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ISOELECTRIC FOCUSING OF SOLUBLE PROTEINS IN THE CHARACTERIZATION OF SPECIES AND ISOLATES OF *NEMATODIRUS* (NEMATODA: TRICHOSTRONGYLOIDEA)

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ABSTRACT: Isoelectric focusing was performed on extracts from *Nematodirus spathiger, Nematodirus filicollis, Nematodirus helvetianus,* and 3 geographic isolates of *Nematodirus battus.* Gender-specific differences were noted within species; however, the overall protein profile of each species and isolate was distinct and reproducible and allowed unequivocal differentiation. A coefficient of similarity (Sm) for males of each species and isolate was calculated, and a dendrogram, based on evaluation of Sm by the unweighted pair-group method with arithmetic means, was produced. Although cluster analysis of the 3 isolates of *N. battus* indicates the North American and Weybridge isolates are similar, interpretation of the relationships and thus the history of introduction based on these data is equivocal. Isoelectric focusing is a robust method for establishing identity and has great utility in diagnostics. However, in the absence of selective histochemical staining, interpretation of identity and homology for specific bands and banding patterns is problematic, thus limiting the utility of this method for phylogenetic inference.

The role of infected animals in the dissemination of helminthic parasites is well documented (Helle, 1969; Andrews, 1973; Mason et al., 1976; Borgsteede et al., 1978; Zimmerman et al., 1986; Smith and Hines, 1987; Suarez et al., 1991; Rickard et al., 1993). One recent example of livestock's role in transporting parasites is the spread of *Nematodirus battus* Crofton and Thomas, 1951, a nematode parasite of sheep. Originally described from domestic sheep in Great Britain (Crofton and Thomas, 1951, 1954), *N. battus* has been identified in sheep in Norway, the Netherlands, France, Denmark, Germany, Switzerland, Italy, Spain, Mexico, the United States, and Canada (Nardi et al., 1974; Borgsteede et al., 1978; Hubert and Kerboeuf, 1985; Hoberg et al., 1986; Smith and Hines, 1987; Smith and McIntosh, 1988; Bauer, 1989; Hertzberg and Wolff, 1990; George-Sanchez and Quiroz-Romero, 1993; Garcia-Romero et al., 1993; Thamsborg et al., 1996). Foci in Norway, the Netherlands, and Canada have been linked to the importation of adult sheep chronically infected with *N. battus* (Helle, 1969; Borgsteede et al., 1978; Smith and Hines, 1987).

The known distribution of *N. battus* in North America includes Oregon, Washington, Maryland, New York, Vermont, Prince Edward Island, New Brunswick, and Nova Scotia (Zimmerman et al., 1986; Smith and Hines, 1987; Smith and McIntosh, 1988). Only the focus on Prince Edward Island has been linked to the importation of sheep from Great Britain (Smith and Hines, 1987). Speculation concerning the mode of introduction of this parasite into Oregon, where it was first identified in North America, included recent importation with animals from endemic areas (Hoberg et al., 1986); however, the source of infection was never determined.

Isoenzyme analysis and isoelectric focusing (IEF) have been used extensively to evaluate isolates and species of parasites (Kumaratilake and Thompson, 1984; Valero et al., 1985; Johnson and Hoberg, 1989; Tenter et al., 1989; Stranden et al., 1990; Rioux et al., 1990; Snabel et al., 1994; Steen et al., 1994). Banding patterns derived from IEF of parasite extracts have been shown to be reproducible, independent of host origin, and capable of distinguishing among species and isolates of the same species (Hermoso et al., 1982; Baldock et al., 1985; Valero et al., 1985; Dixon and Arai, 1987; Johnson and Hoberg, 1989; Steen et al., 1994). As such, IEF of soluble proteins should have practical value in assessing conspecific or subspecific status of morphologically similar parasites (*Nematodirus archari* Sokolova, 1948 and *Nematodirus andersoni* Durette-Desset and Samuel, 1989; see Rickard and Lichtenfels, 1989; Durette-Desset and Samuel, 1989). In addition, this technique could have an advantage as a screening tool in identification of isolates prior to detailed genetic analysis.

The objective of the present study was to evaluate soluble proteins of species and isolates of *Nematodirus* using IEF, determine whether sex of the nematodes influenced the protein banding pattern, and evaluate the usefulness of this technique for diagnostics and as an aid in determining origin of the North American isolates of *N. battus.*

MATERIALS AND METHODS

Parasites and host tissue

Specimens of *N. battus* (*N. battus*—OR), *Nematodirus spathiger* (Railliet, 1896), and *Nematodirus filicollis* (Rudolphi, 1802) were obtained at necropsy from naturally infected sheep whereas specimens of *Nematodirus helvetianus* (May, 1920) were collected from sheep and calves in the Willamette Valley, Oregon, during the period of 1986–1988 (see Rickard et al., 1989). The small intestine was ligated in situ, separated from the remaining viscera, and removed from each animal. It was then divided into anterior and posterior halves. The anterior half was opened longitudinally and the mucosal surface stripped and washed. The contents were sieved using a 100-mesh (150-μm opening) screen and tap water. The material retained on the screen was backwashed with 0.8% saline into a dish. Adult specimens of *Nematodirus* spp. were removed from the dish, identified to species (based on Lichtenfels and Pilitt, 1983; Hoberg et al., 1986), and placed in separate petri dishes of 0.8% saline according to species and sex. Lots of 10–50 nematodes were rinsed in distilled deionized water (ddH_2O), placed in vials, and stored at −70 C.

