is presented in Table IV. The factor scored for the analysis of the 37 population means was used to plot six scatter diagrams or graphs. The graphs which contain the most structure and which are the most pleasing are of factor II versus factor IV (Fig. 5) and of factor II versus factor III (Fig. 6). In both graphs the *H. lagrangei* populations (Nos. 1 and 2), the *H. bispinosa* populations (Nos. 3, 4, and 5), and the *H. hystricis* population (No. 6) separate reasonably well, yet not as clearly as in the analysis of the 28 populations (Fig. 4). Although the separation of parthenogenetic and bisexual populations of *H. longicornis* is not complete, most of the bisexual populations cluster nearer to each other than to parthenogenetic populations. The broken line arrows in the graphs (Figs. 5, 6) indicate positional deviations among *a priori* determined bisexual



5

FIGURE 5. Factor analysis scatter diagram of factor II versus factor IV for the analysis of 37 population means.



6

FIGURE 6. Factor analysis scatter diagram of factor II versus factor III for the analysis of 37 population means.

and parthenogenetic populations. In both graphs the bisexual population No. 27 (HLC-J61) segregates with the parthenogenetic populations. The only other gross deviations are among the five parthenogenetic (Pop. Nos. 9, 10, 11, 12, and 13) and three bisexual (Pop. Nos. 34, 35, and 36) populations which were added to the original 28 populations. In Figure 5, two of the three bisexual populations cluster with the major bisexual group. However, in Figure 6 all three are somewhat removed. In both graphs the five parthenogenetic populations are distinctly separated from the major parthenogenetic group, and in Figure 6 the five parthenogenetic and three bisexual populations appear more or less together in the upper left quadrant of the graph. *Haemaphysalis mageshimaensis* (Pop. No. 37) is included with the bisexual group in both cases.

As stated in the Introduction, Hoogstraal et al. (1968) clearly define *H. bispinosa* and *H. longicornis* as separate and distinct species and note ecological differences between the bisexual and parthenogenetic populations of *H. longicornis*. Clear chromosomal differences also exist between *H. bispinosa* and *H. longicornis* and between bisexual and parthenogenetic races of the latter (Oliver and Bremner, 1968; Oliver et al., 1973, 1974). Our study confirms the above authors' taxonomic recognitions. In both the numerical taxonomic analyses and the factor analyses, *H. lagrangei*, *H. bispinosa*, and *H. hystricis* are clearly differentiated from each other and from *H. longicornis*. Evidence indicates that *H. lagrangei* and *H. bispinosa* are phenetically more closely related to each other than to *H. longicornis*. *H. hystricis* is distinct from all other species included in this study. The evidence also corroborates *Haemaphysalis mageshimaensis* (No. 37) as a distinct species phenetically close to *H. longicornis*. In most analyses it clusters near bisexual *H. longicornis* populations, but not always closely.

Separation of bisexual and parthenogenetic *H. longicornis* was not nearly as clear-cut as separation of the other species. As mentioned, Hoogstraal et al. (1968) were unable to distinguish bisexual from parthenogenetic populations on morphological characters. However, Kitaoka (1961) noted that parthenogenetic individuals are usually slightly larger in average size than bisexual individuals. We can generally confirm that observation, but there are some exceptions which will be discussed later. In our multivariate analysis parthenogenetic and bisexual populations generally form two separate

subclusters. The seven populations consistently clustering together in the bisexual group in all analyses are HLC-J16 (#24), HLC-J68 (#25), HLC-J75 (#26), HLC-J106 (#28), HLC-J52 (#30), HLC-K1 (#32), and HLC-JxK (#33). The HLC-J34 (#21) population clusters with the bisexual group in the numerical taxonomic analyses (phenograms), but not in any of the factor analyses. Conversely, three populations, HLC-J14, HLC-JO5, and HLC-J56 (Nos. 23, 29, and 31, respectively) consistently cluster with the bisexual group in the factor analyses but not in the numerical taxonomic analyses. The latter two populations were determined *a priori* to be bisexual. Another population originally considered to be bisexual, HLC-J61 (#27), clusters with the parthenogenetic group in all analyses except one (Fig. 4). All but one (HLC-J34) of the 28 original populations determined to be parthenogenetic *a priori* cluster together in all analyses.