Specimens of *N. battus* from other populations were also studied. Specimens of the Weybridge isolate (*N. battus*—WEY) were derived from continuous culture maintained by M. B. Lancaster in Weybridge, U.K. in 1988. Additional nematodes of the Nb—WEY population were made available and cultured in March 1991 by E.P.H. Specimens of the Prince Edward Island isolate (*N. battus*—PEI) were derived from eggs collected from naturally infected lambs at Montague, Prince Edward Island, Canada in July and August 1990 by E.P.H. All isolates were established experimentally in lambs at the USDA, ARS, Biosystematics

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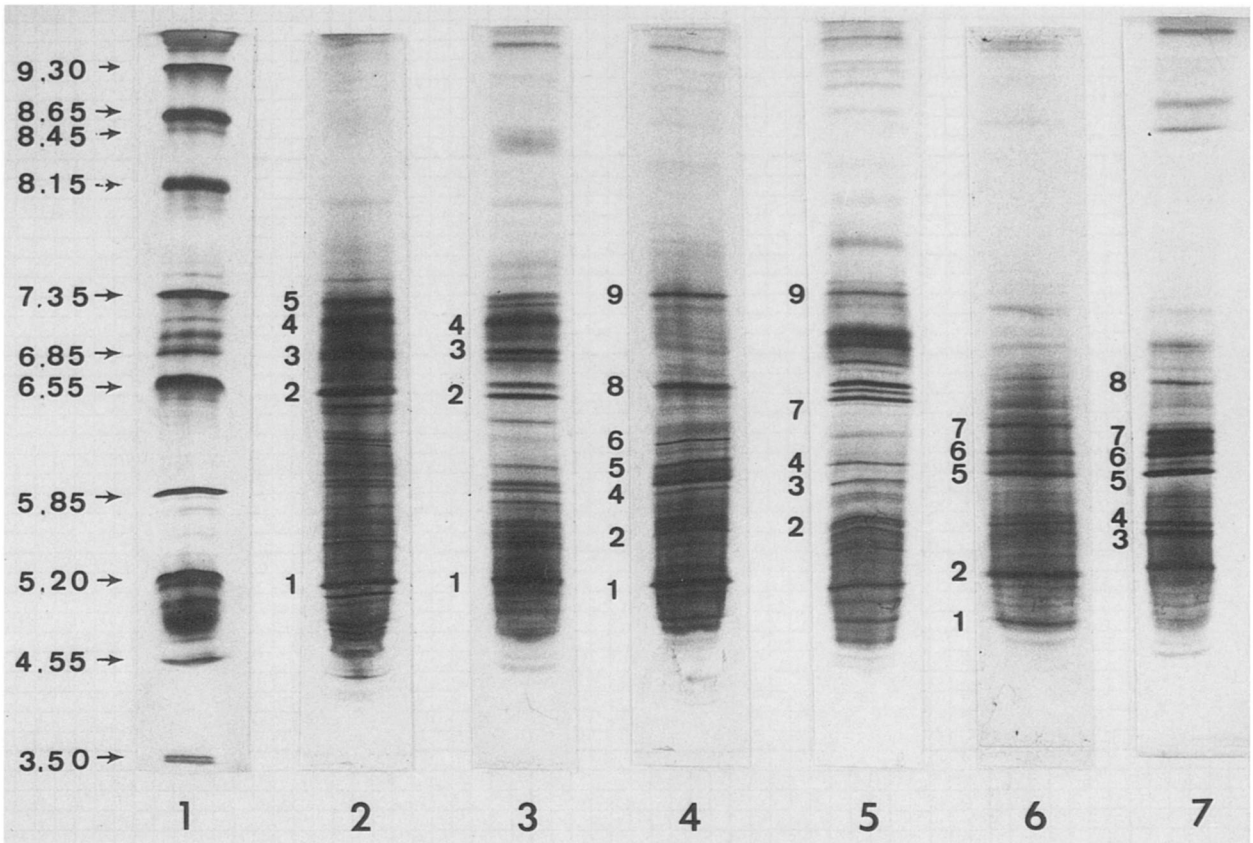

FIGURE 1. Banding patterns for total proteins of three species of *Nematodirus*. Individual lanes are designated as follows: 1 = pI markers; 2 = *N. spathiger*, female; 3 = *N. spathiger*, male; 4 = *N. helvetianus*, female; 5 = *N. helvetianus*, male; 6 = *N. filicollis*, female; 7 = *N. filicollis*, male. Band numbers correspond to dominant peaks in Table I.

and National Parasite Collection Unit, Beltsville, Maryland, USA (BNPCU). At necropsy, nematodes were identified, washed, and stored as described above. Culture techniques for *N. battus*—WEY and *N. battus*—PEI were according to the methods of Lancaster (Ministries of Agriculture, Fisheries and Food, 1986).

Representative specimens of each species were fixed in 10% formalin and deposited in the United States National Parasite Collection with the following accession numbers: *Nematodirus battus*—OR: 86987; *N. battus*—PEI: 70248; *N. battus*—WEY: 70296, 84038; *N. spathiger*: 86989; *N. filicollis*: 86988; *N. helvetianus*: 86990.

Samples of small intestine from infected sheep and calves were collected at the time of necropsy, washed with 0.8% saline, and stored at −70 C until used.

Preparation of samples for IEF

Separate lots of nematodes were thawed and individual nematodes were transferred to separate wells of a coors plate (on ice), containing 20 μl ddH_2O for each male and 60 μl ddH_2O for each female. The specimens were macerated using a rounded end, solid glass rod. The liquid was transferred to centrifuge tubes and centrifuged in an airfuge (Beckman Instruments, Inc., Palo Alto, California) for 2 min at 17,800 *g*. The supernatant was then used for IEF.

Samples of small intestine were thawed, macerated using a tissue homogenizer immersed in ice, and centrifuged as above. The supernatant was then used for IEF.

Protein concentrations for individual nematodes were determined for each lot of specimens, as well as the sections of small intestine, using a Bradford dye-binding assay (BioRad Protein Assay, BioRad Laboratories, Inc., Hercules, California). Regressions were calculated using SigmaPlot (Jandel Scientific, Corte Madera, California). Protein concentrations were 0.35–1.25 mg/ml for the nematode homogenate and 61.8–108.0 mg/ml for the small intestinal homogenate. Protein concentrations for the latter were adjusted to approximately 1 mg/ml prior to use for IEF.

IEF

Isoelectric focusing was performed using a computer controlled-automated electrophoresis system (Pharmacia PhastSystem, Pharmacia Biotech Inc., Piscataway, New Jersey). Preprepared gels of pH 3–9 and 4–6.5 were used. The methods of Johnson and Hoberg (1989) were used with the following exceptions: (1) 4-μl samples were applied to each track on the gel, and (2) silver staining of gels was performed using the PhastGel Silver Stain Kit (Pharmacia Biotech Inc.) according to manufacurer's protocol. The same pI standards (Pharmacia Biotech Inc.) were included on each gel allowing pI values to be assigned to bands of each track. A minimum of 10 individual males of each species and isolate and 5 individual females of each species were used.

Densitometry

Gels were scanned on an LKB UltroScan laser densitometer (LKB Produkter AB, Bromma, Sweden) using a *y*-step of 40 μm and an *x*-width of 1,600 μm. GelScan software (Pharmacia Biotech Inc.) was utilized to evaluate each track as follows: pI markers were used to calculate a standard curve and pI values were then assigned to all bands in each track. Dominant bands, defined as those bands always present regardless of the protein content of the sample used, were then identified. A separate standard curve was calculated for each gel to account for slight variations occurring among gels.

Coefficient of similarity

The coefficient of similarity between paired species and isolates was obtained using the following formula (Ferguson, 1980):