As noted previously, some of the eight *H. longicornis* populations added to the study (3 bisexual and 5 parthenogenetic) do not cluster as expected. Presumably the size factor is responsible for these and other clustering deviations, particularly in the numerical taxonomic analyses. Population means of HLC-J61 (#27), HLC-JO5 (#29), and HLC-J56 (#31) are generally greater for most characters than in the other bisexual populations, and are similar to means obtained from parthenogenetic populations. Conversely, means for HLC-J34 (#21) and HLC-J14 (#23) generally resemble those of the bisexual more than the parthenogenetic populations. This same situation exists among the eight *H. longicornis* populations added later to the study. Population means of all eight are similar; means of the five parthenogenetic populations are less than those of the other parthenogenetic populations and more similar to those of the bisexual group. The *a priori* determination of reproductive types in these eight populations was based on laboratory-reared individuals. Thus, there are definitely exceptions to the generalization that parthenogenetic ticks are larger than the bisexual ones. However, as a group, size differences do seem to be significant (particularly among natural populations).

Overwhelming evidence indicates that parthenogenetic and bisexual races of *H. longicornis* are phenetically different. Still it is not now possible to define those characters which will differentiate parthenogenetic from bisexual populations. A study utilizing discriminant function analyses is in progress in an effort to make that determination. Results of this study will be presented later.

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RESEARCH NOTE . . .

Iron Gallein as a Substitute for Iron Hematoxylin in Parasitological Staining

Lillie, Pizzolato, and Donaldson (1973, Stain Tech. **6**: 348–349) recently pointed out the favorable substitution of iron gallein for iron hematoxylin. With the shortage of hematoxylin being experienced at this time, this substitution, with modification, was used in staining various parasites. *Entamoeba histolytica* trophozoites, from both feces and tissue, *Balantidium coli* trophozoites in tissue, and *Onchocerca volvulus* adults in tissue were used.

Solution A of Lillie's procedure (1 g gallein in 20 ml glycol, followed by 80 ml of absolute alcohol) was used without modification. Solution B (4 g iron alum, 1.6 to 2 ml conc HCl, distilled water to make 100 ml) was modified according to the organism being stained. The two solutions are mixed in equal volumes prior to use.

The best staining characteristics for *E. histolytica* trophozoites in tissue were obtained using a modification of solution B containing 1.6 ml of concentrated HCl for 5 to 10 min. These preparations were counterstained with eosin. *E. histolytica* trophozoites from feces were fixed in Schaudinn's solution and stained 20 min using 3.0 ml of concentrated HCl in solution B and with a counterstain of eosin. The nucleus of *E. histolytica* trophozoites, in fecal smears, stained black, while the cytoplasm stained pink to light red. The nucleus of *E. histolytica* trophozoites, in tissue, stained black, the cytoplasm light purple, and ingested red blood cells light to dark purple. Nuclear detail was excellent in both preparations.

Balantidium coli trophozoites in tissue were stained for 20 min using a modification of solution B containing 2.5 ml of concentrated HCl and lightly counterstained with Van Gieson's stain. The nucleus stained black, the cytoplasm a light brown, and the cilia pink to light brown.

Onchocerca volvulus adults in tissue were stained using the same modification of solution B (2.5 ml of concentrated HCl) for 20 min and Van Gieson's counterstain. The cuticle stained red and the internal structures, including microfilariae *in utero*, stained purple.

The staining procedure is summarized as follows:

1. bring paraffin sections to water
2. stain 5 to 20 min in iron gallein, depending on the organism and the fixation
3. counterstain with eosin or Van Gieson's stain, again depending on the tissue
4. dehydrate quickly, proceed through xylol to mounting.

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