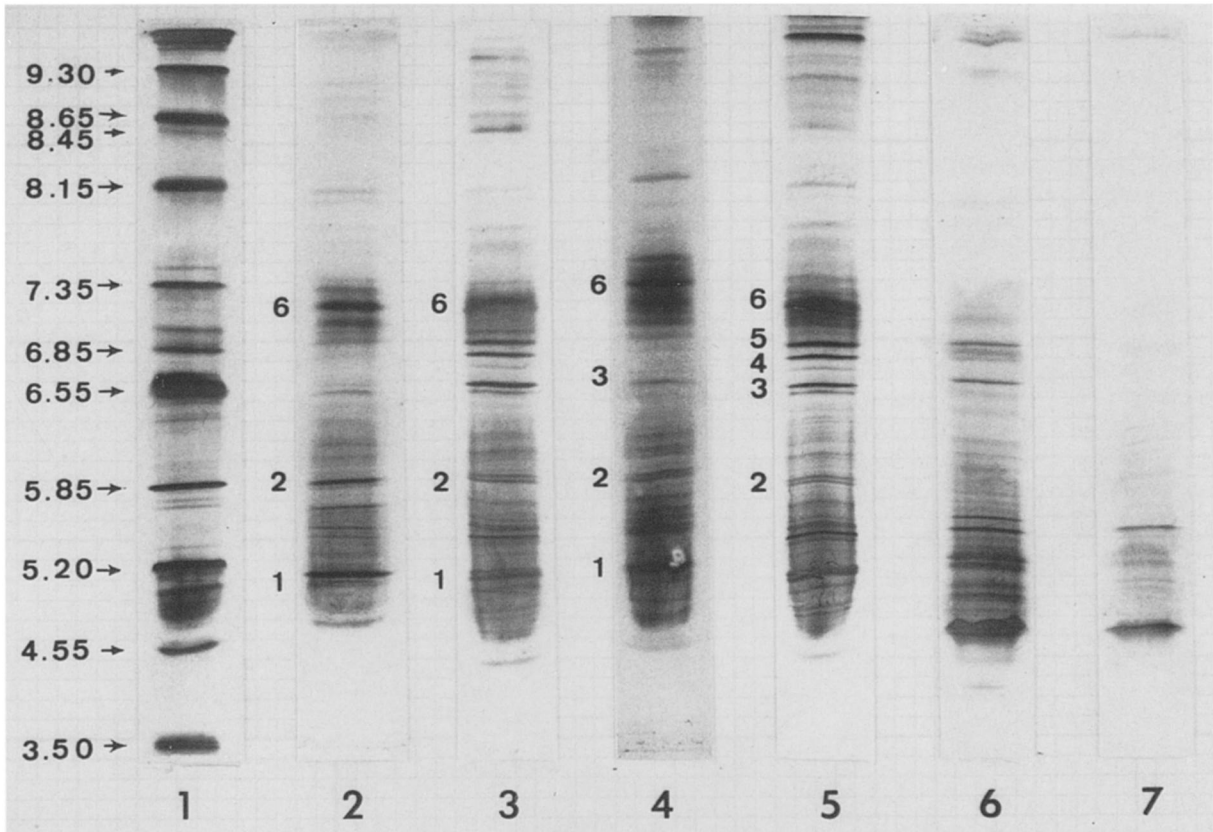

FIGURE 2. Banding patterns for total proteins of three isolates of *Nematodirus battus* and host small intestine. Individual lanes are designated as follows: 1 = pI markers; 2 = *N. battus*—OR, female; 3 = *N. battus*—OR, male; 4 = *N. battus*—WEY, male; 5 = *N. battus*—PEI; 6 = ovine small intestine; 7 = bovine small intestine. Band numbers correspond to dominant peaks in Table II.

Sm = (no. of bands of common mobility
÷ maximum number of bands in an individual)

The similarity between paired species and isolates was determined using male nematodes only because sufficient numbers of females of *N. battus*—WEY and *N. battus*—PEI isolates were unavailable for IEF. A dendrogram from the matrix of similarity coefficients was derived using the unweighted pair-group method with arithmetic means (UPGMA) (Sneath and Sokal, 1973; Ferguson, 1980).

RESULTS

Proteins in all nematodes focused best when the samples were applied in the anodal position and when only 1 position of sample application was used on a gel. Background present at the point of application (pH 4.80–5.20) did not interfere with evaluation. Proteins focused predominantly in the pH range of 4.1–7.2 on gels of 3–9 pH range (Figs. 1, 2). Gels of pH range 4–6.5 were used to increase resolution and to reveal differences that may not have been apparent in gels of the broader pH range. The banding patterns present matched those in the same region of the broad pH range gels, confirming the specificity and differences between species and isolates (data not shown).

Comparisons of *N. helvetianus* from sheep and cattle did not reveal any differences attributable to host influences (data not shown). Consequently, parasite data were combined from both hosts and used in subsequent evaluations. Scans from gels of 3–9 pH range were used to calculate values of pI presented in Tables I and II. Dominant banding patterns for parasites and hosts were replicated irrespective of protein concentrations of samples and intensity of staining.

Banding patterns differed between sexes in each species studied. In total, 21 bands could be distinguished for males of *N. helvetianus* (5 dominant); 26 bands for females of *N. filicollis* (5 dominant) and *N. battus*—OR (3 dominant); 27 bands for males of *N. spathiger* (4 dominant), *N. filicollis* (6 dominant), *N. battus*—WEY (4 dominant), and *N. battus*—OR (3 dominant); 28 bands for males of *N. battus*—PEI (5 dominant) and females of *N. spathiger* (5 dominant); and 31 for females of *N. helvetianus* (7 dominant). Although the total number of bands present differed between sexes, dominant banding patterns were shared between sexes of the same species (Tables I, II). When differences did occur, the additional dominant bands present in 1 sex were found in the opposite sex but were not consistently demonstrable because of low protein concentration. Likewise, low protein concentration of individual bands explains the discrepancies in dominant banding patterns between isolates of *N. battus*. Banding profiles for ovine and bovine intestinal tissue were also distinct from the species and isolates of *Nematodirus* (Figs. 1, 2). Although several nondominant protein bands were shared between parasites and their hosts, only 1 dominant band was present in both host and nematode tissue—band 5 of male *N. battus*—PEI. The IEF banding patterns of the 4 species and 3 isolates of *Nematodirus* were distinct and provided unequivocal differentiation not just between species of *Nematodirus* but for sexes of each species and among isolates as well.

TABLE I. Numerical data for dominant peaks from densitometric scans of IEF gels of *Nematodirus spathiger, Nematodirus helvetianus,* and *Nematodirus filicollis.*

Dominant peak	Females			Males		
	pI value ($\bar{x}$ ± 1 SD)*	pI range†	CV‡	pI value ($\bar{x}$ ± 1 SD)*	pI range†	CV‡
Nematodirus spathiger						
1	5.25 ± 0.03	5.17–5.25	0.57	5.20 ± 0.02	5.16–5.23	0.38
2	6.48 ± 0.10	6.38–6.65	1.54	6.54 ± 0.08	6.36–6.34	1.22
3	6.86 ± 0.06	6.79–6.99	0.88	6.83 ± 0.05	6.76–6.93	0.73
4	7.07 ± 0.06	6.98–7.13	0.85	7.10 ± 0.06	6.99–7.24	0.85
5	7.22 ± 0.06	7.11–7.30	0.83	—§	—	—
Nematodirus helvetianus						
1	5.10 ± 0.06	5.03–5.20	1.18	—§	—	—
2	5.49 ± 0.04	5.44–5.51	0.75	5.49 ± 0.07	5.38–5.52	1.28
3	—§	—	—	5.78 ± 0.07	5.59–5.90	1.21
4	5.91 ± 0.03	5.85–5.94	0.51	5.94 ± 0.09	5.79–6.07	1.52
5	6.03 ± 0.03	5.97–6.06	0.50	—§	—	—
6	6.15 ± 0.05	6.11–6.20	0.81	—§	—	—
7	—§	—	—	6.39 ± 0.07	6.27–6.48	1.10
8	6.53 ± 0.04	6.48–6.57	0.61	—§	—	—
9	7.14 ± 0.08	7.12–7.24	1.12	7.14 ± 0.06	7.05–7.22	0.84
Nematodirus filicollis						
1	4.64 ± 0.06	4.57–4.73	1.29	—§	—	—
2	5.33 ± 0.07	5.23–5.40	1.31			
3	—§	—	—	5.42 ± 0.06	5.36–5.54	1.11
4	—§	—	—	5.49 ± 0.06	5.42–5.62	1.09
5	5.84 ± 0.05	5.79–5.92	0.86	5.91 ± 0.07	5.85–5.96	1.18
6	5.99 ± 0.05	5.94–6.06	0.83	5.98 ± 0.07	5.90–6.13	1.17
7	6.16 ± 0.06	6.10–6.24	0.97	6.05 ± 0.07	6.00–6.20	1.16
8	—§	—	—	6.41 ± 0.06	6.36–6.53	0.94

* Females, n = 5; males, n = 10.
† Minimum and maximum of evaluated pI value.
‡ Coefficient of variation = SD/mean × 100.
§ Bands present but not dominant.

TABLE II. Numerical data for dominant peaks from densitometric scans of IEF gels of isolates of *Nematodirus battus.*

Dominant peak	pI value ($\bar{x}$ ± 1 SD)*	pI range†	CV‡	pI value ($\bar{x}$ ± 1 SD)*	pI range†	CV‡
Nematodirus battus	OR Females			OR Males		
1	5.32 ± 0.08	5.20–5.45	1.50	5.33 ± 0.03	5.31–5.39	0.56
2	5.94 ± 0.06	5.90–6.06	1.01	5.84 ± 0.08	5.69–5.95	1.37
3	—§	—	—	—§	—	—
4	—§	—	—	—§	—	—
5	—§	—	—	—§	—	—
6	7.16 ± 0.04	7.13–7.22	0.56	7.17 ± 0.07	7.05–7.31	0.98
Nematodirus battus	WEY Males			PEI Males		
1	5.35 ± 0.04	5.32–5.43	0.75	—§	—	—
2	5.98 ± 0.05	5.91–6.05	0.84	5.89 ± 0.05	5.85–5.99	0.85
3[e]	6.41 ± 0.04	6.35–6.48	0.62	6.41 ± 0.05	6.33–6.51	0.78
4	—§	—	—	6.71 ± 0.06	6.61–6.81	0.89
5	—§	—	—	6.94 ± 0.10	6.73–6.97	1.44
6	7.20 ± 0.05	7.10–7.30	0.69	7.11 ± 0.06	7.03–7.20	0.84

* Females, n = 5; males, n = 10.
† Minimum and maximum of evaluated pI value.
‡ Coefficient of variation = SD/mean × 100.
§ Bands present but not dominant.

TABLE III. Matrix of similarity coefficients between species and isolates of male *Nematodirus*.

	1	2	3	4	5	6
1. *Nematodirus helvetianus*	—	0.38	0.44	0.44	0.55	0.43
2. *Nematodirus filicollis*	—	—	0.54	0.55	0.52	0.46
3. *Nematodirus spathiger*	—	—	—	0.41	0.45	0.50
4. *Nematodirus battus*—OR	—	—	—	—	0.57	0.64
5. *N. battus*—WEY	—	—	—	—	—	0.70
6. *N. battus*—PEI	—	—	—	—	—	—

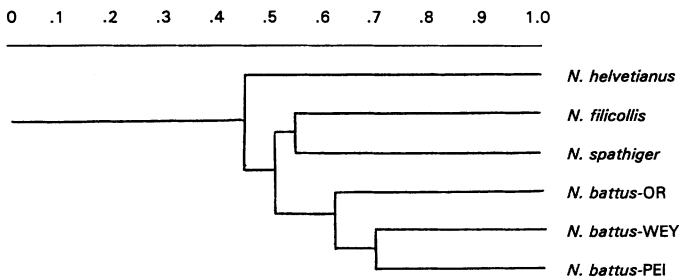

FIGURE 3. Dendrogram showing the relationships of species and isolates of male *Nematodirus* based on unweighted pair-group cluster analysis of similarity coefficients and the data in Table III.

The 3 isolates of *N. battus,* which usually occur in sheep, had the highest coefficients of similarity and clustered together in the dendrogram (Table III, Fig. 3). *Nematodirus filicollis* and *N. spathiger,* which also usually occur in sheep, formed a second group that, in turn, clustered with the *N. battus* isolates. The species with the lowest coefficient of similarity was *N. helvetianus,* which appeared to be the most dissimilar.

DISCUSSION

Automated IEF is a powerful technique enabling the examination of numerous samples in a relatively short period of time. The results of this study demonstrate that individual nematodes can be evaluated using these techniques. This eliminates the potential masking of fine details that can arise when evaluating composite samples. In the present study, individual males of the 3 populations of *N. battus* were readily identifiable as *N. battus* as well as distinct isolates, thus confirming the usefulness of this technique as a diagnostic tool. The ability to evaluate single nematodes, which results in low protein concentrations when processed, further enhances the value of IEF as a diagnostic tool.

In a previous study examining the IEF banding patterns of 3 trichostrongyloid nematodes (Valero et al., 1985), species-specific differences were noted; however, gender differences were not observed. In contrast, in the present study intraspecific banding patterns did differ between sexes. This discrepancy may have 1 of 2 causes. First, gels in the study by Valero et al. (1985) were stained with Coomassie brilliant blue, whereas those in the present study were stained with silver. Silver staining can be up to 100 times more sensitive than Coomassie staining (Hames, 1990); consequently, it is possible that gender differences in dominant banding profiles were detectable because of the more sensitive staining technique. Second, composite samples were used to produce the protein profiles in the study by Valero et al. (1985), whereas, single specimens were used in the present study. As a result, gender-specific differences in dominant banding patterns in the present study occurred because certain proteins in 1 sex, although occasionally demonstrable, were not present in high enough concentrations to be consistently detected. Had several specimens been combined per lane, those bands whose detection was concentration dependent would have possessed sufficient protein content for consistent detection. This would have resulted in identical dominant banding patterns for each sex within a species, as was demonstrated by Valero et al. (1985).

In order to compare studies conducted in different laboratories, previous authors have called for the standardization of techniques when using IEF (Johnson and Hoberg, 1989; Tenter et al., 1989). Suggested protocols include the establishment and reporting of pI ranges for dominant peaks, examination of both broad and narrow pH range gels, and inclusion of standards on each gel to compensate for any variation between gels. The present study indicates that male and female nematode specimens should be evaluated separately whenever possible.

Isoelectric focusing has been used to determine the geographic origin of parasites following translocation and introduction (see Baldock et al., 1985). Although cluster analysis of the IEF patterns of the 3 isolates of *N. battus* indicates the North American and Weybridge isolates are similar interpretation of the relationships, and thus the history of introduction based on these data is equivocal. The relatively limited distribution of *N. battus* in Canada and the United States and a documented history of transcontinental movement with infected hosts indicates that this nematode was not historically present or endemic in North America (Zimmerman et al., 1986; Hoberg, 1997). However the putative relationships depicted in the dendrogram would refute this contention. Thus, current evidence is contradictory and, although it is unlikely that this parasite has been present in the Western hemisphere for an extended period of time, more detailed phylogenetic analyses of molecular sequence data are required to elucidate the recent biogeographic history.

Biochemical and molecular approaches have become standard techniques for reconstructing evolutionary history of organisms (Nadler, 1990). Because organisms have only 1 evolutionary history, studies based on molecular characters should be congruent and additive to studies based on morphological characters. However, cluster analysis of the 4 species examined in the present study does not agree with the phylogenetic hypothesis for several species of *Nematodirus* recently put forth by Hoberg (1997). In that analysis, based on morphological characters of both adult male and female nematodes, *N. battus* is in a clade containing nematodes that are principally parasites of the Cervidae and is only distantly related to species typical of sheep. Among species characteristic of domesticated hosts, Hoberg's (1997) analysis indicated *N. battus* is more closely related to *N. helvetianus* than to *N. spathiger* or *N. filicollis.* In contrast, *N. helvetianus* in the present study had the lowest coefficient of similarity and was the most distant in the dendrogram. This incongruence may be due to different tree-building methodologies, distance versus parsimony, that can be based on divergent evolutionary assumptions (Swofford et al., 1996).

Additionally, these data sets do not include the same taxa and, as a consequence, are not directly comparable.

Beyond these apparent limitations due to comparability of the current data sets, is the problematic nature of interpretating and applying data from IEF directly to elucidation of phylogenetic relationships. Phylogenetic reconstruction is dependent on recognition of homology irrespective of the class of characters that is under evaluation (e.g., morphological, biochemical, or molecular nucleotide sequences). Data from IEF simply may be inappropriate for phylogenetic inference. There can be no supportable assumption about the specific identity (in the absence of selective histochemical staining) and thus the homology of bands, derived from total protein, that have identical isoelectric points (Murphy et al., 1996). Therefore, whereas IEF is a robust method for establishing identity and has great utility in diagnostics, the general applicability of this method in broader studies in evolutionary biology is likely to be limited (Whitmore, 1990). It is clear, however, that the technique of IEF is a valuable adjunct to current methods of identification for these nematodes. Further study is warranted with respect to the phylogenetic and biogeographic history of *N. battus* and associated species of *Nematodirus* from domestic and wild ruminants across the Holarctic and regions where hosts and parasites have been introduced (Hoberg, 1997).

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LITERATURE CITED

ANDREWS, J. R. H. 1973. A host–parasite checklist of helminths of wild ruminants in New Zealand. New Zealand Veterinary Journal **21:** 43–47.

BALDOCK, F. C., R. C. A. THOMPSON, AND L. M. KUMARATILAKE. 1985. Strain identification of *Echinococcus granulosus* in determining origin of infection in a case of human hydatid disease in Australia. Transactions of the Royal Society of Tropical Medicine and Hygiene **79:** 238–241.

BAUER, C. 1989. Infektion mit *Nematodirus battus* (Crofton und Thomas, 1951) und Wide-Eimeriose bei Schaflammern in Deutschland (Fallbericht). Deutsche Tierarztliche Wochenschrift **96:** 382–384.

BORGSTEEDE, F. H. M., J. HENDRIKS, AND W. P. J. VAN DEN BURG. 1978. *Nematodirus battus* in Nederland. Tijdschrift voor Diergeneeskunde **103:** 279–280.

CROFTON, H. D., AND R. J. THOMAS. 1951. A new species of *Nematodirus* in sheep. Nature **168:** 559.

———, AND ———. 1954. A further description of *Nematodirus battus* Crofton and Thomas, 1951. Journal of Helminthology **28:** 119–122.

DIXON, B. R., AND H. P. ARAI. 1987. An investigation of host influence on soluble protein banding profiles of *Hymenolepis* spp. (Cestoidea), using isoelectric focusing. Canadian Journal of Zoology **65:** 2471–2474.

DURETTE-DESSET, M.-C., AND W. M. SAMUEL. 1989. Nematodirinae (Nematoda: Trichostrongyloidea) d'*Antilocapra* et d'*Ovis* en Alberta, Canada. Annales de Parasitologie Humaine et Comparee **64:** 469–477.

FERGUSON, A. 1980. Biochemical systematics and evolution. Halsted Press, New York, New York, 194 p.

GARCIA-ROMERO, C., F. VALCARCEL-SANCHO, M. CORDERO DEL CAMPILLO, AND F. A. ROJO-VAZQUEZ. 1993. Etiologia y epizootiologia de las infestaciones por tricoestrongilidos ovinos en la comarca do Oropesa (Toledo). Investigacion Agraria, Produccion y Sanidad Animales **8:** 155–168.

GEORGE-SANCHEZ, S., AND H. QUIROZ-ROMERO. 1993. Frecuencia de parasitos gastrointestinales, pulmonares y hepaticos en ovinos de la Magdalena Soltepec, Tlaxcala, Mexico. Veterinaria Mexico **24:** 195–198.

HAMES, B. D. 1990. One-dimensional polyacrylamide gel electrophoresis. *In* Gel electrophoresis of proteins: A practical approach, 2nd ed., B. D. Hames and D. Rickwood (eds.). Oxford University Press, New York, New York, p. 1–147.

HELLE, O. 1969. The introduction of *Nematodirus battus* (Crofton and Thomas, 1951) into a new environment. Veterinary Record **84:** 157–160.

HERMOSO, R., A. VALERO, AND M. MONTEOLIVA. 1982. Estudio de isoenzimas y proteinas solubles en *Moniezia expansa* Rudolphi (1810) y *Avitellina centripunctata* Rivolta (1874), por electroforesis y electroenfoque. Revista Iberica de Parasitologia **42:** 109–116.

HERTZBERG, H., AND K. WOLFF. 1990. Welche diagnose stellen sie? Schweizer Archiv fur Tierheilkunde **132:** 331–334.

HOBERG, E. P. 1997. Parasite biodiversity and emerging pathogens: A role for systematics in limiting impacts on genetic resources. *In* Global genetic resources: Access, ownership and intellectual property rights, K. E. Hoagland and A. Y. Rossman (eds.). Association of Systematics Collections, Washington, D.C. (in press).

———, G. L. ZIMMERMAN, AND J. R. LICHTENFELS. 1986. First report of *Nematodirus battus* (Nematoda: Trichostrongyloidea) in North America: Redescription and comparison to other species. Proceedings of the Helminthological Society of Washington **53:** 80–88.

HUBERT, J., AND D. KERBOEUF. 1985. Study of gastrointestinal strongylosis in a sheep flock on permanent pasture. 2. Sheep parasitism in 1978–1979. Annales de Recherches Veterinaires **16:** 29–39.

JOHNSON, M. R., AND E. P. HOBERG. 1989. Differentiation of *Moniezia expansa* and *Moniezia benedeni* (Eucestoda: Cyclophyllidea) by isoelectric focusing. Canadian Journal of Zoology **67:** 1471–1475.

KUMARATILAKE, L. M., AND R. C. A. THOMPSON. 1984. Biochemical characterisation of Australian strains of *Echinococcus granulosus* by isoelectric focusing of soluble proteins. International Journal for Parasitology **14:** 581–586.

LICHTENFELS, J. R., AND P. A. PILITT. 1983. Cuticular ridge patterns of *Nematodirus* (Nematoda: Trichostrongyloidea) parasitic in domestic ruminants of North America, with a key to species. Proceedings of the Helminthological Society of Washington **50:** 261–274.

MASON, P. C., N. R. KIDDEY, R. J. SUTHERLAND, D. M. RUTHERFORD, AND A. G. GREEN. 1976. *Elaphostrongylus cervi* in red deer. New Zealand Veterinary Journal **24:** 22–23.

MINISTRY OF AGRICULTURE, FISHERIES AND FOOD. 1986. Manual of veterinary parasitological laboratory techniques, 3rd ed. Her Majesty's Stationery Office, London, U.K., 160 p.

MURPHY, R. W., J. W. SITES, JR., D. G. BUTH, AND C. H. HAUGLER. 1996. Proteins, isozyme electrophoresis. *In* Molecular systematics, D. M. Hillis, C. Moritz, and B. K. Mable (eds.). Sinauer Associates, Sunderland, Massachusetts, p. 51–120.

NADLER, S. A. 1990. Molecular approaches to studying helminth population genetics and phylogeny. International Journal for Parasitology **20:** 11–29.

NARDI, E., V. PUCCINI, AND L. SOBRERO. 1974. Segnalazione di un nematode reportablie a *Nematodirus battus,* Crofton e Thomas (1951), in ovini Publiesi. Veterinaria Italiana **25:** 590–601.

RICKARD, L. G., E. P. HOBERG, N. M. ALLEN, G. L. ZIMMERMAN, AND T. M. CRAIG. 1993. *Spiculopteragia spiculoptera* and *S. asymmetrica* (Nematoda: Trichostrongyloidea) from red deer (*Cervus elaphus*) in Texas. Journal of Wildlife Diseases **29:** 512–515.

———, ———, J. K. BISHOP, AND G. L. ZIMMERMAN. 1989. Epizootiology of *Nematodirus battus, N. filicollis,* and *N. spathiger* (Nematoda: Trichostrongyloidea) in western Oregon. Proceedings of the Helminthological Society of Washington **56:** 104–115.

———, AND J. R. LICHTENFELS. 1989. *Nematodirus archari* (Nematoda: Trichostrongyloidea) from ruminants in North America with a description of the synlophe and the female. Canadian Journal of Zoology **67:** 1708–1714.

RIOUX, J. A., G. LANOTTE, E. SERRES, F. PRATLONG, P. BASTIEN, AND J. PERIERES. 1990. Taxonomy of *Leishmania.* Use of isoenzymes.

Suggestions for a new classification. Annales de Parasitologie Humaine et Comparee **65:** 111–125.

SMITH, H. J., AND J. G. HINES. 1987. *Nematodirus battus* in Canadian sheep. Canadian Veterinary Journal **28:** 256.

———, AND S. MCINTOSH. 1988. Prevalence of *Nematodirus battus* in sheep in New Brunswick and Nova Scotia. Canadian Veterinary Journal **29:** 385.

SNABEL, V., V. HANZELOVA, AND H.-P. FAGERHOLM. 1994. Morphological and genetic comparison of two *Proteocephalus* species (Cestoda: Proteocephalidae). Parasitology Research **80:** 141–146.

SNEATH, P. H. A., AND R. R. SOKAL. 1973. Numerical taxonomy. W. H. Freeman, San Francisco, California, 573 p.

STEEN, M., S. PERSSON, AND L. HAJDU. 1994. Protostrongylidae in Cervidae and *Ovibos moscatus*: A clustering based on isoelectric focusing on nematode body proteins. Applied Parasitology **35:** 193–206.

STRANDEN, A. M., J. ECKERT, AND P. KOHLER. 1990. Electrophoretic characterization of *Giardia* isolated from humans, cattle, sheep, and a dog in Switzerland. Journal of Parasitology **76:** 660–668.

SUAREZ, V. H., M. R. BUSETTI, M. C. FORT, AND D. O. BEDOTI. 1991. *Spiculopteragia spiculoptera, S. asymmetrica* and *Ostertagia leptospicularis* from *Cervus elaphus* in La Pampa, Argentina. Veterinary Parasitology **40:** 165–168.

SWOFFORD, D. L., G. J. OLSEN, P. J. WADDELL, AND D. M. HILLIS. 1996. Phylogenetic inference. *In* Molecular systematics, D. M. Hillis, C. Moritz, and B. K. Mable (eds.). Sinauer Press, Sunderland, Massachusetts, p. 407–514.

TENTER, A. M., M. R. JOHNSON, AND G. L. ZIMMERMAN. 1989. Differentiation of *Sarcocystis* species in European sheep by isoelectric focusing. Parasitology Research **76:** 107–114.

THAMSBORG, S. M., S. M. GITHIGA, M. LARSEN, P. NANSEN, AND S. A. HENRIKSEN. 1996. Infection med *Nematodirus battus,* en ny parasit hos far i Danmark. Dansk Veterinaertidsskrift **79:** 231–232.

VALERO, A., F. HERMOSO, AND M. MONTEOLIVA. 1985. Characterization of *Ostertagia circumcincta, Ostertagia trifurcata* and *Marshallagia marshalli* (Nematoda: Trichostrongylidae) proteins. Helminthologia **22:** 41–45.

WHITMORE, D. H. 1990. Isoelectric focusing of proteins. *In* Electrophoretic and isoelectric focusing techniques in fisheries management, D. H. Whitmore (ed.). CRC Press, Boca Raton, Florida, p. 81–105.

ZIMMERMAN, G. L., E. P. HOBERG, L. G. RICKARD, J. K. ERNO, D. WILSON, R. K. STRICKLAND, R. R. GERRISH, W. D. PRICHARD, AND E. KNOTT. 1986. Broadened geographic range and periods of transmission for *Nematodirus battus* in the United States. Proceedings of the 90th Annual Meeting of the United States Animal Health Association **90:** 404–412.